ENCYCLOPEDIA OF ANTI-DOPING

IN AN ERA OF EVIDENCED BASED MEDICINE

Scientific literature made available 2006 through 2013

Selected and edited by

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EDITOR’S PREFACE (AND SEARCH ALGORITHM)

The scientific literature also in small medical fields like doping is today enormous – and it is not possible to keep updated unless making strong and focused efforts. The present review is an attempt to make it easier for those fighting doping to keep updated. From the beginning it was foremost an effort to make the reviewer updated, but hopefully it can be used also of others with the same interest, i.e. those who believe in fighting doping with the aim to eradicate it – or at least make doping so difficult that nobody can succeed with it. However, it should be emphazised that this compilation of data will still is a personal review, which means that the selection of presented articles have been up to the reviewer, and probable another author might have made at least some other choices.

The definition of “doping” has varied over the use, not least because it is difficult to combine what really is enhancing performance in sport from a theoretical point of view with what is practical – caffeine, children’s medicine when they have caught a cold, and alcohol are good example of this. Moreover, there is an ongoing evoluation all the time which gives new possibilities but also new problems, e.g. gene doping. Today the used definition of “doping” is very anti-intellectual: “What WADA put on the list of forbidden substances and methods is doping”. In this book the limitis is more generous, as not only the WADA lit is used as an inclusion criterium, but also not forbidden but often discussed substances and methods are covered. The biggest part of the discussed items is dietary supplements, but there are also other substances that might or might not be clasifie as doping substances. Unfortunately the borders of doping are ill defined – and with times it is even more difficult to define. This is why this book has defined anti-doping from the athlete point of view: the aim of this encyclopedia is to include what is discussed as performance enhancing substances and methods, irrespectively if they are working or not.

So, why bother to compile a lot of articles and tease serious individuals interested in sports and sports medicine to wast time on even a superficially look through these texts? The answer to that is that even if each abstract of an article can be rightly criticised the total body of abstracts tell a more important story. This body tells something on what the present issues are in the part of sports medicine that deals with doping, what it is worked with scientifically on these issues in today, and what the thoughts of the researchers were when they wrote their reports. It also tells something where the edge of our knowledge is situated presently, and from that we can better understand what we can expect in the nearest future. So, a recommendation may be to read each paragraph with considerable scepticism, but enjoy them all together as new bricks in our scientific wall and hopefully find that together with the next bricks we will have a chance of reaching the next – higher – level that is more easy to understand. It is all the knowledge together that counts much, much more than every single article.

Moreover, as interested in clinical anti-doping I am proud tog breg that I am interested in a field of medicine that can be included under the heading of evidence based medicine. The anti-dopers are not just beliving and guessing; we can base our recommendations on scientific facts!

There must be made some limitations, otherwise a review in this form should not be possible to write due to lack of time and lack of brain capacity, and probably not possible to read either. Regarding the limitations, first of all the writing here will be based on the abstracts of the articles in almost all cases for practical reasons. This is also in line with the aim of the review: not to report all what has been published, but rather to give an introdutional sample
that hopefully will make the reader eager to read the whole article or articles: this should be regarded as "a taste of anti-doping in the last years".

A second limitation is that most of the selections has been made through PubMed; a few other sources have also been scrutinized, but then more occasional and not systematically. The medical subheadings (MeSHs) always used are (but some more have been used occasionally):

Doping, testing, medical history, biological passports, inadvertent doping, mass spectrometry, liquid chromatography, gas chromatography, isoelectric focusing, anabolic androgenic substances, rhabdomyolysis, designer drugs, AICAR, testosterone, nandrolone, dehydroepiandrosterone (DHEA), stanozolol, boldenone, boldione, bolandiol, methyltestosterone, methyltestosterone, adrenosterone, androstenediol, androstenedione, hydroxyandrostenedione, trenbolone, metandienone, finasterid, norbolethone, phytoecdysteroids, designer steroids, aromatase inhibitors, exemestane, dehydrogenase inhibitor, selective androgen receptor modulator (SARM), estrogens, oral contraceptives, aromatase inhibitors, selective estrogen receptor modulator (SERM), toremiphene, raloxifene, anti-estrogens, tamoxifen, blood doping, red blood cells, total hemoglobin mass, reticulocytes, erythropoietin (EPO), darpoinet, NESP, epoetin delta, hematide, perfluorocarbon, hemoglobin-based oxygen carriers (HBOCs), hemopure, plasma volume expanders, dextran, hydroxyethyl starch, hydroxyethyl starch (HES), glucose solution, saline solution, Ringer lactate solution, albumin, plasma protein fraction, gelatins, alpha-keratose, pullulan, levan, acetyl starch, polyvinylpyrrolidone, glycerol, polyethylene glycol, platelet-rich plasma, growth hormone, insulin-like growth factor i (IGF-1), insulin, human chorionic gonadotropin, clomiphene, luteinizing hormone, mechanical growth factors, AGRP, caffeine, theobromine, ephedrine, pseudoephedrine, methylephedrine, modafinil, methylphenidate, amphetamine, methamphetamine, methylamphetamine (MDMA), mephentermine, metamfepramone, mmephradene, tuaminoheptane, mesocarb, sydnocarb, strychnine, belfluorex, sibutramine, beta2 adrenergic agonists, asthma in sports, exercise-induced bronchoconstriction (EIB), salbutamol, terbutaline, procaterol, clenbuterol, methoxyphenamine, beta-blockers, angiotensin converting enzyme (ACE) inhibitors, diuretics, thiazides, mannitol, epistestosterone, desmopressin, glucocorticosteroids, ACTH, sildenafil, morphine, cannabis, cocain, ecstasy, gamma-amino butyric acid (GABA), gamma-butyrolactone (GBL), gamma hydroxybutyrate (GHB), beta-hydroxy-beta-methylbutyrate (HMB), gamma-oryzanol and ferulic acid, alcohol, nicotine, non-steroidal anti-inflammatory drugs (NSAIDs), peroxisome proliferator-activated receptor, nutritional supplementation, dehydration, exercise-associated hyponatremia, bicarbonate, carbohydrates, mouth rinse, carbohydrate gel, milk, ribose, galactose, fructose, nergy beverages, sport drinks, Red bull, proteins, branched-chain amino acids (BCAA), arginine, ornithine, leucine, tryptophan, taurin, cystein, cystine, theanine, glutathione, glutamine, alanine, methionine, carnosine, citrulline, asparagines, spartame, glycine, alpha-ketoglutarate, alpha-ketoisocaproate, whey protein, fat, medium-chain triglycerides, fish oil, omega-3 fatty acid, calcium, copper, boron, chromium, zinc, magnesium, selenium, vitamins, N-Acetylcysteine, antioxidants, creatine, carnitine, melatonin, methylhexaneameine, luthiatione, lecithin, inoleic acid, gamma-linolenic acid, conjugated linoleic acid, leptin, inosine, glucosamine, dietary nitrates, methylsulphonylmethane, melamine, probiotics, green tea, ginseng, ginko, garlic, macroalgae, quercetin, Teribulus terrestris, Arnica, Rhodiola rosea, Cissus quadrangularis, Cordyceps sinensis, bee pollen, Actovigin, colostrum, gene doping, medical ethics

most often combined with Sport, Sports or Exercise.
This MeSHs will lead to a lack of some articles that might be of interest, e.g. in doping physiology, doping biology and social aspects of doping, but the border has to be set somewhere.

Yet another limitation is that this is a clinical oriented review and the term **human** has been used in the search algorithm. Therefore almost all "preclinical" articles have been neglected; i.e. molecular biology, cell lines studies and whole animal studies are not included except exceptionally (when the authors could not resist the temptation to review also them). This is not because the preclinical issues are not interesting, but because they are so numerous, and because it is much more difficult to evaluate the importance of them.

Note that one aim of this collection of articles has been to file them in a way that the editor and reader can find reports on the same issues close to one another. However, rather often an article may be classified under more than one heading. In these cases the editor has had to make a choise; if it was the best choise possible will be up the reader to judge.

Another "problem" is how to evaluate the quality of the reports. In the best of worlds the reports with highest quality should be given more space, and those with low quality should maybe just get a short note. However, this is an extremely difficult task and this book's editor has almost surrendered; there is almost no quality control, except for those that have an outstandingly poor quality – they have been omitted ruthlessness. However, most of the quality control has been left to the readers.

Why is then all the text on dietary supplements included? There are four reasons for this:

- they are so widely used in the same rings of athletes where doping substances are attempted
- they are not seldomly discussed as if they were part of doping
- most of them is worthless (but rather expensive)
- some of them can be of value in the right dose and right setting (and there is science behind)

When presenting quarterly reviews, annual reports and this encyclopedia I sometimes am criticized for just rewriting what others have just written. I can just agree with them; the only thing I have done is to gather what others have worked with nothing more, nothing less. The only thing I have contributed with is that I have made other's work more available and easier to compare with what others have done. Maybe it is not som much, but I am proud of what I have done.

I am sometimes also asked why it is included so much on dietary supplements, as many of them in without effect and "unscientific." The reason to include them is, however, easy: they are present out there among the athletes and are compared to the doping agents if the antidopers want it or not. Also, one must admit that the border between what is allowed and what is not is not always logic or semantically right. For example water, carbohydrates may be important for athletic performance in some cases, and if too much is given it might even be harmful, i.e. they might fulfill the criteria for doping agents. However, the most important reason to include them is to make them "less mysterious" – if there is any science on the dietary supplements it would be nice to spread that also to them who consider to use them. In most cases it will lead to a drain of money from the pocket of the athletes, in a few cases the dietary supplements will be harmful, and in most cases they are only irrelevant.

All of the text – including tables etc – has been written in Microsoft Words, as this is the most spread mode for communicating with computers. This means that all words and all tables can be search throughout the text; for example the references can most easily be looked for this way. This is also why some of the reference figures have 0 or 00 as the first figures. Also, there is a "Content in the book" but no pages. This is because it is ment to be a book on a computer, and if so different persons and computers make there formatation different.
Also, the book is growing every third month, which makes impractible to give pages, and then change them four times a year would be a waste of time. Please instead use the content-part for selecting the words for the “search” mode – that is how it was planned to be used.

Lastly, the language has not been checked by someone who is English spoken. Instead the text is written by a person who at his very best is speaking Swinglish. The written language could have been presented more properly by a real translater, but the cost for that luxury was too high. Accept therefore that there is a place also for “bad English” in science as long as it can be understood. Maybe some of the sentences or words make you smile a little, but remember than that my Swedish probably still is much better than your English …

This encyclopedia shall be looked upon as a “living” book. This means that it will continuously be added new articles to as they are published and get available – and I hope this can be continued for the foreseeable future. Then I re-edit it by putting articles in a place where they better fit, put in new subheading etc. the most difficult thing is then to compress the text and take away som of what is said twice, three times or more. I love every word, and cry a little bit inside when I have to omit a sentence here or there, just as if they were my friends. Despite that, the compression is in the end necessary and will be done. Gradually, but not without tears. However, the reference list will never have any references taken away, as the meaning of the encyclopedia is to direct the reader to the full articles.

There are chapters that are more difficult to handle regarding re-editing than others. The worst part is by far those that deal with laboratory techniques. The author have difficulties in understanding the issues and even more difficult to classify the different techniques. Also, the chapter on ethical question is difficult to handle, but for another reason: ethic has so little facts and som much of it needs a lot of words – unless you try to describe ethics in black and whit (which this reviewer refuses to do). So, for the moment you have to stand with these drawbacks, or help the reviewer to come up with something better.

This means that the plan is to follow up with quarterly reports during coming years (it is then the quarter when the review was made available through PubMed that counts, not the month it was actually published) and then to include the reports from each quarter in an annual report that is then taken into this encyclopedia – which then is gradually re-edited. This also means that there is probably always a later version by the author than that you have in your hand now. However, if you send me a mail I am happy to send you that latest (not the last) version.

Is this all that has been published on doing? No, certainly not, there is much more out there. However, what is gathered in this book is probably a good deal of what has been published as full articles (no abstracts included), and hopefully all that has been of real importance. If someone find more that should be included, please let me know. If we are of the some opinion I will of course include it as soon as possible.

It I probable that my reporting system can be presented better than what is done here is, but how this is done is not so easy for the author, who like the way he has done it. So, welcome with comments, criticism, or cheers – but if they fail to appear, the next quarters, the next annual report, and the encyclopedia will have the same dispositions as the present. This means that also you have a responsibility for the future!

Welcome to contact!
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EDITOR’S PREFACE (AND SEARCH ALGORITHM)

ABBREVIATION

BACKGROUND FOR TODAY’S DOPING PROBLEMS
Definitions of doping
A background for understanding performance-enhancing drugs in sports
Judging cheaters in different domains

Overview
Little symptoms and signs
Socio-psychological background to doping
To move borders …
Much to win

Ergogenic potential of AAS
Testosterone/epitestosterone (T/E ratio)
Isotope ratio mass spectrometry (IRMS)
Synthetic androgens
Androgen bioassays
Indirect androgen doping
Estrogen blockers
Androgen precursors
Gonadotropins
Boosting in paraolympics
Possible lack of effects of doping
Cognitive doping
Brain stimulation techniques
What can be done with brain stimulation?
Can it be detected? What are the risks?
Ethical issues

Effect of international results during the anti-doping era
100 meter and 5000 meter
100 meter in Olympic Games

Is doping-free sport a Utopia?

DOPING AND ANTI-DOPING HISTORY
Overview
Specific dates
Early (modern) history
History of anabolic steroids
Detection of testosterone
The era of anabolic steroids
US professionals
Anabolic steroid prodrugs in the US
Designer drugs
BALCO

History of blood doping
Early testing strategies for blood doping
History of detection of recombinant erythropoietin and derivates
Background to the athlete biological passport
History of doping with growth hormone
History of doping with caffeine
History of doping with ephedrine
History of doping with cannabis
History of doping with alcohol
History of doping with gamma-Hydroxybutyric acid (GHB)
History of biomarker approach regarding doping
History of gene doping
History of athlete biological passport
Doping in the Olympics
   The ancient Olympic games
   Olympic nationalism
   The first Olympic tests
   Doping during the modern Olympics
   The Olympics in medical journals
   Canada
Introduction of growth hormone
Formation of the IOC Medical Commission
Formation of the World Anti-Doping Agency
Formation of Court of Arbitration (CAS)
Out-of-competition testing
History of therapeutic use exemptions (TUE)
   Anorchia
   Aplastic anemia
   Congenital adrenal hyperplasia (CAH) and hypogonadism
   Stimulation medication
   Danazol
   Glucocorticoids and diuretics
   Beta-blocker
   Recognising the concept of TUEs
   Recent Olympic Games
Legislation
   USA
Industrialized doping
Laboratory testing
   Anabolic steroids
Addendum to the “Prohibited list” in 2011
The 2011 WADA list
The 2012 WADA list
The 2013 WADA list
   Category 0
   Monitoring program
Arne Ljungqvist

ORGANISATION OF ANTI-DOPING
An international background to the anti-doping movement
   Indirect evidence of doping effects: world records
World Anti-Doping Agency (WADA)
   Pre-WADA history
   The code
   The prohibited list
   Sanctions of violations
Anti-doping rules
The rules
Strict liability rule
The whereabouts rule
Separation of power
Non-approved substances

Therapeutic use exemption (TUE)
Abbreviated TUE
Standard TUE
Therapeutic use exemptions (TUEs) at the Olympic Games
Prevalence of use of TUEs in asthmatics

Prioritization in anti-doping
Strategy to reduce illicit drug use in football
Agreements with the pharmaceutical industry

Doping controls in practice
International Sport Federations in the protection of the athlete's health
During the Olympics
Biomarkers
"Exercisemonomics"
Multi-class and multi-analyte test methods
Statistics

Organization in football
FIFA
FIFA Medical Assessment and Research Centre (F-MARC)
FIFA's approach to doping in football.
Future challenges

WADA-accredited doping laboratories

**EPIDEMIOLOGY OF DOPING**
Estimated number of unreported cases
Doping in the community
Epidemiological confounding factors and false consensus effect (FCE)
Elites
Use of drugs during the Olympics
Student athletes
Adolescents
Versus those not participating in sports
Disableds in sports
Issues in paraolympics

Gym users
State-based sports institute
Pharmaco-epidemiology of anabolic steroids
Comparison with a prison population
Drug Information Database
Different countries
Sweden
Denmark
Norway
Finland
UK
Greece
Italy
France
Different sports
Prevalence of use depending on type of sports
Professional ballet dancers
Track and field
Football
Tennis
Table tennis
Combined racket sports
Biking
Bodybuilding
Dancesport
Recreational sports
Prevalence of use by fitness centre members

THEORETICAL ASPECTS ON DOPING-TESTING

Overview
Theories on laboratory testing
Homo economicus: pay-offs and sanctions
Aim of anti-doping
The Goldman dilemma
Forensic intelligence in anti-doping
Colored illicite tablets
Theories on doping in sports
Doping detection
Doping prevention
Explaining the doping behavior
Multiple drug use
Co-operation in drug testing
Methodology for investigation of doping in the society
A support vector machine
Performance profiling
Controls at random
Errors in drug testing
Incongruity of data
Theoretical testing
Self-reporting
Testing as a way of decreasing intent for doping
Fatigue as a limit for test performance
Economical aspects
Chemoinformatics-based classification of prohibited substances
Need of excretion studies
Preanalytical variability
Chemical and physical manipulations of doping tests
Designer drugs in Japan
Testing efficiency
Anti-doping research opportunities
Economy
Measurement uncertainty in anti-doping quantitative analysis
Placebo
  Medical history of placebo
  Powerless placebo
  Lack of effect of flavor
  Ethical issues
  Concluding remarks on placebo
Statistical aspects
  Wald test
  Bayesian statistics
  Decision limit (CCalpha) and detection capability (CCbeta)
Prediction of future doping

ATHLETE BIOLOGICAL PASSPORT (ABP)
Overviews and background
Individualized statistics
Personalized monitoring of biomarkers for doping
  Technical specifications necessary
  Still an interpretation
An international tool
Three complementary items of ABP
Hematological parameters
  Hemoglobin mass
  Animal studies
  Biking
  Possible variables
Erythropoietin
Steroid profile for athlete biological passport
Confounding factors
  Plasma osmolality
  Stability of athlete blood passport parameters during air freight
  Gastroenteritis
Also for research
Law issues
Software
Passports in practice
  Erythropoiesis stimulating agents
  Testing during cycle tournament
  Positive cases in biking
Continuous evaluation of the ABP

SOCIO-MEDICAL ASPECTS ON DOPING
Semantics in doping
Political and economical aspects of anti-doping
Social and socio-medical issues
  Social psychological determinants
  Psychological background
Subjective effects
Expectation of the doped
Rejuvenation
Interaction between athletes and coaches
Sports addiction
Motivational and social cognitive predictors of doping
Motives for use
Elite athletes' attitudes, beliefs, and knowledge
Attitude of elite cyclists on doping
Young athletes attitudes towards doping
Other athletes' attitudes regarding doping
Attitudes against dopers
Body image and attitudes toward male roles
Polypharmacy
Concomittant use of other substances
Neuropharmacy addiction
Connexion to use of dietary supplement
Complementary and alternative medicine (CAM)
Connexion to eating habits
Concomittant symptoms and signs
Performance versus recreational drugs
Health promoting effects of sports
Lifestyle alteration
Prediction of later life-style
Risk factors for doping
Psychiatric comorbidty diseases
Sponsoring of sports
Information to dopers
Medical practitioners' knowledge, attitudes and beliefs
Contagiousness of doping
Economical aspects
Importance of sports medicine as a medical speciality
Impact of national programs
Staffing protocols in high-performance sport
Law issues
Doping + violence
Knowledge in different countries
Illicit drugs around an Olympic game
US position stand on androgen and human growth hormone use

Influence of religion on use of doping
The adolescent athlete
Availability of illegitimate drugs in the society
Internet drug availability
Psychoactive drugs in the society
Organised crime and drugs in sport
Legitimate use of drugs in sports
Over-the-counter medicine
Doping agents as medical treatment
Pharmacists
Compared to general practitioners
Pharmacy students

A cancer risk of doping?
Dependence in clinical practice
Treatment of drug addicts
Educational programs
Social perceptions
Medical risks with illegal drugs
Doping and the respiratory system
Information on doping
    Internet
    Telephone hot-line
    Media medicine
An integrated approach

INADVERTENT DOPING
Sibutramine
Methylhexaneamine
Illicit blue tablets containing anabolic androgen steroids
Dietary supplements containing prohibited anabolic agents
Dietary supplements contaminated with prohormones
Dietary supplements with clenbuterol
Dietary supplements containing prohibited peptide hormones
Designer drugs
Emerging drugs

PREVENTION OF DOPING
Athletes Targeting Healthy Exercise and Nutrition Alternatives (ATHENA)
Telephone counseling
National policy against doping
    Sweden
    Brazil

OVERVIEWS OF GENERAL LABORATORY TECHNIQUES
Olympic laboratories
Practical testing in Brazil
Transportation
Quality of doping testing
Blood sampling and blood samples handling
Handling of urine
    Stability of doping substances in urine
    Diluted urine
    Urinary screening
    Direct injection of urine
    Proteases in doping control analysis
Forensic toxicology
Testing in famous cases
    Katrin Krabbe et al
    Lance Armstrong
Parallel investigations of saliva and urine
    Effects of sample storage condition on salivary hormones
Non-approved substances
Screening methods
    Multi-analytetesting
    “Alternative” specimens
Drug identification
    Blood tests
Laboratory report interpretation
Accuracy of testing
Chromatography
Mass spectrometry
  Small peptides
  Isotope ratio mass spectrometry or carbon isotope ratio
  Vacuum MALDI-linear ion trap mass spectrometry
  Benchtop quadrupole-orbitrap hybrid mass spectrometry
  Chromatographic-mass spectrometry
Liquid chromatography
  Hydrophilic interaction liquid chromatography
  Ultra-high-performance liquid chromatography
  Fast liquid chromatographic/mass spectrometric screening
Liquid chromatography tandem mass spectrometry
  Liquid chromatography-mass spectrometry (LC-MS)
  Metabolite-based liquid chromatography-mass spectrometry
  High-resolution/accurate-mass LC-MS
  With information dependent acquisition
  Liquid chromatography/time-of-flight mass spectrometry
  High performance liquid chromatography retention time of small molecules
  Nano-liquid chromatography/benchtop quadrupole orbitrap tandem-mass spectrometry
Liquid and gas chromatography time-of-flight mass spectrometry
Gas chromatography
  Gas chromatography-microchip atmospheric pressure photoionization-mass spectrometry
Gas chromatography-mass spectrometry
  Gas chromatography-combustion-IRMS (GC-C-IRMS).
  Gas chromatography-triple quadrupole mass spectrometry
  Gas chromatography-QqQ-MS
Carbon isotope (CIR)-based analyses
  IRMS (isotope ratio mass spectrometry)
Isoelectric focusing
Electrospray ionization
Capillary electrophoresis
  Capillary electrophoresis time-of-flight mass spectrometry
Full-capillary sample injection combined with sweeping CE stacking
Hyphenated mass spectrometric techniques
siRNA
Mass spectrometric detection of siRNA
High-resolution liquid chromatography-time-of-flight mass spectrometry
Ultra-high-pressure liquid chromatography-quadrupole time-of-flight mass spectrometry
Isotachophoresis sample stacking
Polar organic chemical integrative samplers (POCIS)
AICAR
Microwave assisted extraction
  Ultrasound and microwave
Liquid/liquid extraction
Two step derivatization
Adsorption to metallic plasmonic nanoparticles
Surface plasmon resonance
Isotope ratio mass spectrometry
ETD and CID tandem mass spectrometry
Protein chips
SIRT1-activating drugs
Bioassay-guided fractionation
In vitro studies
Mammalian reporter gene bioassays
Transcriptome analysis
Compound-specific isotope analysis (CSIA)
Dual-color bioluminescent bioreporter
Two-dimensional gas chromatography with heart-cutting
RNA sequencing
Hydrogen isotope ratio of urinary steroids
Dried blood spots (DBS)
A new hyphenated mass spectrometry
mRNA transcripts
Deuterium/hydrogen ratio
Quantitative structure-retention relationships
Solid phase extraction (SPE) procedure
Solid-phase microextraction
Yeast analysis
Yeast transactivation system
Yeast and mammalian cell-based androgen bioassays
Laser desorption
Non-target metabolomics
FCMIA
In hair
Hair and saliva
Finger nails
In sweat
The potential role of oral fluid in antidoping testing
Virtual screening
Artificial networks
Appearance and Performance Enhancing Drug Use Schedule (APEDUS)

DETECTION OF DOPING AGENTS IN ENVIRONMENT, FOOD AND FOOD SUPPLEMENTS
Analytical strategies
Metabolomics
Screening for hormone residues in drug residues
Anabolic steroids
Nandrolone
Trenbolone
Methenolone acetate in a veal calf
Sexual behavior of fish after trenbolone
Growth hormone
Growth promoters given to livestock
Beta-agonists in pork
Clebuterol
Clenbuterol in muscle
Clenbuterol in pig retina
Clenbuterol in milk
Screening of residues in food
Egg
Bovine
Swine
Fish
Detection of doping agents in waste water
Urine from urinals
Rests in other parts of nature
Beef palatability
Laboratory techniques
In meat
In faeces
Liquid chromatography tandem mass spectrometry
High- and low-resolution mass spectrometry

ANABOLIC ANDROGENIC SUBSTANCES
Overviews
Research activity
Clinical use
Hereditary angiooedema
Effects of androgen deprivation
Abuse
Methods of abuse
Abuse dosage
Recognizing steroid abuse
Different actions due to ways of administration
Different anabolic androgenic steroids with different specific actions
Possible use in children and adolescents
Clinical effects
Prevalence of adolescent anabolic-androgenic steroid use
Use of testosterone precursors by adolescents
Use in women
Prevalence
Effects
Steroid precursors (androstenedione and dehydroepiandrosterone)
Anabolic steroids in young females
Estrogen antagonists
Genetic influence
Doping tests and genetic confounders
UDP-glucuronosyltransferase and UGT2B17 genotype
Genetic polymorphism
Association with renal disease and gene polymorphism
Chemistry
Steroid hormones
Testosterone and its modifications
General metabolism
Steroids from musk deer
Physiology
Mechanism of action
Chemical structure versus function
Myotrophic action of androgens
Endogenous androgens
Androgen disposition and genetic variation
Steroid hormone binding globulin (SHBG)
Hydroxysteroid dehydrogenase
Modulation of follistatin and myostatin propeptide
Chromosomal damage
Androgen receptors
Androgen receptor polymorphism
Antagonists of the androgen receptor
Physiological and clinical effects and side effects
Commonest AASs in use worldwide, according to main effect
Molecular function
Physiological cellular effects of androgens
Episodical secretion
Circadian rhythm
Endogenous steroids
Effect of smoking
Influence of alcohol on steroid metabolism
Effects of dietary components on testosterone metabolism
Testosterone plus an ornithine decarboxylase inhibitor
Salivary hormones
Salivary testosterone
Effects on cognitive functions
Effects on training
Morphology versus performance after use of anabolic steroids
Effects on training in males
Effects on training in females
Effects on stress
Effects on muscles and tendons
Influence on immunological function
Influence on endurance
Influence on strength
Influence of sprint
Influence of bodybuilders' fasting periods
Flywheel ergometer workouts
Watching a previous victory
Effects of magnesium supplementation
Effects of training on salivary levels
Effects of androgens on IGF-1
Interactions with opioids
Induction of nitric acid
Lack of influence of NSAID
Longitudinal steroid profiling
Long-time effects of anabolic steroids
Multi-parametric steroid profiling
Prevalence of misuse
Dependence
Overview of detection of AAS abuse
Ratio between testosterone and epitestosterone
Specific laboratory techniques for anabolic steroids
Bayesian based screening
Purity certified reference materials
Steroid profiling
Stacking method of repetitive large volume sample injection
ELISA
Liquid chromatography
Gas chromatography-triple quadrupole mass spectrometry
Liquid chromatography-tandem mass spectrometry method (LC-MS/MS)
Ligand and structure-based virtual screening
3-Oxo-steroidal agents
Stable isotope dilution liquid chromatography electrospray ionization
Microflow tile technology and LC-MS/MS
Effects of sample storage condition steroid hormones in saliva
IRMS
Gas chromatography coupled to IRMS
Gas chromatographic/time-of-flight mass-spectrometric
Carbon isotope ratio (CIR)
Capillary electrophoresis
Prediction of metabolic pattern of new derivatives of AAS
Mass spectometry
GCxGC-TOFMS
UHPLC-HRMS
Single-stage-Orbitrap-MS
Full-capillary sample injection combined with a sweeping CE stacking method
Oxidizing adulterants’ effect on the steroid profile of human urine
Electrospray ionization tandem mass spectrometry (ESI-MS/MS)
Glucurononoconjugated metabolites
Relative retention times
Variability in the $^{13}$C/$^{12}$C ratios
Principal components analysis
Androgen receptors assay
Androgen bioassays
Protein assays
Enzyme-immunoassay kit
Triptorelin test
Serum inhibit B as a potential marker of testosterone doping
Two-dimensional gas chromatography
Designer drugs
Molecularly imprinted polymer filaments (MIPFs)
Urinary steroids
In saliva
In hair
Faecal analyses
In food
Musk extracts
A user-friendly library
Reference values from South America
Experimental
Markers for anabolic steroids
AICAR
Activity on carboanhydrases
Purchase over the Internet
Anabolic steroid use and condom use
Case reports
Experimental

- GABA type A receptors
- Effect of subcutaneous testosterone on emotionality
- Effect of testosterone in castrated guinea pigs
- Apoptosis and NOS2 (nitric-oxide synthase 2)
- Androgen-induced cardiac autonomic dysfunction
- Interaction of testosterone with cocaine

SIDE EFFECTS OF TESTOSTERONE AND OTHER ANABOLIC STEROIDS

Overviews
- Short-term side effects
- Anesthesia risk

Different effects of different anabolic steroids

Toxicokinetics
- Impurities in illicit samples of anabolic steroids

Anabolic steroids’ impact on the cardiovascular system

- Overview
- Metabolic syndrome
- Coronary artery calcifications
- Myocardial infarction
- Cardiac arrhythmias
- Maximal heart rate
- Heart rate recovery
- Myocardial fibrosis caused of anabolic steroids
- Left ventricular myocardial dysfunction and cardiac hypertrophy
- Cardiac (autonomic) dysfunction
- Vascular reactivity
- Cholesterol changes
- Impaired exercise-induced cardioprotection of antioxidant enzymes
- Sudden death
- Altered lipid profile
- Abnormal plasma lipoprotein
- Trombocyte function
- Hypercoagulability
- Arterial thrombosis
- Thrombosis
- Pulmonary embolism
- Endothelial cells
- Increased ilintima-media thickness
- Aortic elasticity
- Arterial hypertension
- Other vascular effects
- Experimental
- Cardiovascular effects of low androgens
- Summaries of effects of anabolic steroids on the heart

Increased risk of diabetes

Effects on the brain
- Brain development
- Cerebral infarction
- Brain nerve growth factor
- Decreased memory
- Mania
- Effects on GABA
Rewarding systems
Monocygotic twins
Sleeping pattern
Psychologic and psychiatric effects
Agression and violence
Association with criminality
Suicide risk
Addiction
Withdrawal effects
Treating psychiatric effects of steroid use
Cognitive deficits

Liver changes due to sex hormones (anabolic steroids and oral contraceptives)
Overview
Metabolism of anabolic steroids in the liver
Enzyme elevations
Cholestatic liver disease
Non-alcoholic fatty liver disease (NAFLD)
Peliosis
Hepatocellular adenoma and carcinoma
Hepatocellular necrosis.
Spontaneous hepatic rupture

Anabolic steroids and reproductive system and male infertility
Physiology of reproduction-endocrinology
Anabolic steroid-induced hypogonadism in young men
Azospermia
Histopathology
Impact on semen quality
Aneuploidies and ultrastructural changes in spermatozoa
Case report

Reproductive-endocrine effects in women
Skin
Acne
Hirshutism
Androgenic alopecia (baldness)

Effects on the immune system
Infection risks after injections of anabolic steroids
Injection policies

Bone
Effect on bone density

Influence on muscle and tendon injury and injury healing
Muscle healing in power-lifters
Tendon adaptation
Compartment syndrome in upper limb

Rhabdomyolysis
Renal effects
Gynecomastia
Other side effects
Thyroidal effects
Hematological
Hypercalcemia
Effect on gingival tissues
Effect on inflammation
In basket ball players
Side effects in elderly
Other infections
Genotoxicity (cancer risks)

Fatal events
Multiple organ failure

Side effects of topical anabolic steroids
Influence on reaction on pain
Long-term effects on social-medicine demography
Swedish data
Increased mortality in former users of anabolic steroids
Tour de France (1947-2012)

TESTOSTERONE
Theoretical, overviewing, aspects
Normal values

Problems in evaluating serum testosterone values
Half life
Testosterone/epitestosterone concentration ratio (T/E)
Free testosterone/cortisol ratio
Time-course of testosterone action
Stability in the urine
Testosterone prohormones
Seasonal variations
Ethnic differences in steroid-related diseases
Urinary levels of testosterone and epitestosterone in a Korean male population
Status of lean elite athletes
Levels in male Olympics
Older men
Women
Testosterone after use of chlorinated swimming pools
Testosterone prohormones
Salivary testosterone (and testosterone to cortisol ratio)

Biological action
Hypothalamic-pituitary-gonadal (HPG) axis
Brain
Heart
Tendon
Muscle
Breast
Liver
Testosterone deficiency
Opioid-induced androgen deficiency (OPIAD)

Effect of magnesium on testosterone levels
Testosterone and motivation to compete
Psychological influence on testosterone levels
Influence of psychosocial environment
Victory or defeat
Home or away game
Spectators

Influence of stress on testosterone (and other anabolics) levels
Hormone profile in men
Hormone profile in women
Influence of fasting (Ramadan)
Circadian rhythm
Genetic influence (polymorphism)
Influence of exercise on testosterone levels
   In trained and in not-trained
   Effects of hard exercise
   Effect of endurance training
   Effect of resistance training
   Effect of different types of training
   Effect of different short-time types of exercise
   Explosive performances
   Active recovery versus passive recovery
   Influence of red color
   Pre-game free testosterone concentrations and outcome
   Pre-game testosterone level: home advantage
   Effect of testosterone on myoblasts
   Testosterone levels after concussions
   Effect of soccer
   Effect of golf
   Overreaching
   Older men
   Females
   Effects in a young female
   Adolescents
   Biking
   Response to marathon running
Effects on immune system
Testosterone and age
   Aging athletes
Effects of long flights
   Influence of space flights
Effects of diet on testosterone metabolites
   Influence of zinc
In hypogonadal men
Testosterone level as an indicator of gender
Transdermal testosterone
   Topical preparations
   Transdermal preparations
   Gels and solutions
Effect of postexercise ethanol ingestion
Effect on cocaine’s vascular effects
Interaction with NSAIDs
Influence on gastric ulcer precursors
Side effects
   Cardiovascular disease
   Diabetes
Laboratory techniques
   Testosterone versus epitestosterone
   TLC-densitometry method
   Mobility spectrometry separations
   Analysis of the transcriptome
Experimental

NANDROLONE
Physiology

Effects on mitochondria
Effect on dynorphin A in the brain

Metabolism

Protein disulphide isomerase (PDI)
Diagnostic metabolites
Effects of the menstrual cycle
Influence on hypothalamic-pituitary-adrenal axis

Effect on muscles
- Effect of training
- Effect on healing of muscle injuries
- Positive effect on nerve regeneration

Side effects
- Effect on male fertility
- Cardiotoxic effects
- Harmful effects on brain axons
- Harmful effects on learning capacity

Effect on hypertension
Effect on peripheral nerve injury
Effect on growth hormone
Effect of small doses on stress response
Aortic adaptations to exercise
Nandrolone-mediated testosterone reduction during alcohol intoxication
Impact of nandrolone on biosynthesis of steroids
Effects of nandrolone on recovery of denervated muscle

Genotoxic effects
Upregulation of aromatase expression
Endogenous or exogenous origin of nandrolone
Significance of 19-norandrosterone in athletes' urine

Laboratory techniques
- Liquid chromatography/tandem mass spectrometry
- Cyclic, differential pulse and square-wave voltammetry
- Gas chromatography-tandem mass spectrometry

Experimental

**BOLDENONE, BOLDIONE AND BOLANDIOL**

Overviews
Boldenone potency compared with other anabolic steroids
Effect on reproductive functions
Possible endogenous origin
Urinary samples contaminated with faecal boldenone
Influence of renal function
Metabolism of 1-ene-steroids
- Stenbolone
- Quinbolone
- Boldione

Experimental
Laboratory techniques
- Conjugated and unconjugated
- In cattle

**DEHYDROEPiANDROSTERONE (DHEA)**

Physiology
Metabolism
Molecular and cellular mechanism of DHEA
Effects of DHEA on body composition, bone metabolism, and skin
Role of DHEA in sexual function

Normal values related to age
Influence of menstrual cycle
In women

Efficacy in performance enhancement
Effects of walking training

Effects on asthma
DHEA metabolites activate estrogen receptors
DHEA in vascular disease

Adverse effects
5-androstene-3beta,7beta,17beta-triol
Laboratory techniques
Current medical recommendations

OTHER SPECIFIED ANABOLIC ANDROGENIC STEROIDS
Oxandrolone
Stanozolol
Methyltestosterone
Biomarkers
Side effects
Laboratory techniques
Desoxymethyltestosterone (DMT)
Metabolism
Methyltestosterone
Testing
Fluoxymesterone
Dihydrotestosterone (DHT, androstanolone)
Dehydroepiandrosterone
Diurnal secretion
Deposterone
Dromastanolone
Adrenosterone
Androstenediyl
Metabolism of 1-androstenediol and 1-androstenedione
3alpha-Androstanediol
Androstenedione
Hydroxyandrostenedione
Androst-4-en-3-one-based steroids
Dehydrochloromethyltestosterone
Fluoxymesterone
Desoxymethyltestosterone
Keto-androgens
Trenbolone
Experimentally
1-Testosterone
Metabolism
Metenolone
Metandienone
Metabolism
Laboratory tests
Mestranolone
Norbolethone
Phytosterols
Phytoecdysteroids
17-hydroxyandrosta-3,5-diene ("Syntrax Tetrabol")
Yeast transactivation system
Designer steroids
Tetrahydrogestrinone (THG)
YK11
Delta6-methyltestosterone
Methylstenbolone
Methyl-1-testosterone (M1T)
Laboratory techniques
Reductase inhibitors
Finasterid
Aromatase inhibitors
Exemestane
Anastrozole and exemestane
Formestane
Metabolism of keto and hydroxy steroids
Dehydrogenase inhibitor
Transsexuality

SELECTIVE ANDROGEN RECEPTOR MODULATOR (SARM)
Background
Steroidal ligands and their clinical applications
Arylpropionamide-based SARMs
SARMs produced by fungus
Andarine
New SARM compounds
Laboratory techniques

ESTROGENS AND FEMALE SPORTS
Ethnic variations
Urinary estrogens and androgens during pregnancy
Endogenous versus exogenous estrogens
Sex-hormone binding globulin
Testosterone
Prolonged aerob exercise
Urinary steroids in exercise
Salivary testosterone in exercise
Hyperandrosteronism in women
Effects of menstrual cycle
Effects of exercise on the female reproductive system and sex hormones
Effects of strength training
Comparison of baseline free testosterone between elite and non-elite female athletes
Effect of stretching
Effect of estrogens on muscle regeneration
Influence of IGF-1
Influence of age on muscle mass in women
Gestrinone
Estrogen blockers
Effects in men
Experimental
Oral contraceptives
  Effect of bone mass
  Influence of oral contraceptives on tendons and ligament
Influence of SERMs on steroid metabolism
Folinic acid supplementation
Laboratory techniques
  Selective estrogen receptor modulator (SERM)
    Toremiphene
    Raloxiphene
    Laboratory techniques

AROMATASE INHIBITORS
  Tamoxifen
  Exemestane
  Aminoglutethimide
  Formestan

BLOOD DOPING
  Overviews
  Prevalence of blood doping
  Biochemical markers of hypoxia
  Theoretical aspects on energy transfer in the body
    Hemoglobin mass and physical performance
Erythropoiesis
  Effects of marathon
  Effects of training in juniors
Red blood cells
  Effect of iron on performance
  Testing of blood
  Freezing red blood cells
  Effect of endurance training on erythrocyte deformability
Hemoglobin and hematocrit
  Seasonal variations of hemoglobin
  Regulation of red blood cell mass
  Circadian rhythm
  Stability of hemoglobin under testing conditions
  Statistical models for blood cell survival
  Preanalytical mixing of whole-blood specimens
  Blood parameters during endurance exercise
  During flights
Total hemoglobin mass
  Within-subject variation in hemoglobin mass
  Influence of exercise
  A stable parameter
  Influence of travelling
  Influence of erythropoietin
  Laboratory technique
Reticulocytes
  Sports anemia
  Influence of erythropoietin
  Blood drawing
Measurement
diurnal variation
biological variability
kinetics of reticulocytes production in humans
reported reticulocyte values in athletes
gender effects on reticulocytes
stability of reticulocytes
effects of exercise on reticulocytes
reticulocytes in doping
neocytolyis

polycythemia
blood conservation and transfusions
blood transfusions in general
autologous transfusion
adverse events
plasma markers for testing
screening for homologous blood transfusions
screening for autologous blood transfusions
mRNA
dNA testing
response to foreign erythrocytes
effects of blood withdrawal and reinfusion on biomarkers
laboratory problems
robustness of measurements after storage of blood
reticulocytes
whole blood transfusion
detection of homologous blood transfusion
capillary electrophoretic separation
intravascular hemolysis
evaluation of blood parameters

erthropoietin (EPO)
overview
physiology
different ways of action
erythropoietin and the heart
a neuroprotective agent
neovascularization
psychological effects
influence of N-acetylcysteinin
interactions with androgens
effects of exercise on erythropoietin molecules
effect of endurance training
effect on performance
effects of erythropoietin on serum hepcidin and serum iron bioavailability
new biomarkers
theoretical aspects of detection blood doping with erythropoietin
micro dosing
excessive dosing
enzymatic desialylation
laboratory techniques
hepcidin as a marker
microRNAs
confounding factors for erythropoietin detection
Available erythropoietins
CERA, a continuous erythropoietin receptor activator
Darpoietin/NESP
Epoetin delta (Dynepo®)
Hematide®
Non-EPO-related erythropoiesis stimulating agents
Synthetic peptide-based EPO receptor agonist
EPO-Fc fusion protein
New erythropoietin-like drug principles
Adverse effects of erythropoietin
Novel erythropoietin doping strategies
Detection rate
Detection of misuse of erythropoietin in competitive sports and laboratory testing

Effects outside the bone marrow
Economy
Experimental

Blood substitutes, in general
Perfluorocarbon emulsions
Efaproxiral
Ex vivo erythrocyte generation
Irradiation of red blood cells
Enhancement of oxygen transfer
2,3 DPG
Hypoxia-inducible factor
Hemoglobin-based oxygen carriers (HBOCs)
Allosteric modulators of hemoglobin
New products
Laboratory techniques
Hemopure®
Hematide/Peginesatide
Hypoxia-inducible factor (HIF) stabilizers
Cobalt salt as erythropoietic agent

Phthalates
Granulocyte colony-stimulating factor (G-CSF)
Other blood products
Actovegin

Intravenous fluid use in athletes
Discussion on dangerous dehydration in sports
Oral versus intravenous fluids
Exertional muscle cramp prevention
Preexercise fluid requirements
IV volume expanders
Rehydration

Plasma volume expanders
Dextran and hydroxyethyl starch
Hydroxyethyl starch (HES)
Glucose solution
Saline solution
Ringer lactate (or acetate) solution
Hypertonic crystalloid solution
Albumin
Plasma protein fraction
Plasma
Gelatins
alpha-Keratose
Combined solutions (hypertonic crystalloid/colloid)
Pullulan
Levan
Acetyl starch
Polyvinylpyrrolidone
Mannitol
Glycerol
Polyethylene glycol
Newer plasma expander

Intravenous rehydration
Hyponatremia

CREATED HYPOXIA
Nomenclature
Physiological mechanisms
Cellular mechanisms
Training at high altitude
Recovery
Effects on sleep

Hypoxic tents
Living high, training low
Short-term normobaric hypoxia

Effect of intermittent hypoxic training on cycling performance
Effects on laboratory parameters of intermittent hypoxic training
Position statement on hypoxic training

Aviability of Hb_{mass} in altitude training
For players of which team sports might altitude training be relevant?
What is the optimal altitude dose to be used?
Correlation between Hb_{mass} and altitude training result
Physiological markers worthwhile monitoring to identify altitude responders?

The ethics of hypoxic training

PLATELET-RICH PLASMA
Platelets in PRP
A randomized study
Rotator cuff
Tendopathies
Hamstrings
The knee
Achilles tendon
Muscle injuries

GROWTH HORMONE
Overviews
Recombinant human growth hormone
Epidemiology and demographic factors
Adolecents
Children
Women

Prevalence
Side effects

Physiology
- Molecular and metabolic mechanisms
- Promoters of secretion
- Influence of nutrition
- Influence of testosterone on GH
- Immunofunctional and traditional growth hormone
- Effects on circulation
- Fett-substrate metabolism
- Glucose-substrate metabolism
- Effects on muscle mass and strength,
- Body fat, extracellular water, and thermoregulation.
- Interactions with thyroid hormones and sex steroids
- Hypothalamo-pituitary-adrenal (HPA) axis
- Growth hormones secretagogues
- The GH-IGF-I axis and exercise in normal subjects
- Supraphysiological GH and exercise performance
- Influence of training on GH response
- Influence of carbohydrates and proteins
- Influence on glucose homeostasis
- Influence on cardiovascular system
- Effect on IGFBP-4 and -5
- Influence of alcohol
- Effects on other variables
- Effects on respiration

Side effects
Clinical use
- Delivery methods
Use in weightlifters
- Biomarkers for use (other than IGF-1)
  - GH2000
  - Intra-individual variability
  - Effect of training of the analyse results
- Theories for detection of doping with growth hormones
  - Isoform approach to detection abuse
Possible ethnic differences
- Pharmacokinetics and pharmacodynamics of GH
Metabolism of GH
- Effect on red blood cells
- Positive association with estrogens in men
- Negative effects on collagen synthesis
- Idiopathic adult growth hormone deficiency
- Effect on memory
- Gene expression in peripheral blood
- GH-receptor antagonists
- Growth hormone releasing peptides
Legal framework
- Effect of dietary supplements on GH levels
- Effects of hypertemia on GH levels
- Effects of exercise on GH levels
  - High intensity versus high volume endurance training
  - Eccentric exercise
  - Effects in immobilisation
Effects of GH on performance in sports
   Endurance
   Resistance exercise
   Sprint
   Muscle strength
   With and without carbohydrate + protein
   Slow eccentric exercise velocity
   Tendons
   Meta-analysis
   In women

Impact of injuries
Boxers
Horse growth hormones
Detection of different brands
Laboratory techniques
   The isoform methods
   With nano-technology
   Stability of GH during testing procedures
   Monoclonal antibodies
   Electrophoresis and mass spectrometry
   Immunoaffinity purification
   Immunoassays
   Freeze-thaw cycling
   Intra- and inter-laboratory validation
   Tests for GH deficiency

Ethics of use of growth hormone in sports

INSULIN-LIKE GROWTH FACTOR I (IGF-1)
Polymorphism
Physiology
In peripubertal females
Effects of training
   Resistance exercise
   Training with calorie restriction and sleep deprivation
   In Kenyans
Local administration
IGF-1 in deer antler
Markers for IGF-1
Laboratory techniques
Detection
   Variation in measurements
   In saliva

INSULIN
Amino acid-stimulated insulin secretion
In weightlifting
   Effects of leucine on post-exercise muscle protein synthesis
   Protein hydrolysates
   Whey
   Effects of insulinotropic nutritional mixtures on insulin secretion
   Effects of insulinotropic nutritional mixtures on post-exercise muscle anabolism
   Protein shake
   Effects of post-exercise hyperinsulinaemia on fat oxidation and de novo
Anti-inflammatory effects of insulin
Laboratory techniques

**OTHER DEFINED PEPTIDE HORMONES**

*Gonadotropins*
- Human chorionic gonadotropin
- Chorionic gonadotrophin (CG) and luteinizing hormone (LH)
- Clomiphene
- Release of GnRH
- Luteinizing hormone
- Natural agonists

*Secretagogues: gonadotrophin-releasing hormone*

*Myostatin*
- Effect of psychological stress
- Experimental

*Mechanical growth factors*
- Stretching
- Antibodies against human MGF E-peptide
- Fibroblast growth factor (FGF) and mechano growth factor (MGF)

**AGRP**

**CAFFEINE**

*Overviews*
- History of coffee drinking
- Presence of caffeine in society
- Epidemiology of use
  - Self-reported consumption
  - Emergency medicine residents' use of psychostimulants

*Athletes' knowledge of effects of caffeine*

*Factors influencing serum caffeine concentrations*

*Bioavailability of coffee*

*Caffeine dosages versus effects*
- Duration of effect
- Administration mode
- Dosage versus endurance cycle time
- Use as a flavor
- Potential adverse effects

*Physiological effects of caffeine*
- Effect on EMG frequency variables
- Effect on energy expenditure
- Effect on glucose homeostasis

*Molecular effects*
- Blocking of adenosine receptors
- Coffee polyphenol caffeic acid

*Analgesic effect*

*Users versus non-users*

*Metabolism*

*Mobilization of fat*
- Effects on performance after a fat meal

*Effects on blood pressure*

*Effect on cardiac blood flow*
- Caffeine-reduced myocardial blood flow during exercise
Dose-dependent neuromuscular effects
Effect of caffeine on delayed onset muscle soreness
Effects on performance
  Effect versus measurable levels
  Effects on the brain
  Effects on mood and vigilance performance
  Effects on endurance
  Effects on short term performance
  Effects on skill performance
  Effects on resistance training
  Effects of performance of chronic use
  Effects on cycling
  Effects on muscles
  Effects of neural recovery
  Effects in children
  Effects on tennis
  Effects on rugby
  Effects on weight-lifting
  Effects on rowing
  Effects on iron-man
  Effect on field hockey
  Effect on sedentary men
  Effects on basket
Caffeine in central fatigue
  Ratings of perceived exertion
Caffeine levels before and after the removal of caffeine from the doping list
Caffeine in diabetes
Combination with creatinine
Combination with ephedrine
Caffeine versus pseudoephedrine
Combination with albuterol
Combination with sodium bicarbonate
Combination with sodium citrate
Combination with carbohydrates
  Caffeine at low muscle glycogen availability
  Caffeinated mouth-rinse
  Effect on individuals with negative energy balance
Combination with epigallocatechin
Combination with taurine
Combination with ecstasy
Caffeine versus theobromine
Caffeine in asthma
Influence on circadian rhythms
Influence on biomonitoring data
Impact on pain perception
Impact on testosterone levels
Impact in hot environments
Impact on immunology
  Immunoendocrine effects
Effect on hydration
Effect on urea formation
Caffeine with carbohydrates
Caffeine with phosphatidylserine
Lack of effect on oxidative stress
Modulation of oxidative stress markers in the liver of trained rats
Impact on the inflammatory response
Impact on glycogen accumulation
Impact on ventilation
Impact on sweating
Impact on delayed-onset muscle soreness
Impact on postexercise oxygen consumption
Impact on potassium levels
Impact on glutamine acid
Impact on sex-hormone binding globulin
Effects after a withdrawal period
Effects on arousal
Moderated effect of anxiety
Psychological effects
Caffeinated versus decaffeinated coffee
Hematological side effects
Experimental
Caffeine and taurine
Interaction with amitriptyline
Muscle metabolism
Effect of fat free weight
Practical recommendations
Caffeinated “energy shots”
Caffeinated chewing gum
Caffein gel
Guarana

OTHER STIMULANTS
Ergogenic effects
Theobromine
Theobromine and theophylline
Ephedrine
Chinese preparations
Use in women
Legal affairs
Physiological effects
Side effects
Ephedrine in sports
Laboratory techniques
Pseudoephedrine
Dose-response in cycling
Influence of preexercise food intake
Methylephedrine
Synephrine
Preynlamine
Octopamine
Dopamine
Phenylethylamine
Modafinil
Combined with cannabis
Combined with cocaine
Adrafinil
Armodafinil
Experimental
Methylphenidate
Transdermal
Laboratory techniques
Amphetamine
Side effects
Amphetamine in sports
Physiologic effects of amphetamine
Amphetamine in sport
In hair
Famprofazone
Laboratory techniques
Methamphetamine
Treatment of dependence
L-methamphetamine
Methyamphetamine
Mephentermine
Metamfepramone
Cathinones
Laboratory techniques
Methylenedioxypyrovalerone (MDPV)
Sudden death
Mephedrone
Long-term effects
A fatal case
Tuaminoheptane
Mesocarb
Sydnocharb
Strychnine
Befluorex
Laboratory techniques

SIBUTRAMINE

BETA₂ ADRENERGIC AGONISTS
Impact of WADA regulations
Finland
Therapy or doping?
   Improves swim ergometer sprint performance?
Doping efficiency of beta₂-agonists
Physiology
Asthma and sympathomimetica
Definition of asthma in sports
   Extended diagnostic criteria
Diagnosis of asthma
   How should bronchial hyperreactivity be defined?
   Testing of asthma in athletes
   Predictive values of tests
Asthma in sports
   Hard training as a cause of bronchial hyperreactivity
   Prevalence of asthma in sports
In elite swimmers
In canoe- and kayak athletes
Misdiagnosis of exercise-induced bronchoconstriction in professional soccer players

Use of antiasthmatic medicine in athletes
Allergic rhinitis in athletes
During Olympic games
Prevalence of use beta-2-agonists

Exercise-induced bronchoconstriction (EIB)
Prevalence of exercise-induced bronchoconstriction
Eucapnic voluntary hyperpnea
In children

Comparison of blood and urin levels between routes of administration
Ergogenic effects
In chronic pulmonary disease
In non-asthmatics

Treatment
Meta-analysis of beta_2-agonists effects
Designer beta_2-agonists
Salbutamol
Effect on performance
Neuromuscular function
After intense exercise in endurance athletes
Renal elimination
Acute effects on muscles
Side effects
Experimental
Laboratory techniques

Terbutaline
Urine and serum concentrations of inhaled and oral terbutaline

Formatterol
Procaterol
Clenbuterol
Induction of IGF and myostatin
Clenbuterol in food
Clenbuterol in hair
Clenbuterol and heart problems
Supression of bacterial phagocytosis
Salbutamol and clenbuterol
Laboratory technique
Experimental
Illegal in cattle
In horses

Methoxyphenamine
Laboratory techniques
Experimental

**BETA-BLOCKERS**

**ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORS AND ANGIOTENSIN II TYPE RECEPTOR ANTAGONISTS**
Telmisartan
Experimental
DIURETICS
Rapid weight loss
Thiazides
Mannitol
Laboratory techniques

OTHER MASKING AGENTS
Epitestosterone
Proteases
Desmopressin
Other chemical and physical manipulation of doping tests

GLUCOCORTICOSTEROIDS
Overview
Stimulation of myocytes
Effects on exercise performance
Adrenal insufficiency after use of corticosteroids
  Corticosteroid-induced adrenal insufficiency in elite cyclists
ACTH
Synachthen®
Budesonide
Methylprednisolone
Dexamethasone
Cortisol value dururing an exercise season
Prednisolone in human urine
Topical administration of prednisolone
Effect on muscles
Glucocorticoid-induced skeletal muscle atrophy
Transfer factor
Use in football
Endogenous prednisolone
Microbial transformation of cortisol to prednisolone
Ophthalmological aspects
Side effects
  Heart
Laboratory techniques
  Measurement in hair
Experimental

MELATONIN
As marker for training
Effect on performance
Effects on strenuous exercise
  Experimental
Jet lag
Salivary
Experimental
  Influence on plasma glucose
  Combination with imipramine

SILDENAFIL (VIAGRA®)
MORPHINE
Decreased sensitivity after exercise
Dermorphine

RECREATIONAL DRUGS: CANNABIS, COCAINE, ECSTASY AND GAMMA-BUTYROLACTONE (GBL)
Cannabis
- Overviews
- Long-term use
- Epidemiology of use of cannabis
- Mechanism of action
- Synthetic cannabinoids
- Medical use
- Duration of detection in blood
- Effects
- Influence on psychomotor performance
- Cannabis use in sports
- Influence of nandrolone on cannabinoid dependence
- Cannabis in urine
- Does cannabis have a doping effect?
- Endogenous
- “Spice”
- Occasional or frequent smokers
- Side effects
- Laboratory technique

Cocaine
- Environmental factors important for abuse
- Effects
- Cocaine in sport
- Side effects
- Laboratory techniques

Methylenedioxyxymethamphetamine (MDMA; Ecstasy)
- Together with Ecstasy
- Metabolites
- Laboratory testing

gamma-Aminobutyric acid (GABA)
- Influence of anabolic steroids

gamma-Butyrolactone (GBL)
- gamma-Butyrolactone and gamma-butyrolactone
- gamma-Butyrolactone and 1,4-butanediol

gamma Hydroxybutyrate (GHB)
- Metabolism and neuromodulatory properties
- Positive aspect of GHB
- Cardiovascular and respiratory effects
- Central nervous system effects
- GHB in obstetrics
- Sexual enhancing effects of GHB
- GHB use in alcohol and opiate withdrawal
- Interaction with alcohol
- Pharmacokinetics and pharmacodynamics
Other negative aspect of GHB
GHB abuse
GHB detection
Laboratory technique
beta-Hydroxy-beta-methylbutyrate (HMB)
Metabolism
HMB safety
Effects on skeletal muscle damage, protein breakdown, and recovery
Effects of training status
Duration of supplementation, dose, and timing
The effects on skeletal muscle hypertrophy in healthy untrained and trained adults
HMB in athletes training in an energy restricted state
HMB supplementation in youth and adolescent populations
HMB supplementation in aging and masters athletes
HMB improves indices of aerobic performance, fat loss, and energy metabolism
Proposed mechanisms of action
Skeletal muscle regeneration
Skeletal muscle proteolysis
HMB in young trained subjects
HMB in trained versus untrained subjects
HMB mixed with other molecules in young trained subjects
HMB mixed with other molecules in young untrained subjects
Dose and safety of treatment
HMB versus glucocorticoids
Attenuation of muscle loss during sustained energy deficit
Influence of HMB on protein synthesis
Influence on free acids
Laboratory testing
Experimental
Summarised aspects of HMB in sports
1,4-Butanediol (1,4-BD)
gamma-Oryzanol and ferulic acid

ALCOHOL
Epidemiology
Motives and attitudes for alcohol use by athletes
Mechanism of action
Studies on alcohol and performance
Practical effects of energy drinks on alcohol priming
Effects of ethanol on glycogen metabolism
Hydration and thermoregulatory function
Effects of acute alcohol consumption on neuromuscular function.
Effects of acute alcohol consumption on recovery from a match
Effects on brain’s white matter
The aftermath of alcohol use
Effects of alcohol on injury and incapacity
Influence of sex
Impact of alcohol on steroid profiles
Cardioprotective effects
Carbohydrate deficient transferrin (CDT)
In football
Consumption in US college sports
Sponsorship
Prevention
Alcohol control strategies at large sports events

NICOTINE
Mechanism of action
Effect on performance
Detection

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDS)
Mechanism of action
Ketoprofen
Topical NSAID
How safe are NSAIDs?
  Gastrointestinal
  Cardiac
  Renal and hypertension
  Asthma
  Pregnancy
  Comparison with other analgesics
Use in handicapped athletes
In ultramarathon runners
Side effect
Recommendations
Medical ethics

AICAR (5-AMINO-4-IMIDAZOLECARBOXYAMIDE RIBONUCLEOSIDE)

ANTICONVULSANTS

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR
Genetics
Effect of lactate
Pioglitazone
Interaction with beta-adrenergic blocker

PHOSPHODIESTERASE-4 INHIBITORS

NUTRITIONAL SUPPLEMENTS: GENERAL ASPECTS
Governance around sports supplementation
  Role of sports medicine specialists
Legislation
Theoretical aspects
Scientific nutrition strategy
Attempts to measure effects of supplements
  Measurements of enzyme levels
  Molecular markers in dietary supplement research
  Analysis of actual content in supplements
  Quality assurance of dietary supplements
Perceptions of nutritional supplementation
  Motives for use of supplements
  Attitudes towards dietary supplements
  Stacking
For injury prevention
Effects of energy conditioning on food preferences and choice
Side effects of dietary supplements
Appropriate regulations
Quantitation of use of supplements in sports
Combination with anabolic steroids
Germany
Spain
Greece
Italy
Slovenia
Poland
Singapore
Canada
USA
Saudi Arabia
Oman
Judo
Sailing
Football
Track and field
Rowing
Males and females
Use of nutritional supplements among master athletes
Use of dietary supplements among adolescents
Recovery after sports
Individualized nutrition as doping
Side effect
Cholestatic jaundice
Contaminated supplements
Anabolic steroids
Ephedrine
Hepatotoxicity
How to deal with supplements in the field of practice
Recovery time after sports
Self-reported recovery
Practical nutritional recommendations
Adolescents’ nutrition
Self-regulation concepts
Excess of protein and fat
Female needs in nutrition and hydration
A triad
Energy need
Carbohydrate need
Protein need
Hydration during sports
Calcium
Iron
Creatine
Nutrition in the aged athletes
Dietary recommendation
Impact of dietary recommendations
Energy intake
For team sports
For power sports
For endurance sports
For young soccer players
Response to long distance running (energy needed)
Food provision during Olympic Games
General recommendations
  - Nutrition for sprinters
  - Nutrition for runners
  - Nutrition for triathlon and marathon
  - Nutrition for football players
General recommendations regarding the use of dietary supplements
Recommendation of the International Society for Sports Nutrition
  - Energy need
  - Carbohydrate
  - Protein
  - Strategic eating and refuelling
  - Vitamins
  - Minerals
  - Water
  - Convenience supplements
  - Muscle building supplements
  - Creatine
  - Essential amino acids (EAA)
  - Beta-hydroxy beta-methylbutyrate (HMB)
  - Branched chain amino acids (BCAA)
  - alpha-Ketoglutarate (alpha-KG)
  - alpha-Ketoisocaproate (KIC)
  - Ecdysterones
  - Growth hormone releasing peptides (GHRP) and secretagogues
  - Ornithine-alpha-ketoglutarate (OKG)
  - Zinc/Magnesium aspartate (ZMA)
  - Glutamine
  - Isoflavones
  - Sulfo-polysaccharides (myostatin inhibitors)
  - Boron
  - Chromium
  - Conjugated linoleic acids (CLA)
  - Gamma oryzanol (Ferulic acid)
  - Prohormones
  - Tribulus terrestris
  - Vanadyl sulfate (Vanadium)
  - Green tea extract
  - Phosphatidyl choline (Lecithin)
  - DHEA and 7-Keto DHEA

NUTRITIONAL.SupPLEMENTS: WATER, CARBOHYDRATE, PROTEIN, AND FAT

Hydration
  - In adolescents
  - Effects on environment and persons around for drinking habits
  - Sweating
  - Hydration assessment
  - Dehydration
Hyperhydration
Muscle problems in hypohydration
Modifying factors for hypohydration
Diet
Fluid replacement before exercise
Fluid replacement during exercise
Drinking pattern during different parts of activity
Different commercial available sport drinks
Dehydration despite favorable conditions for fluid intake
Gastric emptying
Fluid restriction increases GI permeability
Effects of hypohydration on performance
Drinking pattern during sports
Hydration in marathon
Water and carbohydrates
Water and electrolytes
Alcoholic beverage for treatment of dehydration
Oral salt supplementation

Exercise-associated hyponatremia
Use of NSAID
Pathophysiology
Arginine vasopressin (AVP)
Sweating
Clinical features
Prevention of EAH
Therapy of EAH

Bicarbonate
Physiology
Effect on exercise
Effect on symptoms from the stomach
Sodium bicarbonate and sodium citrate

Energy need
Meal frequency

Carbohydrates
Glucose homeostasis in athletes
Effect on perceived exertion
Effect on endurance exercise
Effect of pre-exercise carbohydrate-loading on endurance performance
Effect on combat sports
Effect on tennis
Effect on football
Effect on sleep onset
Mouth rinse with carbohydrate solutions
Influence on testosterone levels
Intake recommendations
Can low carbohydrate high fat provide fuel for sport?
Carbohydrates and protein
Carbohydrates plus whey
Carbohydrates with fat
Milk carbohydrate and protein
Carbohydrates and caffeine
Carbohydrates and electrolytes
Influence of age and pubertal status on substrate utilization
Carbohydrate gel
Milk
Chocolate milk
Raisens
Fruits and vegetables
Cornstarch
Hydroxypropyl-distarch
Honey-sweetened beverage
Low-carbohydrate diets and performance
Carbohydrate loading effect of menstrual-cycle phase
Effects of carbohydrate beverage ingestion on the salivary IgA
Carbohydrate in young adolescents
Glucose polymers
Carbohydrate effect on oxidative changes
Increased lactate (decreased lipolysis) due to carbohydrates
Lactate
Sucrose
Ribose
Galactose
Galactose and fructose
Multiple transportable carbohydrates
Carbohydrates in tennis

Energy beverages/Sport drinks
Science on “sports drinks”
With caffeine
With carbohydrate, protein and antioxidants
With other nutrients
Effect on exercise performance
Energy drinks and their role in energy expenditure and weight loss
Gastric emptying
Safety considerations
Consumption pattern
Beverage after aerobic activity
In school children
Adverse effects
Sweet, non-alcoholic beverages
Red bull
Canada
USA
Effects of cold drinks

Proteins
Recommended daily allowance in athletes
High protein feeding
Egg white protein
Whey
Wheat germ
Soy-protein
Combination of sago and soy-protein
Wheat gluten hydrolysate
Building muscles in fed and fasted state

Protein drinks
Effect on endurance and muscles
The effects of protein intake timing in relation to strength training
Proteins before exercise
Protein-rich intake for recovery after exercise
Needs in older, training subjects

Peptides
Amino acids
Branched-chain amino acids (BCAA)
  BCAA plus arginine
Neutral amino acids
  Effect on performance
  Effects on muscle soreness
Arginine
  Combined with ornithine
  Combined with citrullin
Leucine
  4-hydroxyisoleucine
L-tryptophan
Taurin
Cystein and cystine
Cystine/theanine
Glutathione and glutamate
Glutamine
  Studies in intensive care units
  Glutamine, athletes, and immunity
  Glutamine, muscle function, and athletic performance
Alanine
  L-alanine
  beta-Alanine
  Alanine plus creatine
Methionine
Threonine
Tryptophan
Tyrosine
Valine
CBEX
Carnosine (beta-alanyl-l-histidine)
  With and without sodium bicarbonate
  Effect of training
Citrulline
Aspartate and asparagine
Aspartame
Glycine
Glycine-arginine-alpha-ketoisocaproic acid
alpha-Ketoglutarate
alpha-Ketoisocaproate
Prolin
Amino acids and creatine
Fat
  Medium-chain triglycerides
  Maximal lipid oxidation
  In cyclists
Fish oil
  Omega-3 fatty acid
Multi-ingredient performance supplements (MIPS)
NUTRITIONAL SUPPLEMENTS: TRACE ELEMENTS, VITAMINS AND OTHER OXIDANTS

Iron
Boron
Calcium
Cobalt
Copper
Chromium
Magnesium
Potassium
Selenium
Vanadium
Zinc
Zinc monomethionine aspartate
Phosphate
Nitrate
Vitamins, in general
Vitamin B
Vitamin D
Vitamin D and muscle tissue
Vitamin D recommendations (intake and desirable levels)
Vitamin D status of athletes
Vitamin D and athletic performance
Summer versus winter
Experimental
Vitamin A
Coenzyme Q10
Folate
Vitamin K
Vitamin C
Optimal vitamin C intake for athletes
Vitamin C and E
Effects on running
Side effects
Vitamins A, C, and E
Vitamin E
Dietary antioxidants, in general
Theoretical aspects on the cellular level
Redox state in athletes
In rugby players
Resveratrol
Resveratrol and quercetin
N-Acetylcysteine
Allopurinol
Other antioxidants
Cognitive performance
Flavonoids (including quercetin)
Astaxanthin
In handball
alpha-Tocopherol, ascorbic acid, and beta-carotene
Catechins
In chronic obstructive pulmonary disease
Experimental
Hydroxycitrat
Applephenon

NUTRITIONAL SUPPLEMENTS: OTHER DEFINED CHEMICAL SUBSTANCES AND METHODS

Creatine
Effects of creatine supplementation on physical performance
Effects of creatine on anaerobic exercise
Effects of creatine on aerobic exercise
Effects of creatine supplementation on skeletal muscle hypertrophy
Effect on plasma levels of pro-inflammatory cytokines
Effects of creatine on glycogen stores
Effects on blood lactate
Effects on kidney function
Effects on inflammatory markers
Side effects of creatinine
Dosing protocols applied in creatine
Commercially available forms of creatine
Creatine in combination with other supplements
Safety and side effects of creatine
In muscle wasting
Effects of statins
Creatine monohydrate and conjugated linoleic acid
Creatine kinases
Influence on glucose metabolism
Creatine and whey protein
Cognitive effects of creatine
Use in women
Effect on myostatin
Children
Creatine purity
In taekwondo
Effect on tennis
Effect on ice-hockey
Effect on sprint running
During sleep deprivation
Side effects
Creatine and bicarbonate
Experimental

Carnitine
Effect on post-resistance-exercise (RE)
Side effects

Methylhexaneamine (DMAA)
Glutatione
Choline bitartrate plus acetylcholine
Chondroitin/glucosamine
Lecithin
Linoleic acid
gamma-Linolenic acid
Conjugated linoleic acid
Leptin
Inosine
Glucosamine
“Fat burners”
L-arginine alpha-ketoglutarate (AAKG)
Blend supplements
Nutritional support to maintain proper immune status during intense training
Electrolytes
Dimethylglycine
Dietary nitrates
Dihydroxyacetone phosphate and pyruvate
Methylsulphonylmethane
Melamine
Nootkatone
Octacosanol and policosanol
Superoxygenated water
Non-pharmacological therapy
Acupuncture
Cryostimulation
Cooling
Sublingual, ergogenic spray

**NUTRITIONAL SUPPLEMENTS: FROM PLANTS AND ANIMALS**

**Herbs**
- Ergogenic theory
- Herbal weight loss supplements

**Prebiotics**
**Probiotics**
- Probiotic intervention studies in athletes

**Bluberries**
**Wolfberry (goji berry)**
**Green tea**
**Black tea**
**Ginseng**
**Ginkgo biloba**
**Garlic**
**Macroalgae**
**Spirulina (microalgae)**
**Phosphatidylserine**
**Quercetin**
**Capsaicin**
- Soldiers
- Mice

**Pycnogenol**
**Kava kava (Kava)**
**St. Johns wort**
**Yucca**
**Teribulus terrestris**
**Arnica**
**Rhodiola rosea**
- Lack of effect on marathon running

**Cissus quadrangularis**
**Hydroxycut**
**GENE DOPING**

**Overviews**
- The science behind gene doping
  - Definitions and history
  - Gene transfer approach to therapy
  - Therapeutic gene transfer vectors introduced into human beings
  - Reversing the effects of transplanted genes
- Medical uses of gene therapy
- Animal use of gene doping
- Genes versus environmental influence
- Gene therapy versus gene doping
- Epigenetics
- Evolving definitions
  - RNA interference
- Genetic enhancement
  - In vivo gene doping
  - Ex vivo gene doping
- Possibilities for gene doping
  - Gene-based doping for muscle function
  - Gene-based doping for oxygen delivery to exercising tissues
  - Further possibilities of detecting gene doping
- Doping targets
  - Glucose metabolism
  - Red blood cell activity/delivery
  - Skeletal muscle size, strength and endurance
  - Vascular endothelial growth factor
  - Fibroblast growth factor
  - Preventing pain (endorphin and enkephalin)
  - alpha-Actinin 3
  - Peroxisome proliferator-activated receptor-delta
  - Cytosolic phosphoenolpyruvate carboxykinase
  - Gene doping with intracellular molecules
- Options for gene doping
  - Gene doping targets
- Candidate genes for athletic gene doping
Hematopoietic/vascular systems
Hypoxia inducible factors
Vascular endothelial growth factor
Actin-binding peptides
Angiotensin-converting enzyme
Insulin-like growth factor
Myostatin
Peroxisome proliferator-activated receptor delta
Endorphins
Erythropoietin gene transfer
Polymorphism
Experimental
Sport as a case study
Risks and complications of gene doping
  Gene silencing
  Immune reaction
  Integration
  Infection of germ cells
  Expression
  Storage and usage
  Long term
  Uses of gene therapy
Potential strategies for detection of gene doping
  Detection of erythropoietin gene doping
  Muscle biopsy
  Blood monitoring
  Genetic activity tests
  Protein fingerprints
  Genetic barcodes
Laboratory techniques for detection of gene doping
  Direct methods
  Indirect methods
  Transgene and nonviral vectors in blood
  PCR-based detection of gene transfer vectors
Regulation of gene doping
  The ethics of gene doping
    Bioethical concerns
    Definition of enhancement
    Genetic enhancement
    The thin line between therapy and enhancement
    Specific ethical questions

ETHIC ISSUES IN DOPING AND ANTI-DOPING
The history of anti-doping ethics
War on doping versus war on drug
  Doping is here to stay
  "Everybody take doping drugs"
Arguments for doping and their ethical anti-arguments
  Human nature
  Regulation could improve safety
  Spirit of sport
  Argument against doping in sports
Escalating problems
Engineered athletes
Bans can work
What justifies anti-doping?
Alternatives to current anti-doping strategy
A continuing discussion is needed
The reasons for anti-doping rules
Sports as part of the society
Doping as a sign of dehumanization
Making sport possible
Technology introduced but changing the sport
Increased participation and spectatorship
Alternate conceptualizations
Ethical aspects of “harm” in sports
Harm to the athlete
Harm to other athletes
Harm to society
Ethics of the athletes regarding doping
Sports Federations’ attitudes
Litigation in sports medicine
Physician’s role in doping
The challenge of working in sports medicine
Environmental factors
Case reports of the doctor’s fault but athlete’s verdict
Rumanian gymnast
Serbian handball player
Russian basketball player
French basketball player
Spanish basketball player
Regulations
Aims of the regulations
Legislative structures on enhancement
Courtroom medicine
Evidence-based doping?
Motherhood goes with gold?
Legitimacy of ban on cannabis and cocaine
The complexity of anti-doping
The ethics of doping
Education in moral judgment of participants in team sports
Professionalism and the ethics of the sports physician
Conflicts of interest: the need to minimize
Need to recognize the limits of athlete’s autonomy
The need to maintain informed consent
Confidentiality in doping
Patient confidentiality
Fairness in sports
Criticism of the fairness of the testing procedure
The WADA mission
Sport and the history of ethics in sports medicine
Multiple obligations
Attitudes on anti-doping
Hypotheses on background of doping
Youth and adolescents
Environmental factors
Anti-doping programmes
The life-cycle model of performance enhancement
A gold medal but also death in 5 years’ time?

Prevention of athletes from harm
On cheating
Different shades of blood doping
The grey zone of undiscovered doped
Enforcement of anti-doping policy today
Smart drugs (“brain doping”)
Performance-enhancing drugs create an uneven playing field
Everybody else is taking them
Performance-enhancing drugs are dangerous
Drug use would be almost impossible to control
Danish ban on gyms not adhering to doping-tests

Cannabis
Gender as more than a binary quantity
On how to make illegal drugs less dangerous
Hidden assumptions and inherent contradictions in anti-doping policy
Setting a good example
A level playing field
Protecting the health of athletes
Preserving the integrity of sport
Policy implications

Research on doping substances
WADA’s statement

Ethical codes
A code of ethics with a foundation in evidence
The complex environment of elite sport
Multiple obligations
Sharing personal information about athletes with others
Risk taking by athletes
Aims for a new code of ethics
Practicalities and negotiating the politics

Scientific cooperation
The formal ethics of research with human subjects
The ethical principles used by review bodies
Further amendments

Sport as an occupation
Legislation of drugs aimed for doping
“Naturalness” in sports
An example for the society

Law versus ethics
International doping policy: the WADA and the WADC
Public law versus sport’s law
Guilt, negligence and liability
Privacy

Physician’s responsibility
Self-reporting on doping

Hypoxic tents
Pain medication
Injection therapy with local anaesthetics
Injection therapy with local corticosteroids
Concluding remarks
Publication ethics

VETERINARY
Bacterial hydrolysis of urine without doping substances
Contaminated food for animals
Screening
Anabolic steroids, general
  Anabolic steroids in dogs
    In hair
  Differentiation between endogenous steroids and synthetic homologues in cattle
Testosterone
  Testosterone and nandrolone
19-nortestosterone
Nandrolone
1-Testosterone
Stanozolol
Methyltestosterone and mestranolone
Mesterolone
Methandienone
Boldione, boldenone and boldenone esters
Trebolone and estradiole
Fluoxymesterone
Growth hormone
Selective androgen receptor modulators (SARMs)
Erythropoietin
  Horse
  Dog
Darbepoetin
Insulin
Seven peptide hormones
Relaxine
Thyroid hormones
Hydrocortisone
  Topical glucocorticoids
Clenbuterol and hydrocortisone
Clenbuterol
THG
Theobromine
Flurbiprofen
Phenylbutazone
Salicylic acid
Prednisolone
Bupivacain
Levamisole
3,4-Methylenedioxypyrovalerone (MDPV)
Glycopyrrolate
Dermorphine
Capsaicin
Acepromazine
Antioxidants
Vitamin E
Myo-inositol trispyrophosphate (ITPP)
Alpha-cobratoxin
Quaternary ammonium drugs
Ethanol

Laboratory techniques
  Direct-injection differential-gradient LC-LC coupled to hybrid tandem MS/MS
  Stable carbon isotope analysis
  Solid-phase extraction and liquid chromatography-mass spectrometry
  Liquid chromatography – Orbitrap mass spectrometry
  Proteomics et al
  In hair
  Identifying individual horses urine by single-nucleotide polymorphisms (SNPs)
  TB-500
  Primary hepatocytes as a bioassay
  Molecularly imprinted polymer applied to the selective isolation of urinary steroids
  Single-nucleotide polymorphism assay
  Orbitrap
  Protein biomarkers

Other aspects on forensic medicine
Horses (in general)
  Drug metabolism in horses

Report from Iran
Live stock
Intersex conditions

**FUTURUM**
Continuing research
Criminals
Economy and techniques
Intelligens
What if current zero-tolerance anti-doping policy continues?
Social, policy, and public health perspectives on new psychoactive substances
Technology advancement
More complex challenges
New classes of drugs
  Synthol
  Tolperisone
  5-hydroxytryptamine (5-HT) agonist
Prediction of futures anabolic androgenic steroids

**REFERENCES**
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AA</td>
<td>anabolic agents</td>
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<tr>
<td>AA</td>
<td>amino acid</td>
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<td>adrenergic agonists</td>
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<td>aromatic amino acid</td>
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<td>androsterone acetate</td>
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<td>analytical adverse findings</td>
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<td>L-arginine alpha-ketoglutarate</td>
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<td>Athlete Biological Passport</td>
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<td>Abnormal Blood Profile Score</td>
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<td>Anti-Doping And Management System</td>
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<td>androsta-1,4-dien-3,17-dione</td>
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<td>antidoping rule violation</td>
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<td>AOPP</td>
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<td>AOR</td>
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<td>APED</td>
<td>appearance- and performance-enhancing drug</td>
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</table>
APMU | athlete passport management unit
APO | apomorphine
APPI | atmospheric pressure photoionization mode
AQUA | Allergy Questionnaire for Athletes
AR | androgen receptor
AR | adrenergic receptor
ARB | angiotensin receptor blocker
ARC | arcuate nucleus
ARCI | Association of Racing Commissioners International
ARE | androgen responsive element
ARG | arginine
AS | average speed
AS | anabolic steroids
AS | antioxidant supplementation
AS | affinity tests
ASADA | Australian Sports Anti-Doping Authority
ASAP | Atmospheric Solids Analysis Probe
ASAT | aspartate transaminase
ASC | colloid acetyl starch
ASCA | Anabolic Steroid Control Act
ASCM | American College of Sports Medicine
ASE | accelerated solvent extraction
ASIH | anabolic steroid-induced hypogonadism
ASP | athlete steroidal passport
ASP | athlete support personnel
AST | aspartate aminotransferase
AST | astaxanthin
AT | angiotensin
ATD | 1,4,6-androstatriene-3,17-dione
ATHENA | Athletes Targeting Healthy Exercise and Nutrition Alternatives
ATG | autophagy-related protein
ATP | adenosine triphosphate
ATP-PC | adenosine triphosphate-phosphocreatine
ATR | attenuated total reflection
AT₁-R | angiotensin II receptor
aTUE | abbreviated TUE process
ATUE | abbreviated Therapeutic Use Exemption
Au | gold
AUC | area under the curve
AUDIT | Alcohol Use Disorders Identification Test
AUDIT-C | Alcohol Use Disorders Identification Test-alcohol consumption
AuNP | gold nanoparticle
AVP | arginine vasopressin
AVS | antioxidant vitamin supplementation
BA | beta-alanine
BALCO | Bay Area Laboratory Co-operative
BAP | biological antioxidative potential
BBB | blood-brain barrier
BCAA | branched-chain amino acids
BCKD | branched-chain ketoacid dehydrogenase
BD | 1,4-butanediol
1,4-BD | 1,4-butanediol
BDI | Beck Depression Inventory
BE base excess
BES benzoate
17beta-NT 17beta-19-nortestosterone
B-FGF basic fibroblast growth factor
bFGF basic fibroblast growth factor
BFR blood flow restriction
BFU burst colony forming units
BFU-E burst forming unit-erythroid
BGE background electrolyte
BH bronchial hyperreactivity
BHB beta-hydroxybutyrate
BHK baby hamster kidney
BHR bronchial hyperresponsiveness
BIAT Brief Implicit Association Test
BICA bicalutamide
BioMS bioaffinity liquid chromatography-mass spectrometry
BJR Bezold-Jarisch reflex
BL baseline
BL bioluminescent
BM body mass
BMC bone mineral content
BMD bone mineral density
BMI body mass index
BML body mass loss
BMC bone mineral content
BMD bone mineral density
BMP bone morphogenetic protein
BMSC bone marrow stem cells
BMX bicycle moto cross
BNST bed nucleus of the stria terminalis
BOL boldenone (17-hydroxy-androsta-1,4-diene-3-one)
BP bench press
BP blood pressure
BPA bisphenol A
BPH benign prostatic hyperplasia
Bpm beats per minute
BRIC benign recurrent intrahepatic cholestasis
BPT bromo pentane
BR bronchial reactivity
BRUMS Brunel Mood State Inventory
BS breeding stallions
BS box squat
BSA bovine serum albumin
BSC bovine satellite cell
BSTFA N,O-bis(trimethylsilyl)trifluoroacetamide
BT bench throw
BT bioavailable testosterone
BUD budesonide
BUN blood urea nitrogen
BV blood volume
BW body weight
BWPLV bioenhanced whey protein
BWS best-worst scaling
CERA continuous erythropoietin receptor activator
CES carbohydrate-electrolyte solutions
CES-D Center for Epidemiological Studies Depression Scale
CE-TOF/MS capillary electrophoresis time-of-flight mass spectrometry
CETP cholesteryl ester transfer protein
CF cognitive function
CFA complete Freund's adjuvant
CFTR cystic fibrosis transmembrane conductance regulator
CFIRMS continuous-flow isotope ratio mass spectrometry
CFU-E colony forming unit-erythroid
CG chromatography
CG chorionic gonadotropin
C6G codeine-6-glucuronide
CHCA cyano-4-hydroxycinnamic acid
CHD coronary heart disease
CHF chronic heart failure
CHF congestive heart failure
CHO Chinese hamster ovary
CHO carbohydrate
CHO-E carbohydrate-electrolyte
CHO-P carbohydrate-protein
CHOPA carbohydrate-protein-antioxidant beverage
CHT oxidative capacity
CI confidence interval
C150 concentration causing 50 percent inhibition
CID collision-induced dissociation
CIR carbon isotope ratio
CIR circuit training
CIR circumference
CK creatine kinase
cIEF capillary isoelectric focusing
CIR carbon isotope ratio
CK creatine kinase
CK-BB brain creatine kinase
CK-MB myocardial creatine kinases
CK-MM muscle creatine kinases
CL confidence limit
CLA conjugated linoleic acid
CLB clenbuterol
CLEN clenbuterol
CMBCD carboxymethyl-beta-cyclodextrin
CM creatine monohydrate
Cmax maximum concentration
Cmin minimum concentration
CMJ counter-movement jumping
CML chronic myelogenous leukemia
CMO chief medical officer
CMS carboxymethyl starch
CMV cytomegalovirus
CNB caloric nutritional beverages
CNDP carnosinase
Cne cholestane
CNS central nervous system
CNTF  ciliary neurotrophic factor
CO   cardiac output
CO   carbon monoxide
Co   cobalt
COLL  collagen
COMT  cathecol-O-methyltransferase
CON  control
COPD  chronic obstructive pulmonary disease
CoQ  coenzyme Q10
COX  cyclooxygenase
COX-2  selective cyclooxygenase-2
CP  carbonylated proteins
CPC  colostrum protein concentrate
CPK  creatine phosphokinase
cps  counts per second
CPX  cardiopulmonary exercise test
CQR  Cissus quadrangularis
CR  creatine
Cr  creatine
CRE  cyclic AMP response element
CRM  certified reference materials
CrM  creatine monohydrate
CRM  certified reference materials
CRP  C-reactive protein
CS  citrate synthase
CS  cage-switching stress
CS  creatine supplementation
CSA  cross-sectional area
CSIA  compound-specific isotope analysis
CT  computed tomography
CT  calcaneal tendon
CTAB  cetyl trimethyl ammonium bromide
CTX  C-terminal telopeptide of type-I collagen
CV  coefficient of variance
CV  cardiovascular
CVD  cardiovascular disease
CVF  collagen volumetric fraction
CVP  central venous
CVST  cerebral venous sinus thrombosis
CW  Caucasian women
CWI  cold-water immersion
CYP  cytochrome P
CZE  capillary zone electrophoresis
CZE-UV  capillary zone electrophoresis-UV absorbance detection
D  deletion
DA  dopamine
Da  Dalton
DAD  diode-array detector
DALDA  Daily Analysis of Life Demands for Athletes
DAM  N,N-dimethylamphetamine
D-AMPH  d-amphetamine
DART  direct analysis in real time
DAT  drug and alcohol testing
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<td>dopamine transporter</td>
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<td>DBD</td>
<td>DNA binding domain</td>
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<td>dried blood spots</td>
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<td>dcELISA</td>
<td>direct competitive enzyme-linked immunoabsorbent assay</td>
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<td>dimethyl-arginine dimethylaminohydrolase</td>
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<td>desmopressin</td>
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<td>DDR</td>
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<td>Drug Enforcement Administration</td>
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<td>decanoate</td>
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<td>DEHP</td>
<td>di-(2-ethylhexyl)phthalate</td>
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<td>Diabetes Exercise and Sports Association</td>
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<td>DESI</td>
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<td>dextrose</td>
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<td>dexamethasone</td>
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<td>difluoromethylornithine</td>
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<td>DFSA</td>
<td>drug facilitated sexual assault</td>
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<td>DFT</td>
<td>deep flexor tendon</td>
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<td>DFT</td>
<td>density functional theory</td>
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<td>2DG</td>
<td>2-deoxyglucose</td>
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<td>D/H</td>
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<td>dihydroDMA (7alpha,11beta-dimethyl-19-nortestosterone)</td>
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<td>DHA</td>
<td>3,4-dihydroxyamphetamine</td>
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<td>DHAP</td>
<td>dihydroxyacetone phosphate</td>
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<td>DIONE</td>
<td>androstenedione</td>
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<td>DLLME</td>
<td>dispersive liquid-liquid microextraction</td>
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<td>DMA</td>
<td>7alpha,11beta-dimethyl-19-nortestosterone</td>
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<td>DMMA</td>
<td>methylexaneamine (or 1,3 dimethylamylamine)</td>
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<td>DMBCD</td>
<td>heptakis(2,6-di-O-methyl)-beta-cyclodextrin</td>
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<td>DME</td>
<td>drug metabolizing enzymes</td>
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<td>DMG</td>
<td>N,N-dimethylglycine</td>
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<td>DMH</td>
<td>dorsomedial hypothalamus</td>
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<td>DMI</td>
<td>Doppler myocardial imaging</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
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<td>DMT</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>Declaration of use</td>
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<td>DPV</td>
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<td>Dietary reference intakes</td>
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<td>Deconvolution reporting software</td>
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<td>Dietary supplements</td>
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<td>DS</td>
<td>Dilute-and-shoot</td>
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<td>DSC</td>
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<td>EAA</td>
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<td>EAC</td>
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<td>EAHE</td>
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<td>EAPH</td>
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<td>EASIA</td>
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<td>eCB</td>
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<td>EDC</td>
<td>endocrine disrupting chemicals</td>
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<td>2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine</td>
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FTICR  Fourier transform ion cyclotron resonance
FT-ICR/MS  Fourier transform ion cyclotron resonance mass spectrometry
FTIR  Fourier transform infrared
F20TPP  meso-tetrakis(pentafluorophenyl) porphyrin
FV  fruits and vegetables
FW  fat weight
FW  flavored water
FWHM  full-width at half maximum
GA  gastrocnemius
GABA  gamma-aminobutyric acid
GABAAR  gamma-aminobutyric acid receptor
GAKIC  glycine-arginine-alpha-ketoisocaproyl acid
GAL  galactose
Gas  musculus gastrocnemius
GASP-1  growth and differentiation factor-associated serum protein-1
GBL  gamma-butyrolactone
GBR  gamma-aminobutyric acid (GABA)/benzodiazepine receptor complexes
GBS  global positioning system
GC  glucocorticoid
GC  gas chromatography
GCC  gas chromatography combustion
GC/C/IRMS  chromatography/combustion/isotope ratio mass spectrometry
GC/GC/TOF/MS  two-dimensional gas chromatography coupled to time-of-flight mass spectrometry
GC/HRMS  gas chromatography-high-resolution mass spectrometry
GC/GC  two-dimensional gas chromatography
GC/GC/TOFMS  two-dimensional gas chromatography with time-of-flight mass spectrometry
GC/IT/MS  gas chromatography-ion trap-mass spectrometry
GC/MS  gas chromatography/mass spectrometry
GC/MSD  gas chromatograph-mass spectrometer
GC/MS/MS  gas chromatography-tandem mass spectrometry
GC-NCI-MS/MS  gas chromatography-negative chemical ionization-tandem mass spectrometry
GCoaTOFMS  gas chromatography/electron ionization orthogonal acceleration time-of-flight mass spectrometry
GCS  glutamylcysteine synthetase
GCS  Glasgow Coma Scale
G-CSF  granulocyte colony-stimulating factor
GC/TOF/MS  gas chromatography time-of-flight mass spectrometry
GD  gastrointestinal distress
GET  gas exchange threshold
GF  growth factor
GFP  green fluorescent protein
GFP  general factor of personality
GFPQ  General Factor of Personality Questionnaire
GFR  glomerular filtration rate
GGT  gamma-glutamyl-transferase
GH  growth hormone
GHB  gamma-hydroxybutyric acid
GHBP  growth hormone binding protein
GHD  growth hormone deficiency
GHR  growth hormone receptor
GHRH  growth hormone-releasing hormone
GHRP  growth hormone releasing peptides
GHS  growth hormone secretagogue
GHV  gamma-hydroxyvalerate
GI  gastrointestinal
Gl  glycemic index
GIH  glycerol-induced hyperhydration
GIP  glucose-dependent insulinotropic polypeptide
GL  glycemic load
GLA  gamma-linolenic acid
GLC  gas-liquid chromatography
Gln  glutamine
GLP  glucagon-like peptide
Glu  blood glucose
GLUC  glucose-maltodextrin
GLUT  glucose transporter
GLUT4  glucose transporter 4
GMFI  geometric mean fluorescence intensity
GnIH  gonadotropin inhibitory hormone
GNP  gold nanoparticle
GnRH  gonadotrophin-releasing hormone
GOS  galacto-oligosaccharides
GP  General Practitioner
GP  glucose polymer
GP  growth promoters
GPA  growth-promoting agents
GPA  grade point average
GPCR  G protein coupled receptors
GPER  G protein-coupled estrogen receptor
GPLC  glycine-propionyl-L-carnitine
GPR30  G protein-coupled receptor-30
GPx  glutathione peroxidase
GR  glutathione reductase
GPS  global positioning systems
GPx  glutathione peroxidase
GR  glutathione reductase
GR  glucocorticoid receptor
GS  glycogen synthase
GS/GS/TOFMS  gas chromatography coupled two time-of-flight mass spectrometry
GSH  glutathione
GSH-Px  glutathione peroxidase
GS-MS  gas chromatography-mass spectrometry
GSSG  glutathione disulphide
GT  glutamyl-transferase
GTC  green tea catechins
GTE  green tea extract
GTN  gastrocnemius
GVL  gamma-valerolactone
GXT  graded exercise tests
HA  hepatic adenomas
HABA  2-(4-hydroxyphenylazo)benzoic acid
HAD  hydroxyacyl-CoA dehydrogenase
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<td>maximum inspiratory pressure</td>
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<td>MRPL</td>
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<td>M1T</td>
<td>methyl-1-testosterone</td>
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mTb  mean body temperature
MTB  mountain bike
MTBE methyl tert-butyl ether
MT  methylestosterone
MTC multiple transportable carbohydrates
mtDNA mitochondrial DNA
MTFA N-methyltrifluoroacetamide
mTOR mammalian target of rapamycin
MTS methyltestosterone
mtTFA mitochondrial transcription factor A
MU methylprednisolone undecanoate
MuRF1 muscle RING finger 1 protein
MVC maximal voluntary contractions
MVIC maximal voluntary isometric contractions
MyHC myosin heavy chain
MYOPRO myostatin propeptide
MV muscle volume
MVC maximal voluntary contraction
MWCNT multi-walled carbon nanotubes
MVIC maximum voluntary isometric contractions
MW molecular weight
6MWD 6-min walk distance
6MWV six-minute walk test
myoFSR myofibrillar fractional synthesis rate
m/z mass-to-charge ratio
NA nandrolone
NA noradrenaline
NA norandrosterone
19-NA 19-norandrosterone
NAC N-acetylcysteine
NAC nucleus accumbens
NaC₄H₅O₇ sodium citrate
nAChR neural nicotinic acetylcholine receptor
NADO national anti-doping organisations
NAED 19-Norandrostenedione
NaHCO₃ sodium bicarbonate
NAN nandrolone
NANA N-acetylnorleucaminic acid
NAS norandrosterone sulfate
NCAA National Collegiate Athletic Association
NCOLA noncola soft drink intake
NC normochromatric erytrocyte
ND nandrolone decanoate
NE norepinephrine
NE neuroenhancement
19-NE 19-noretiocholanolone
NEAA nonessential amino acids
NECA N-ethylcarboxamidoadenosine
NEFA non-esterified fatty acids
Neo5Gc N-glycolyl-neuraminic acid
NESP novel erythropoiesis stimulating protein
NET noradrenaline transporter
NEV non-ecologically valid
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<td>participation in sports, athletics or exercising</td>
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<td>polyvinyl chloride</td>
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PVC  premature ventricular contractions
PVD  peripheral vascular disease
PVE  plasma volume expanders
PVP  polyvinylpyrrolidone
PWC  physical working capacity
PWCT  physical working capacity at fatigue threshold
PYK2  proline-rich tyrosine kinase 2
PYY  peptide YY
Q10  coenzyme Q10
QAD  quaternary ammonium drugs
QC  quality control
QD  quantum dot
QED-MS/MS  quantitation-enhanced data-dependent MS/MS
qRT  quantitative real-time
qRT-PCR  quantitative reverse transcriptase polymerase chain reaction
QSAR  quantitative structure-activity relationships
QSR  quantitative structure-retention relationship
QqQ  triple quadrupole
QTSR  quantitative structure-activity relationship
QTSRR  quantitative structure-retention relationship
QTc  corrected QT interval
QTOF  quadrupole-time of flight
QTof  quadrupole time-of-flight
QTOFMS  quadrupole time-of-flight mass spectrometry
QTRAP  quadrupole linear ion trap
RAAM  reagent array analysis method
rAAV  recombinant adeno-associated viral vector
RAB  risk assessment behaviors
RAC  ractopamine
RAS  renin-angiotensin system
RAST  running anaerobic sprint test
RAT  reactive agility test
Rb  retinoblastoma
RBC  red blood cell
RBC#  absolute red blood cell
RBCM  red blood cell mass
RBL  residual bilinearization
RCP  respiratory compensation point
RCT  respiratory compensation threshold
RCT  randomised controlled trials
RCV  red cell volume
RD  recreational drugs
RDA  recommended dietary allowances
RE  resistance exercise
REE  resting energy expenditure
reGH  recombinant equine growth hormone
REI  relative energy intake
REM  rapid eye movement
rEPO  recombinant erythropoietin
RER  respiratory exchange ratio
RES  resistance exercise scheme

79
RES  resveratrol
RET  resistance exercise training
Ret  reticulocytes
Ret %  reticulocytes percentage
RET#  absolute reticulocyte
RETN  resistin
rFst  recombinant follistatin
RGA  reporter gene assays
rGH  recombinant growth hormone
rhCG  recombinant human chorizon gonadotropin
rhEPO  recombinant human erythropoietin
rhGH  recombinant human growth hormone
rhIGF  recombinant human insulin like growth factor
rhIGFBP  recombinant human insulin-like growth factor-I binding protein
RHS  rebound hypersomnolence
rhSHBG  recombinant human sex hormone-binding globulin
rHuEPO  recombinant human erythropoietin
RIA  radioimmunoassay
RIM  racing intact males
RLX  relaxin
1-RM  one repetition-maximum
Rmax  maximum rate of oxidation
RMR  resting metabolic rate
RMSSD  root mean square of successive differences
RMST  reactive motor skills test
rMV  relative mobility values
RNA  ribonucleic acid
RNI  recommended nutrient intake
ROM  range of movement
ROM  reactive oxygen metabolites
RONS  reactive oxygen and nitrogen species
ROS  reactive oxygen species
RP  relative potencies
RP  reversed-phase
RPE  ratings of perceived exertion
RPF  ratings of perceived fatigue
RP-HPLC  reversed phase high performance liquid chromatographic
RP-LC-HRMS  reversed-phase interaction chromatography systems
RQ  respiratory quotient
RR  Rhodiola rosea
rRE  resistance exercise
RRT  randomized response technique
RRT  relative retention time
RS  restraint stress
RSA  roentgen stereophotogrammetric analysis
RSA  repeated sprint ability
RSb  best repeated sprint
RSD  relative standard deviation
RSH  repeated sprint training in hypoxia
RSI  reactive strength index
RSI  reactive strength index
RSm  mean repeated sprint
RST  repeated-sprint tests
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT</td>
<td>resistance training</td>
</tr>
<tr>
<td>RT</td>
<td>reaction time</td>
</tr>
<tr>
<td>RT</td>
<td>reverse-transcriptase</td>
</tr>
<tr>
<td>RTD</td>
<td>ready to drink supplements</td>
</tr>
<tr>
<td>RTIPESAR</td>
<td>sarcosyl</td>
</tr>
<tr>
<td>RTLM</td>
<td>right thigh non-osseous fat-free mass</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real-time polymerase chain reaction</td>
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<tr>
<td>RUCAM</td>
<td>Roussel Uclaf Causality Assessment Method</td>
</tr>
<tr>
<td>RV</td>
<td>right ventricular hypertrophy</td>
</tr>
<tr>
<td>RVH</td>
<td>right ventricular hypertrophy</td>
</tr>
<tr>
<td>RVP</td>
<td>rapid visual information processing</td>
</tr>
<tr>
<td>RVSD</td>
<td>right ventricular systolic dysfunction</td>
</tr>
<tr>
<td>RVSP</td>
<td>right ventricular systolic pressure</td>
</tr>
<tr>
<td>RWL</td>
<td>rapid weight loss</td>
</tr>
<tr>
<td>SA</td>
<td>synthetic androgen</td>
</tr>
<tr>
<td>sAA</td>
<td>salivary alpha amylase</td>
</tr>
<tr>
<td>SABA</td>
<td>short-acting beta-2-agonists</td>
</tr>
<tr>
<td>SAGE</td>
<td>Serial Analysis of Gene Expression</td>
</tr>
<tr>
<td>SAL</td>
<td>salbutamol</td>
</tr>
<tr>
<td>SaO₂</td>
<td>oxygen saturation</td>
</tr>
<tr>
<td>SARM</td>
<td>selective androgen receptor modulator</td>
</tr>
<tr>
<td>SAR-PAGE</td>
<td>sarcosyl polyacrylamide gel electrophoresis</td>
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<tr>
<td>SB</td>
<td>sodium bicarbonate</td>
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<td>SBMA</td>
<td>spinal and bulbar muscular atrophy</td>
</tr>
<tr>
<td>SC</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SC</td>
<td>synthetic cannabinoids</td>
</tr>
<tr>
<td>SCA</td>
<td>cross-sectional area</td>
</tr>
<tr>
<td>SCAD</td>
<td>severe combined auto-immuno deficiency</td>
</tr>
<tr>
<td>SCD</td>
<td>sudden cardiac death</td>
</tr>
<tr>
<td>SCG</td>
<td>sodium cromoglycate</td>
</tr>
<tr>
<td>SCI</td>
<td>spinal cord injury</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
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<tr>
<td>SCL</td>
<td>skin conductance level</td>
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<tr>
<td>SCL-90</td>
<td>Symptoms Check List-90</td>
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<tr>
<td>SCRTT</td>
<td>choice serial reaction time task</td>
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<tr>
<td>SD</td>
<td>socially desirable</td>
</tr>
<tr>
<td>SDMA</td>
<td>symmetrical dimethylarginine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SDS/SAR-PAGE</td>
<td>sodium dodecyl sulfate/sarcosyl polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SDT</td>
<td>signal detection task</td>
</tr>
<tr>
<td>SDT</td>
<td>self-determination theory</td>
</tr>
<tr>
<td>Se</td>
<td>selenium</td>
</tr>
<tr>
<td>SEC</td>
<td>size exclusion chromatography</td>
</tr>
<tr>
<td>SELDI/TOF</td>
<td>surface-enhanced laser desorption/ionization time-of-flight</td>
</tr>
<tr>
<td>SEM</td>
<td>structural equation modeling</td>
</tr>
<tr>
<td>SEP</td>
<td>synthetic erythropoiesis stimulating protein</td>
</tr>
<tr>
<td>SERM</td>
<td>selective estrogen receptor modulator</td>
</tr>
<tr>
<td>SERS</td>
<td>surface-enhanced Raman scattering</td>
</tr>
<tr>
<td>SERT</td>
<td>serotonin transporter</td>
</tr>
<tr>
<td>SF</td>
<td>serum ferritin</td>
</tr>
<tr>
<td>sFe</td>
<td>serum ferritin</td>
</tr>
<tr>
<td>SFR</td>
<td>structure-fragmentation relationship</td>
</tr>
</tbody>
</table>
SSA  succinic semialdehyde
SSB  sugar-sweetened beverages
SSE  steadystate endurance exercise
SSH  sex steroid hormones
ST   smokeless tobacco
ST   stanozolol
STAN  stanozolol
STAT  signal transducers and activators of transcription
STB  standardbred
sTfR  soluble transferrin receptor
sTP  saliva total protein
STR  short tandem repeat
STR  striatum
s-TRAP  serum antioxidant potential
SUD  substance use disorder
SULT  sulphate transferase
SUM  substance use and misuse
SUP  supplement
SV  stroke volume
SVM  support vector machine
SPE  solid-phase extraction
SPR  surface plasmon resonance
SVR  systemic vascular resistance
SWNT  single-wall carbon nanotubes
S\textsubscript{z}  stanozolol
T\textsubscript{4}  thyroxine
T\textsubscript{3}  triiodothyronine
TA  tibialis anterior
TAA  total antioxidant activity
TAA  total amino acids
TAC  total antioxidant capacity
tACS  transcranial alternating current
TAFLD  toxicant-associated fatty liver disease
TAG  triglycerides
TAP  tapering period
TAS  total antioxidant status
TASH  toxicant-associated steatohepatitis
TAT  tyrosine aminotransferase
TAU  taurine
TB  thoroughbred
TB  trenbolone
TBA  trenbolone acetate
TBA  trenbolone acetate
TBARS  thiobarbituric-acid-reactive substances
TBG  thyroxine binding globulin
TBI  traumatic brain injury
TBLM  total body non-osseous lean mass
TBME  tert-butyl-methyl-ether
TBO  trendione
TBOH  trenbolone
TBW  total body water
TC  total cholesterol
T/C  testosterone/cortisol ratio
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>TPP</td>
<td>testosterone phenyl propionate</td>
</tr>
<tr>
<td>TQ-MS/MS</td>
<td>triple quadrupole mass spectrometry</td>
</tr>
<tr>
<td>TRA</td>
<td>Theory of Reasoned Action</td>
</tr>
<tr>
<td>TRE</td>
<td>time to running exhaustion</td>
</tr>
<tr>
<td>TREN</td>
<td>trenbolone- enanthate</td>
</tr>
<tr>
<td>TRT</td>
<td>testosterone replacement therapy</td>
</tr>
<tr>
<td>Ts</td>
<td>team sports</td>
</tr>
<tr>
<td>TSB</td>
<td>tryptic soy broth</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulation hormone</td>
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<tr>
<td>TSP</td>
<td>total suspended particulate matter</td>
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<tr>
<td>TST</td>
<td>tail suspension test</td>
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<td>TST</td>
<td>total sprint times</td>
</tr>
<tr>
<td>TT</td>
<td>total testosterone</td>
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<tr>
<td>TT</td>
<td>time trial</td>
</tr>
<tr>
<td>Tty</td>
<td>tympanic temperature</td>
</tr>
<tr>
<td>TTE</td>
<td>time to exhaustion</td>
</tr>
<tr>
<td>TTF</td>
<td>time to fatigue</td>
</tr>
<tr>
<td>TU</td>
<td>testosterone undecanoate</td>
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<tr>
<td>TUE</td>
<td>therapeutic use exemption</td>
</tr>
<tr>
<td>TUEC</td>
<td>therapeutic use exemptions committee</td>
</tr>
<tr>
<td>TW</td>
<td>total work</td>
</tr>
<tr>
<td>TWD</td>
<td>total work done</td>
</tr>
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<td>TYMP</td>
<td>tympanic temperature</td>
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<tr>
<td>UA</td>
<td>uric acid</td>
</tr>
<tr>
<td>UAMS</td>
<td>unmodified acid/alcohol-modified cornstarches</td>
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<tr>
<td>UCI</td>
<td>International Cycling Union</td>
</tr>
<tr>
<td>UCLA</td>
<td>University of California at Los Angeles</td>
</tr>
<tr>
<td>UCP</td>
<td>uncoupling protein</td>
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<tr>
<td>UDP</td>
<td>uridine diphosphophate</td>
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<tr>
<td>UEFA</td>
<td>Union of European Football Associations</td>
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<tr>
<td>uEPO</td>
<td>urinary erythropoetin</td>
</tr>
<tr>
<td>UGT</td>
<td>uridine diphospho-glucuronosyl transferases</td>
</tr>
<tr>
<td>uhEPO</td>
<td>urinary erythropoetin</td>
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<tr>
<td>UHPLC</td>
<td>ultra-high pressure liquid chromatography</td>
</tr>
<tr>
<td>UHPLC/MS</td>
<td>ultra high-performance liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>UHPLC-ESI-MS/MS</td>
<td>ultra-high pressure liquid chromatography coupled to electrospray ionization tandem mass spectrometry</td>
</tr>
<tr>
<td>UPLC</td>
<td>ultra performance liquid chromatography</td>
</tr>
<tr>
<td>UPLC-MS-MS</td>
<td>ultra-performance liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>UHPLC/TOFMC</td>
<td>ultra-high performance liquid chromatography combined with time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>UHPLC-QTOF-MS</td>
<td>ultra-high-pressure liquid chromatography coupled to a quadrupole time-of-flight mass spectrometry</td>
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<tr>
<td>UL</td>
<td>upper limits</td>
</tr>
<tr>
<td>UNESCO</td>
<td>United Nations Educational, Scientific and Cultural Organisation</td>
</tr>
<tr>
<td>UOLC/MS/MS</td>
<td>ultraperformance liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>UPLC</td>
<td>ultraperformance liquid chromatography</td>
</tr>
<tr>
<td>UPLC</td>
<td>upper-body power and lower-body strength</td>
</tr>
<tr>
<td>U-PLC</td>
<td>unfolded partial least-squares</td>
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<td>UPLC-MS</td>
<td>ultra-performance liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>UPLC-MS/MS</td>
<td>ultra high performance liquid chromatography tandem mass spectrometric</td>
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<tr>
<td>UPLC-QqQ-MS</td>
<td>ultrahigh-performance-LC-electrospray ionization-triple-quadrupole-MS</td>
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<tr>
<td>URTI</td>
<td>upper respiratory tract infection</td>
</tr>
<tr>
<td>US</td>
<td>ultrasonography</td>
</tr>
<tr>
<td>USADA</td>
<td>United States Antidoping Agency</td>
</tr>
<tr>
<td>USG</td>
<td>urine specific gravity</td>
</tr>
<tr>
<td>USP</td>
<td>unique selling proportion</td>
</tr>
<tr>
<td>USPIO</td>
<td>ultrasmall superparamagnetic particles of iron oxide</td>
</tr>
<tr>
<td>UTI</td>
<td>urinary tract infections</td>
</tr>
<tr>
<td>UTPT</td>
<td>Unique Trait Personality Theory</td>
</tr>
<tr>
<td>UUN</td>
<td>urine urea nitrogen</td>
</tr>
<tr>
<td>UPS</td>
<td>underperformance syndrome</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UVB</td>
<td>ultraviolet B</td>
</tr>
<tr>
<td>VAS</td>
<td>visual analog scale</td>
</tr>
<tr>
<td>VCT</td>
<td>Vogel conflict test</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VHIR</td>
<td>very high intensity running</td>
</tr>
<tr>
<td>VISA-A</td>
<td>Victorian Institute of Sport Assessment – Achilles</td>
</tr>
<tr>
<td>VL</td>
<td>vastus lateralis</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein cholesterol</td>
</tr>
<tr>
<td>VLT</td>
<td>velocity at lactate threshold</td>
</tr>
<tr>
<td>vMALDI</td>
<td>vacuum matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>VO&lt;sub&gt;2peak&lt;/sub&gt;</td>
<td>peak oxygen consumption</td>
</tr>
<tr>
<td>VO&lt;sub&gt;2max&lt;/sub&gt;</td>
<td>maximal oxygen uptake</td>
</tr>
<tr>
<td>4-VP</td>
<td>4-vinylpyridine</td>
</tr>
<tr>
<td>VPDP</td>
<td>Vienna PeeDee Belemnite</td>
</tr>
<tr>
<td>VRM</td>
<td>visual recognition memory</td>
</tr>
<tr>
<td>VT</td>
<td>ventilatory threshold</td>
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<tr>
<td>WAA</td>
<td>whey amino acids</td>
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<tr>
<td>WADA</td>
<td>World Anti-Doping Agency</td>
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<tr>
<td>WAC</td>
<td>Wingate Anaerobic Capacity</td>
</tr>
<tr>
<td>WADC</td>
<td>World Anti-Doping Code</td>
</tr>
<tr>
<td>WAnT</td>
<td>Wingate anaerobic test</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cells</td>
</tr>
<tr>
<td>WBGT</td>
<td>wet bulb globe temperature</td>
</tr>
<tr>
<td>WBPT</td>
<td>whole-body protein turnover</td>
</tr>
<tr>
<td>WBPTO</td>
<td>whole-body protein turnover</td>
</tr>
<tr>
<td>WBS</td>
<td>whole-body strength</td>
</tr>
<tr>
<td>WBSF</td>
<td>Warner-Bratzler shear force</td>
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<tr>
<td>WCID</td>
<td>whole column imaging detection</td>
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<td>WG</td>
<td>Wingate test</td>
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<td>WGA</td>
<td>wheat germ agglutinin</td>
</tr>
<tr>
<td>WGH</td>
<td>wheat gluten hydrolysate</td>
</tr>
<tr>
<td>WGO</td>
<td>wheat germ oil</td>
</tr>
<tr>
<td>WHI</td>
<td>water-induced hyperhydration</td>
</tr>
<tr>
<td>W&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum power output</td>
</tr>
<tr>
<td>WM</td>
<td>white matter</td>
</tr>
<tr>
<td>WMD</td>
<td>weighted mean difference</td>
</tr>
<tr>
<td>Short Form</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
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<td>WOC</td>
<td>World Championships</td>
</tr>
<tr>
<td>vVO&lt;sub&gt;2max&lt;/sub&gt;</td>
<td>minimum speed associated with VO&lt;sub&gt;2max&lt;/sub&gt;</td>
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<tr>
<td>WP</td>
<td>whey protein</td>
</tr>
<tr>
<td>WPH</td>
<td>whey protein hydrolysates</td>
</tr>
<tr>
<td>XCS</td>
<td>cross-country skiing</td>
</tr>
<tr>
<td>XIC</td>
<td>extracted ion current</td>
</tr>
<tr>
<td>XO</td>
<td>xanthine oxidase</td>
</tr>
<tr>
<td>YAS</td>
<td>yeast androgen receptor reporter system</td>
</tr>
<tr>
<td>YPHV</td>
<td>years from peak height velocity</td>
</tr>
<tr>
<td>ZH</td>
<td>zilpaterol hydrochloride</td>
</tr>
<tr>
<td>ZMA</td>
<td>zinc monomethionine aspartate</td>
</tr>
<tr>
<td>ZMA</td>
<td>zinc/magnesium aspartate</td>
</tr>
<tr>
<td>Zn</td>
<td>zinc</td>
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</table>
BACKGROUND FOR TODAY’S DOPING PROBLEMS

More than two thousand years ago, naked athletes competed at the Olympic games in ancient Athens for eternal fame and an olive branch. Today, most athletes run, jump or swim not only for fame and honour but also for money – after all, a gold medal is the ticket to lucrative advertising contracts. Not surprisingly, professional sports now resemble high-tech races in which any technological trick is used to gain milliseconds to set the next record. This involves not only designing faster bobsleds, racing bikes or shark-skin swimsuits but also pushing the physical abilities of athletes by using the latest medical and biological research.

Since ancient times, unethical athletes have attempted to gain an unfair competitive advantage through the use of doping substances, even though the methods and drugs have varied over time. Nowadays a list of doping substances and methods banned in sports is published yearly by the World Anti-Doping Agency, and what is on that list is defined as doping irrespectively if enhance performance or not. A substance or method might be included in the list if it fulfills at least two of the following criteria:

- enhances sports performance
- represents a risk to the athlete’s health
- violates the spirit of sports.

This list, yearly updated to reflect new developments in the pharmaceutical industry as well as doping trends, enumerates the drug types and methods prohibited in and out of competition. Among the substances included are steroidal and peptide hormones and their modulators, stimulants, glucocorticosteroids, beta2-agonists, diuretics and masking agents, narcotics, and cannabinoids. Blood doping, tampering, infusions, and gene doping are examples of prohibited methods indicated on the list. From all these, hormones constitute by far the highest number of adverse analytical findings reported by the antidoping laboratories. Although to date most are due to anabolic steroids, the advent of molecular biology techniques has made recombinant peptide hormones readily available. These substances are gradually changing the landscape of doping trends. Peptide hormones like erythropoietin, human growth hormone, insulin, and insulin-like growth factor I are presumed to be widely abused for performance enhancement. Furthermore, as there is a paucity of techniques suitable for their detection, peptide hormones are all the more attractive to dishonest athletes.

The national attention and economic gains that come with success in professional sports has produced tremendous pressure on modern athletes to win at all costs. In 1994, it was completed a survey of 198 Olympic level athletes. They were asked, if they were guaranteed of winning an Olympic medal and not getting caught would they take a banned substance. One hundred and ninety five of 198 athletes answered yes. When presented with the same scenario, with a guarantee of winning every competition for the next five years, but they would eventually die from adverse effects of the substance, more than 50 percent of the athletes reported that they would still use the substance.

Over the last 20 years systematic doping has become a major threat for elite sport. So far, there is no clear information about the daily practice of doping. Repeated scandals and recent personal statements have added to our knowledge. Several more recent doping agents like Erythropoietin (EPO) and, probably, growth hormone (GH) enhance performance in a highly effective way and, together with the well-known anabolic steroids (AAS), belong to...
the major doping categories. The introduction of EPO has really changed the paradigm in endurance sports allowing a good middle class athlete to become a champion. It is evident that doping practices are influenced by the possibilities of the anti-doping control system. Unethical, criminal medical doctors play a decisive role in the ongoing practice of major doping. Apart from the already mentioned substances AAS, EPO and GH several novel drugs appear on the horizon. They are highly effective and there is no doubt that they will be used in attempts to improve performance. During the last years, doping control systems have also been improved: EPO can now be detected in urine samples and the detection of AAS has also become much more sensitive. However GH hormone detection is not possible at the moment and this remains a major weakness of doping control. Other problems are the control procedures which are far from being optimal. In the future the quality of doping controls will be decisive and not only the quantity; controls will have to be "intelligent". The effective fight against doping in the next years will decide about the survival of elite sport [07003].

The list of drugs prohibited by the World Anti-Doping Agency (WADA) has grown in the last decade. The newer entries into this list include gonadotropins, estrogen antagonists, aromatase inhibitors, androgen precursors, and selective androgen receptor modulators. The use of mass spectrometry has revolutionized the detection of various compounds; however, challenges remain in identifying newer designer androgens because their chemical signature is unknown. Development of high throughput bioassays may be an answer to this problem [10043].

Success in sports is often defined by winning, which drives athletes to use performance-enhancing drugs (PEDs) to gain an advantage over opponents. Over the past 20 years, use of PEDs by Olympic and professional athletes has led to public discussion regarding potential negative health effects and ethical implications of their use. Unfortunately, PEDs are not isolated to professional athletes, as PED use in adolescents has increased dramatically. Many professional organizations, including the American Academy of Orthopaedic Surgeons, have taken a stance against PED use in sports. The AAOS believes neither anabolic steroids nor their precursors should be used to enhance performance or appearance, and that these substances should be banned in all sports programs. Pediatricians and orthopedists are often the first physicians to see these young athletes. It is critical for these physicians to recognize the significance of the problem, have the knowledge to inform adolescents, dissuade them from future use, and provide viable alternatives for meeting performance goals [10435].

Doping incidents infesting high prestige sport events such as the 1998 Tour de France, which was dubbed as the "Tour of Shame" or the 2004 Athens Olympic Games with a sudden double number of positive cases; and the reaction to them (i.e. establishing national anti-doping agencies) indicate that these events may only be the tip of the iceberg. Whilst the adverse analytical findings (positive results) in tests conducted by the World Anti Doping Agency (WADA) remain low around 2 percent, other occasions have revealed an elevated level of substance use. For example, the presence of some kind of drug or supplement was evidenced in 45 percent of the athletes who participated and were tested in the Tour de France 2000. However, the problem seems to be rooted more deeply. The literature supports the assumption that the consideration of and actual use of doping starts well before the athlete reaches his/her best career years as the prevalence of doping, particularly the use of anabolic steroids, is well documented among adolescents and even among pre-adolescent athletes where a steady increase in doping use was observed over the period of four years from age 11 to 15 [07004].

Ergogenic aids (from the Greek, ergon, meaning work) are ingested to enhance energy
utilization in athletes. While there is no scientific evidence to support the usage of these agents for enhancing performance in children and adolescents, using supra-physiological doses may be associated with undesired side effects [07005].

Doping is a problem that has plagued the world of competition and sports for ages. Even before the dawn of Olympic history in ancient Greece, competitors have looked for artificial means to improve athletic performance. Since ancient times, athletes have attempted to gain an unfair competitive advantage through the use of doping substances. A Prohibited List of doping substances and methods banned in sports is published yearly by the World Anti-Doping Agency. Among the substances included are steroidal and peptide hormones and their modulators, stimulants, glucocorticosteroids, β₂-agonists, diuretics and masking agents, narcotics, and cannabinoids. Blood doping, tampering, infusions, and gene doping are examples of prohibited methods indicated on the List. Apart from the unethical aspect of doping, as it abrogates fair-play's principle, it is extremely important to consider the hazards it presents to the health and well-being of athletes. The referred negative effects for the athlete's health have to do, on the one hand, by the high doses of the performance-enhancing agents and on the other hand, by the relentless, superhuman strict training that the elite or amateur athletes put their muscles, bones, and joints [11556].

The fact that athletes routinely use a wide array of substances is well documented as are the potential reasons for use. Whilst performance-enhancing substances are recognised in global as well as local anti-doping prevention programmes, other substances such as alcohol, tobacco and psychoactive drugs constitute a somewhat neglected area in the current idealised anti-doping educational effort. This prevailing approach creates an artificial divide between athletes' lives as sportspersons and private individuals. In reality, athletes constantly navigate their athletically active years on a tightrope between the different expectations they face both as athletes (often being in the spotlight) and as ordinary citizens, and know that failing in one part of their lives could easily result in failures in the other and vice versa. Substances in sports are mainly used for the following reasons:

- enhancing physical capacities (e.g. enhancing endurance, strength, or recovery between exercise sessions)
- psycho-stimulation (e.g. as a way of dealing with psychological stress)
- improving physical appearance (e.g. for achieving a lean figure)

Contemporary sport legislation recognises two types of substances used in sports: non-controlled substances, such as the majority of nutritional supplements, and products that contain prohibited substances (the use of which is often referred to as doping). Nutritional supplementation is defined as a preparation intended to provide nutrients, such as vitamins, minerals, fibre, fatty acids or amino acids, which are otherwise missing or not consumed in a sufficient quantity in the athlete's diet. It is generally accepted that substance use and misuse (SUM) in sports is more common in physically demanding sports (e.g. weightlifting or cycling) than in sports that require advanced specific motor skills (e.g. diving, sailing, table tennis or curling). However, to our knowledge, such generalisation is not sufficiently supported by any systematic comparative analyses of SUM across a variety of sports. Instead, the association of doping with particular types of sports has mostly come from anti-doping testing and the consequential public perception about doping in certain sports such as professional cycling, track and field or weightlifting. The majority of sport activities take place outside of controlled environments, leading to substance use without medical advice or supervision. The mismatch in targets in the anti-doping prevention and deterrence programmes coupled with the limited concern over substances such as alcohol and social drugs raises questions about the suitability of the current anti-doping policy. Whilst both arms of WADA's anti-doping effort represent heroic measures to keep doping out of sports, laboratory statistics shows no
significant change between 2003 and 2009 with the proportion of adverse and atypical findings ranging between 1.50 and 2.12 percent. Self-reports, alternative analyses and epidemiologic estimations indicate that the actual prevalence of doping is greater than this official statistic and ranges up to 40 percent. Although it is difficult to make a direct comparison between the latter and the WADA laboratory report, a recently published report evaluating 7,289 blood samples from 2,737 track and field athletes in the athlete testing pool, using the Athlete Biological Passport approach, estimated the prevalence of blood doping to be at 14 percent overall and between 1 and 48 percent for sub-populations, which supports the results from the epidemiologic studies. Anti-doping policy focuses on preventing selected substance use in situations where such behaviour has been deemed to result in increased athletic performance giving an unfair advantage. Drugs such as anabolic steroids that have long lasting effects and are considered “training drugs” are prohibited both in and out of competition. Other substances, such as alcohol, marijuana and opiates, have only an in situ effect on performance and, thus, are only prohibited in competition. Furthermore, the detection-based doping policy sanctions athletes if there is evidence of a prohibited drug in their body whilst completely disregarding whether the substance found has any performance-enhancing effect on the individual. This narrow view fails to address health concerns that might arise from SUM that happens outside the regulated domain. The main pillars of the current anti-doping approach are fair play, level playing field and equal chance; only those substances that violate these principles are considered with health being secondary. The detection- and sanction-based approach to prevent doping reinforces the priority given to protecting the sport instead of protecting the athletes' health. Future anti-doping policies should address the gaps that currently exist between the testing pool and all athletes including emerging (thus not yet selected for the testing pool) athletes and those training and competing at the sub-elite level; A holistic approach to substance use and misuse that considers athletes' substance use behaviour as a whole should be used in order to prevent doping and preserve not only the integrity of sports but also the athletes' health. Critical analysis of one of the three pillars of the doping ban, namely the protection of the health of athletes, points to the health risks inherently present in elite level sports along with the widespread use of acceptable substances that can also pose health risks. Furthermore, that excessive alcohol or social drug use does not pose infringements upon the anti-doping rules if their use happens outside of competition is a concerning phenomenon among athletes and gym patrons. Both experts and athletes concerned agree that tailored and innovative ways are needed to deliver relevant information on performance-enhancing and illicit drugs to athletes and key stakeholders [11557].

Semantics and definitions of doping

The International Olympic Committee's (IOC) definition of doping is the "use of an expedient (substance or method) which is potentially harmful to athletes' health and/or capable of enhancing their performance, or the presence in the athletes' body of a prohibited substance or evidence of the use thereof or evidence of the use of a prohibited method". There is no mention of intent or of how the substance entered the body. If the substance is in the athlete's body, then he or she is responsible. That is the basis of sanctions for testing positive for a prohibited substance. Sir Arthur Porritt, first chairman of the IOC Medical Commission, noted: "To define doping is, if not impossible, at best extremely difficult, and yet everyone who takes part in competitive sport or who administers it knows exactly what it means. The definition lies not in words but in integrity of character". Such agents are also known as "performance enhancing substances" (PES) or "performance enhancing drugs" (PED). The American Academy of Pediatrics defines these agents as: "...any substance when taken in non-pharmacological doses specifically for the purposes of improving sport performance. A
substance should be considered performance enhancing if it benefits sports performance by increasing strength, power, speed or endurance (ergogenic) or by altering body weight or body composition [10001].

The WADA has defined doping in their World Anti-Doping Code (the “Code”). Under the Code, a violation of one or more of the following rules is considered doping and may result in sanction:

- the presence of a prohibited substance or its metabolites or markers in an athlete’s bodily specimen
- the use or attempted use of a prohibited substance or a prohibited method
- possession of prohibited substances and methods
- administration or attempted administration of a prohibited substance or prohibited method to any athlete
- assisting, encouraging, aiding, abetting, covering up or any other type of complicity involving an anti-doping rule violation or any attempted rule violation.

A substance or method is considered for inclusion on the WADA’s prohibited list if the WADA determines that the substance or method meets any two of the following three criteria:

- medical or other scientific evidence, pharmacological effect, or experience that the substance or method has the potential to enhance or enhances sport performance.
- medical or other scientific evidence, pharmacological effect, or experience that the use of the substance or method represents an actual or potential health risk to the athlete.
- determination by the WADA that the use of the substance or method violates the spirit of sport as described in the “Introduction to the Code”

The current emphasis of prohibition also appears to be based on four factors:

1. Substances within the athlete’s body.
2. Methods that enhance oxygen transfer through blood doping or artificial measures.
3. Altering collected body fluid samples.
4. Genetic manipulation.

The interpretation of the Code is a legal one and largely untested. The broad interpretation of the principles behind the Code would seem to be related to any substance or method that (potentially or actually) enhances sport performance, becomes a health threat to the athlete, or is against the spirit of the sport. If the motivation, and indeed, the imperative, of an athlete is to constantly seek creative ways to improve the sporting performance, what then is the spirit of sport, and what actions are deemed to have violated that spirit? Using a biopsychological perspective, issues of what are acceptable levels of naturally occurring endogenous compounds, what is a method that is not considered doping, and what is the spirit of sport may be discussed especially regarding unnatural amounts of natural substances? Evidence-based medicine requires large sample sizes and preferably randomised-controlled trials to provide statistical evidence of significant effects. The issue of doping, however, is as much an interpretation of the law, as it is about the scientific and statistical evidence. The Code states that “…regardless of whether the expectation of performance enhancement is realistic…” [12015].

The WADA characterises spirit of sport under the Code as:

1. ethics, fair play and honesty;
2. health;
3. excellence in performance;
4. character and education;
5. fun and joy;
6. teamwork; dedication and commitment;
7. respect for rules and laws;
8. respect for self and other participants;
9. courage;
10. community and solidarity.

While these objectives are noble and worthy principles for sport, their abstract nature sometimes creates confusion when applied to the day-to-day realities that athletes face. The Code cites specific issues in the context of the spirit of sport, but these add to the confusion. The WADA states in the Code that the [121015]:
“... use of genetic transfer technology to dramatically enhance sport performance should be prohibited as contrary to the spirit of sport even if it is not harmful...”
and that
“... the potentially unhealthy abuse of certain substances without therapeutic justification based on the mistaken belief they enhance performance is certainly contrary to the spirit of sport regardless of whether the expectation of performance enhancement is realistic...”

**A background for understanding performance-enhancing drugs in sports**

Performance-enhancing drug use is a prevalent problem in sports. It is a problem that has captured the world's attention as the media highlights story after story of athletes who have transformed their bodies over a short period of time, those who have simply defied the aging process in an attempt to prolong a career and those whose careers have been tarnished because of drug use. The baseball investigations and the Mitchell Report of 2007 opened the US’s eyes and gave a glimpse of a secretive underground world. This "world" is much more intelligent and sophisticated than it is given credit for. It was the goal of one article to increase the awareness of the medical provider about the types of steroids and other medications used, the influence these substances have on the athletes, and how and why they use them [12018].

**Judging cheaters in different domains**

The present study examines how individuals judge others who use performance-enhancing drugs in two different domains – the athletic domain and the academic domain. Approximately 1,200 males in their freshman year of college completed a questionnaire that included two scenarios. One scenario described an athlete who misused anabolic steroids to help him succeed at a sporting event. The other described a college student who misused Adderall to help him succeed on his midterm exams. Participants rated the extent to which they thought the target had cheated and the extent to which they felt the substances were necessary for success. Results showed participants believed the athlete was more of a cheater than the student, and this difference got larger as past prescription stimulant misuse increased. Results also demonstrated that participants felt Adderall was more necessary than anabolic steroids for bringing about success. Contributions to the literature on zero-sum and non-zero-sum domains are discussed. Implications for future research and efforts to prevent substance misuse are described [12019].
Overviews

Doping is a phenomenon which in the past years through the various incidences in for example professional cycling has come more and more into the focus of the public interest. Whilst in the young past the problems were to define the term "doping" exactly, today's problem is to prevent adolescents and children of doping. This may partly be achieved by carrying out controls and serious sanctions for doping violations. However, scientific research has also proved that doping usage can be avoided by broad specific prevention measures. In general, the earlier the athletes dope the higher the risk to become addicted later on in life to other legal or illegal drugs. To get closer to a doping-free sport it is important to analyse the prevalence of doping regarding youth-, competitive-, high performance and recreational sports to find more specific ways for prevention. Moreover it may be rewarding to examine also other aspects of doping abuse, risks of addiction, the legal situation, current strategies in the fight against doping to enhance chances of further doping prevention opportunities. By means of this data an all-embracing view should be given over the current situation, problems and prospects [10002].

The drive toward success in sports and the need for a cosmetically acceptable appearance have driven many adolescents to take a wide variety of doping substances. The consumption of these chemicals in the hope and hype of improved sports performance, fueled by the easing of government restrictions on their proof of safety and efficacy, has resulted in an explosion of so-called ergogenic products available to the youth. Agents that have been used include anabolic steroids, anabolic-like agents, designer steroids, creatine, protein and amino acid supplements, minerals, antioxidants, stimulants, blood doping, erythropoietin, beta-blockers, and others. The use of these agents has considerable potential to cause physical and psychological damage. Use and misuse of drugs in this sports doping process should therefore be discouraged. Clinicians providing sports medicine care to youth, whether through anticipatory guidance or direct sports medicine management, should educate their young patients about the hype and hyperbole of these products that may keep them out instead of in the game at considerable financial cost to the unwary consumer [10003].

Testosterone is the principal male sex hormone. As with all natural steroids, it is biosynthesized from cholesterol. Phase I metabolism employs some very specific enzymes and pathways. Phase II metabolism and excretion follow more general patterns. The effects of testosterone are twofold: anabolic and androgenic. Because of its anabolic effects, testosterone is frequently abused in sports. Because of its endogenous nature, testosterone doping is difficult to detect. The standard procedure is based on the evaluation of the urinary steroid profile. Conspicuous samples then are submitted to compound-specific \(^{13}\text{C}/^{12}\text{C}\) analysis. Synthetic and endogenous steroids differ in this measure. However, numerous xenobiotic compounds have been derived from testosterone. The modifications typically aim at a reduction of the androgenic properties while maintaining the anabolic potential. Most of these compounds have been withdrawn from the legal market but are despite that found to be illicitly added to otherwise inefficient nutritional supplements. These products represent a major problem to doping control. Recently, also clinical trials with selective androgen receptor modulators have been started in a hope to get more anabolism and less androgenic effects [10004].

Little symptoms and signs

Abuse of doping substances has become increasingly widespread among athletes also at sub competitive and recreational level, due in part to the lack of controls in form of doping tests. Hypertension and the many other side effects related to the illicit use of prescription
drugs pose a substantial but often underestimated threat to public health. The symptoms are recognized late and are then mostly repressed or misjudged. Since the abuse is concealed to the doctor when help is finally sought, it might result in extensive and expensive tests that can seldom lead to an effective treatment. Two case reports were presented to elaborate on this issue [10005].

**Socio-psychological background to doping**

Sporting competition in the society has become the spectacle that mobilises and brings together the greatest number of people throughout the world, with the corresponding cultural and economic influence that this implies. As a result, the desire for athletic prowess has led sportspersons to undergo intense training programs and to consume substances that improve their performance, at times having recourse to doping techniques. At present, doping is the result of a combination of social, individual, physiological and cultural factors, which affect not only professional, but also amateur sportspeople. In order for the control and eradication of doping to be efficient, it is necessary to understand the problem and the substances that are most employed, amongst which special mention is merited by hormonal substances due to the complexity of detecting them and their possible repercussions on health [06012].

**To move borders …**

Human performance, defined by mechanical resistance and distance per time, includes human, task and environmental factors, all interrelated. It requires metabolic energy provided by anaerobic and aerobic metabolic energy sources. These sources have specific limitations in the capacity and rate to provide re-phosphorylation energy, which determines individual ratios of aerobic and anaerobic metabolic power and their sustainability. In healthy athletes, limits to provide and utilize metabolic energy are multifactorial, carefully matched and include a safety margin imposed in order to protect the integrity of the human organism under maximal effort. Perception of afferent input associated with effort leads to conscious or unconscious decisions to modulate or terminate performance; however, the underlying mechanisms of cerebral control are not fully understood. The idea to move borders of performance with the help of biochemicals is two millennia old. Biochemical findings resulted in highly effective substances widely used to increase performance in daily life, during preparation for sport events and during competition, but many of them must be considered as doping and therefore illegal. Supplements and food have ergogenic potential; however, numerous concepts are controversially discussed with respect to legality and particularly evidence in terms of usefulness and risks. The effect of evidence-based nutritional strategies on adaptations in terms of gene and protein expression that occur in skeletal muscle during and after exercise training sessions is widely unknown [08002].

The creed of the Olympics states: “The important thing in the games is not winning but taking part. The essential thing is not conquering, but fighting well”. As noble a goal as this is, it has little to do with the reality of the modern sports world. Athletes today are expected and encouraged to seek every possible way to improve their performance, including specialised training, hi-tech design of equipment and apparel, scientific and medical support, including the use of nutritional supplements [08003]. Being a high performing athlete is nowadays a profession that requires dedication, long-term commitment and sacrifice [08004], whereas a great concern to sport governing bodies is the chemical alteration of athletic performance. The intriguing question is then what compels athletes to risk their health or reputation for
outstanding sports performance, how high price the athlete is willing to pay, and what factors make athletes vulnerable to doping and at which point of their careers [08005]. New, more powerful and undetectable doping techniques and substances are now abused by professional athletes, while sophisticated networks of distribution have developed. Professional athletes are often the role models of adolescent and young adult populations, who often mimic their behaviors, including the abuse of drugs [08006].

Breaking of borders
To explore how current substance use, including the use of sports supplements and illicit drugs, may impact upon a person’s future intentions to use anabolic-androgenic steroids (AAS) 214 exercising males (mean age, 30 years; range, 17-61 years) recruited from 5 gymnasia in Sydney, Australia, completed a web-based survey. The survey contained questions relating to sport supplement use, illicit substance use, reasons for currently not using AAS, and reasons for intending to use AAS in the future. Participants completed a structured interview schedule that included questions regarding licit and illicit substance use, reasons for non-AAS use, and, where appropriate, reasons for intended future AAS use. Sixteen percent of the sample indicated that they would use AAS in the future. Reasons for future AAS use included increasing muscle size (80 %), improving appearance (74 %), and increasing strength (57 %). Four-fifths (80 %) of the sample reported use of sports supplements, with vitamins and protein supplements commonly reported (83 % and 67 %, respectively); more than one-third (36 %) reported use of creatine in the past 6 months. Half (52 %) of the sample reported use of illicit substances in the preceding 6 months, with amphetamines and cannabis commonly reported (66 % and 62 %, respectively). Significant predictors of intending to use AAS included past 6-month use of creatine and knowing AAS users. The authors concluded that the use of sport supplements and/or illicit substances may remove barriers for the future use of such drugs as AAS [09011].

Much to win
Athletes are rewarded for winning at virtually every level of competition. Second place is viewed as the “first loser”. A coach’s job security is directly related to his team’s success, not that they are simply “fighting well”. Given this reality, it is not surprising that athletes and coaches will sacrifice and risk a great deal in order to obtain a competitive edge and enhance performance at all costs. Performance enhancement in Olympic and professional sport has now become a medical, ethical, and legal problem for modern athletes and athletic organizations. This is primarily due to the amount of money associated with winning in today’s sports industry. Multimillion dollar contracts, appearance fees, international endorsement and sports merchandising represent a billion dollar industry that offers today’s athletes, their sponsors and entourage previously unheard of financial gains. Driven by the millions of dollars now routinely available for winning a sporting event, unethical pharmacists, medical professionals, trainers and sports organizations have worked secretly, and at times without their athletes’ consent, to develop sophisticated doping programs where performance is optimized, often at the risk of the athletes’ health. Now, these same doping programs are moving out of the professional sports market to our youth and other at-risk populations at alarming rates [08006].

Modern sports and the media’s misplaced fixation on fame, fortune and winning at all costs have unintentionally created a growing market for doping substances. These substances, once only abused by elite athletes, are clearly spreading into schools and health clubs worldwide. They are being accepted by a whole new generation of young customers who see reports daily in the newspapers of sports icons accused of abusing drugs only to continue playing, breaking records and claiming fortunes. These same performance-enhancing drugs...
are also abused by adolescents and weekend athletes and non-athletes who have wider behavioral and health risk problems. In addition, these drugs are now being abused by male and female adolescents for cosmetic purposes in an attempt to achieve the "cut" and sexy look promoted by the media [08006].

Ergogenic potential of AAS

It was in 2005 described physiological changes that occurred in a Tour de France cyclist as he matured from 21 to 28 years of age during the period of 1992 to 1999. This cyclist has recently admitted to using performance-enhancing drugs: erythropoietin, red blood cell reinfusion, testosterone, cortisone, and human growth hormone. The main physiological improvements he displayed over this 7-year period during which the author was testing him were an improved gross mechanical efficiency and a reduced body weight. It is also worth noting that four of the five laboratory-based physiological testing sessions were performed in the precompetitive season or with reduced training, although one session was conducted at the end of the competitive season. It is not possible to know the extent to which his drug use might have improved his gross mechanical efficiency because there have not been direct studies conducted to the author’s knowledge. Erythropoietin and/or red blood cell reinfusion would seem to be taken acutely during the competitive season to boost blood volume during a race, although it is conceivable that erythropoietin could be taken as a training aid. However, it seems unlikely that this cyclist had elevated blood volume at the time of physiological laboratory testing. Furthermore, it is unlikely that an elevated blood volume would improve gross mechanical efficiency, because studies that have acutely infused red blood cells into athletes have not reported changes in efficiency measured from open circuit spirometry. Since publication of the 2005 paper, there have been several reports of champion athletes displaying improved efficiency of movement. The world record holder in the women’s marathon, Paula Radcliffe, displayed a remarkable 15 percent improvement in running economy between 1992 and 2003. Therefore, there is growing evidence that mechanical efficiency can improve with chronic training. However, we cannot be absolutely certain that the improved gross mechanical efficiency and reduced body weight displayed in the subject of the 2005 paper was not somehow influenced by his reported drug use [0021].

Although AAS have been used in sports for ergogenic purposes for decades, many believed that the improved performance seen with AAS was due to their influence on motivation and aggression. It took landmark studies from Bhasin et al to prove that testosterone dose-dependently increases muscle mass, maximal voluntary strength, and power and that these improvements are correlated with circulating testosterone concentrations. There are multiple mechanisms that lead to this anabolic response. Androgens are known to increase fractional muscle protein synthesis and increase the size of both type I and type II muscle fibers. Studies have also shown that testosterone directs the pluripotent mesenchymal stem cell toward myogenic lineage rather than adipogenic lineage. Some clinical trials of testosterone replacement in hypogonadal elderly men have also shown improvement in muscle strength. Because androgens improve maximal voluntary muscle strength, it is understandable that a high rate of androgen use is seen among weight lifters and other power athletes. However, the use of androgens in endurance events, e.g. bicycling, is not based on scientific evidence because androgens have not been shown to improve whole body endurance. There are two types of androgen doping: direct and indirect. Direct doping involves exogenous administration of both natural and synthetic androgens. Indirect doping refers to using compounds that increase the production of endogenous testosterone (estrogen receptor antagonists or aromatase inhibitors), androgen precursors (dehydroepiandrosterone (DHEA), androstenedione), and gonadotropins [13003].
Testosterone/epitestosterone (T/E ratio)

To circumvent the detection of synthetic androgens, athletes have resorted to doping with testosterone. Hence, the detection of illegal use depends upon distinguishing between endogenous and exogenous testosterone. There are two main detection methods available. Epitestosterone (17alpha-hydroxy-4-androsten-3-one) is a 17-epimer of testosterone that is also secreted by the Leydig cells of the testes. It was first described in 1947 as an androgen metabolite; however, the first direct evidence of its urinary excretion was seen in the 1960s. It is biologically inactive, and there is no interconversion between testosterone and epitestosterone. Although its production rate is less than 5 percent of testosterone, its urinary excretion is 33 percent that of testosterone. It is mainly excreted in the urine as a glucuronide; however, a small amount is also excreted as a sulfate. Due to its rapid clearance, excretion rates of testosterone and epitestosterone are similar, making the urinary T/E ratio approximately 1. Because there is no interconversion between the two compounds, administration of exogenous testosterone results in an increase in the T/E ratio. Measurement of T/E ratio in a large number of athletes has shown that generally the ratio remains below 6. This initially led the IOC to adopt the cutoff threshold of 6 to consider the test as positive. However, it was later recognized that genetic differences in various populations may influence T/E ratio. Some athletes have low endogenous epitestosterone production rate; hence, their T/E ratio always exceeds 6. To the contrary, a deletion polymorphism in uridine diphosphate glucuronyl transferase 2B17, an enzyme that facilitates epitestosterone excretion, lowers T/E ratios (especially in Asian populations). Considering these genetic variants, the IOC recently lowered the T/E cutoff ratio to 4. Hence, any value above 4 is considered suspicious. To complicate matters, some athletes may mask the use of exogenous testosterone by coadministration of epitestosterone. In such cases, the exogenous use of epitestosterone is detected by elevated concentrations of its urinary metabolites. Generally, in athletes not using AAS, the T/E ratio remains fairly constant. Hence, monitoring T/E ratio serially in the same athlete is occasionally performed to detect any change that may suggest illicit use [13003].

Isotope ratio mass spectrometry (IRMS)

If the T/E ratio is abnormal, the WADA requires additional confirmation by gas chromatography combustion IRMS, which involves measurement of $^{13}$C/$^{12}$C isotope ratio in testosterone. This method is based on the fact that the percentage of $^{13}$C (a naturally occurring isotope of carbon) in endogenous testosterone (synthesized in the body from carbon sources derived from animals and plants) is higher than in synthetic testosterone. Hence, synthetic testosterone has a lower $^{13}$C/$^{12}$C ratio. During the IRMS, the steroids are separated by gas chromatography and oxidized to carbon dioxide in a combustion chamber. The ratio of $^{13}$CO$_2$/$^{12}$CO$_2$ is monitored in an isotope ratio mass spectrometer. A lower $^{13}$C/$^{12}$C ratio suggests exogenous testosterone administration. In addition to testosterone, IRMS is also used to distinguish endogenous from exogenous nandrolone, dihydrotestosterone, and DHEA [13003].

Synthetic androgens

The availability of synthetic androgens has not only provided more options for the athletes but at the same time circumvents their detection by the doping authorities because chemical signatures of many of these compounds are not readily available. Norbolethone has been credited as the first designer androgen that was identified in the 1960s. In 2002, its chemical signature was identified after it was detected in an athlete’s urine. In 2003,
tetrahydrogestrinone was identified. It is a derivative of gestrinone (a progestin); hence, it is both a potent androgen and progestin. In 2005, a third designer androgen was identified as desoxymethyltestosterone. In addition to these androgens, SARMs have recently gone to clinical trials and have the potential for abuse in various sports. Various bioassays are being employed in the detection of these designer androgens [13003].

**Androgen bioassays**

Bioassays are functional assays that are employed to determine the bioactivity of a compound. Hence, they measure potency of a substance. The unit of bioactivity is defined as the lowest concentration at which a functional response is measured. This unit is then compared with known bioactivity of a standard compound (such as testosterone or dihydrotestosterone). The main advantage of these bioassays lies in their ability to detect even those androgens whose chemical structure is unknown, hence making this an ideal tool to detect novel designer androgens (unlike RIA and mass spectrometry that require the chemical signature of the steroid to be known). Bioassays can be divided into in vivo and in vitro assays. The former involves evaluation of in vivo responses of androgenic stimulation such as the growth of capon comb or changes in weight of androgen-dependend tissues (levator ani, seminal vesicles, etc.) in castrated male rats. These assays are time consuming and expensive. Hence, in vitro assays are used more commonly because they are faster, relatively inexpensive, and can simultaneously screen a large number of test samples. The in vitro assays are of three types [13003]:

- **Receptor binding assays**: These assays evaluate the ability of a substance (test sample) to bind to the androgen receptor in the presence of a known radioactive ligand for the receptor. Hence, the displacement of the radioactive ligand from the androgen receptor by the test sample correlates with its bioavailability.

- **Cell proliferation assays**: These assays involve measurement of bioactivity of an unknown substance by its ability to proliferate androgen-dependent cell lines, e.g. human prostate cancer cell line (LNCap). Although sensitive, these assays are not entirely specific for androgens.

- **Reporter gene assays**: In these assays, the bioactivity of a compound is measured by the degree of expression of the reporter gene. These assays can be performed in either yeast (usually Saccharomyces cerevisiae) or mammalian cells. In short, the process entails choosing a host cell line that does not express an endogenous androgen receptor. The next step involves introduction of two plasmids into the cell line: an expression plasmid for constitutive expression of androgen receptor in the cells and a reporter plasmid in which the androgen response element sequences drive the expression of a truncated form of firefly (Photinus pyralis) luciferase. The latter serves as the reporter gene. The amount of luciferase expression is proportional to the bioactivity of the compound being tested in the sample and is measured by luminometry. These assays have now been optimized for high throughput screening. Hence, a large number of samples can now be tested with reporter gene assays.

**Indirect androgen doping**

Indirect doping refers to strategies employed by athletes that result in a sustained increase in endogenous testosterone production and is tailored to circumvent the banning enforced on the administration of natural or synthetic androgens by WADA. These strategies include [13003]:

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99
- estrogen blockers such as estrogen receptor antagonists (antiestrogens) or aromatase inhibitors
- androgen precursors such as DHEA and androstenedione
- gonadotropins.

**Estrogen blockers**

The rationale for using estrogen antagonists and aromatase inhibitors stems from the fact that estrogen is a more powerful negative regulator of hypothalamic-pituitary-gonadal axis than testosterone itself. This is evident in men with congenital aromatase deficiency where both androgens and gonadotropins are elevated and exogenous estrogen replacement suppresses them. Although less than 1 percent of daily testosterone produced is aromatized to estradiol, it has 100-fold higher molar potency than testosterone. Hence, the reflex rise in endogenous testosterone as a result of estrogen blockade may result in myotrophic effects, although the rise in serum testosterone concentrations is modest (50-65 %). Hence, both estrogen receptor antagonists and aromatase inhibitors have been put on the list of banned drugs by WADA. The original antiestrogens were the nonsteroidal drugs like clomiphene and tamoxifen that bind to both estrogen receptor alpha and beta. Since then, newer nonsteroidal agents have become available such as raloxifene, toremifene, and droloxfene. The newest steroidal estrogen analog that has come to market is fulvestrant. Aromatase is an enzyme that is a product of CYP19 gene and is responsible for converting testosterone to estradiol. Aromatase inhibitors completely and irreversibly inhibit this enzyme, which results in a decrease in estradiol synthesis. Aminoglutethimide was the first steroidal drug in this class. Since then, more specific and potent steroidal aromatase inhibitors have become available such as testolactone, atamestane, and exemestane. The nonsteroidal aromatase inhibitors include anastrozole, letrozole, and vorozole. Recently, mass spectrometric methodologies have become a formidable tool in identifying both estrogen blockers and aromatase inhibitors. Hence, a sensitive and robust assay is now available to circumvent indirect doping by these agents [13003].

**Androgen precursors**

This class of agents includes drugs like androstenedione and DHEA. Androstenedione is a prohormone that is produced in the gonads and the adrenals in both sexes. It is synthesized from DHEA and is converted to testosterone by 17beta hydroxysteroid dehydrogenase. On the other hand, DHEA is predominantly secreted by the adrenal glands. Both these compounds were sold over the counter in an uncontrolled fashion as dietary supplements for almost two decades under the Dietary Supplement Health and Education Act. It was only recently that the US Congress added androstenedione to the list of banned steroids. To the contrary, the IOC prohibited the use of these agents more than a decade ago (DHEA in November 1996 and androstenedione in December 1997). Androstenedione at a dose of 300 mg results in a significant increase in serum testosterone levels, whereas a dose of 1500 mg results in an increase in lean body mass and muscle strength. Similarly, one study showed that DHEA at high doses (100 mg) also results in an increase in muscle strength. Hence, the banning of both these prohormones by the IOC is justified because they do carry ergogenic potential. Each of these hormones results in an increase in T/E ratio, which is then followed by confirmation of $^{13}\text{C}/^{12}\text{C}$ ratio with IRMS. In the case of androstenedione, another mode of confirmation is by measuring its metabolite, 4-hydroxyandrostenedione, by mass spectrometry or IRMS [13003].

**Gonadotropins**
Another form of indirect doping is the use of gonadotropins such as LH or human chorionic gonadotropin (hCG), both on the WADA’s list of prohibited drugs for male athletes. The latter is a dimeric glycoprotein containing an alpha- and a beta-subunit that is normally produced by the human placenta. Its alpha-subunit is similar to other glycoprotein hormones such as FSH, LH, and TSH. It undergoes glycosylation with sialic acid residues that prolongs its half-life and makes it a long-acting analog of LH. Clinically, recombinant LH or hCG is used to stimulate spermatogenesis and endogenous testosterone production in men who have central hypogonadism and desire fertility. Not only are hCG and LH expensive, requiring injections several times per week, but the evidence that they improve muscle strength is sparse. A small randomized placebo-controlled study of older men showed that hCG therapy resulted in an increase in serum testosterone levels and lean body mass; however, it failed to improve upper or lower body strength (61). In addition to its presumed ergogenic effects, subjects abusing AAS also use hCG to avoid detection of exogenous testosterone by stimulating endogenous testosterone production and preventing testicular atrophy. Because hCG doesn’t alter T/E ratio, this methodology is not useful in the detection of indirect doping with hCG. The detection of hCG is initially performed with immunoassays, and if positive, confirmation is carried out with immunoextraction and mass spectrometry. hCG is not banned in female athletes because there is no evidence suggesting that it improves muscle mass and strength. Furthermore, there is an ethical dilemma because positive hCG may reveal underlying pregnancy and is therefore considered an invasion of privacy. Detection of LH is accomplished by immunoassays [13003].

GnRH is synthesized in the hypothalamus, and it is transported from there via the hypothalamic-pituitary portal venous microcirculation to stimulate the gonadotrophs, resulting in LH and FSH production. For this, GnRH must be secreted in a pulsatile fashion (peaks every 60–90 min). Continuous administration of GnRH desensitizes the gonadotrophs, resulting in down-regulation of GnRH receptors. This results in suppression of testosterone to castrate levels (after an initial flare) and forms the basis of therapy for prostate cancer. Pulsatile administration of GnRH via pumps is performed in clinical settings in men who have a hypothalamic cause of hypogonadism; however, serum testosterone levels achieved are generally within the therapeutic range. Hence, GnRH administration doesn’t result in sustained production of supraphysiological testosterone levels. For these reasons, GnRH and its analogs are not banned by WADA. However, mass spectrometry-based assays have been developed to detect the use of exogenous GnRH. Under normal circumstances, only fragments of GnRH may be detectable in the urine. Hence, a urinary concentration of intact GnRH of at least 20 pg/ml is considered unambiguous evidence for surreptitious use of the hormone [13003].

For decades, scientists have been trying to dissect out the anabolic and androgenic properties of androgens. The discovery of SARMs was a result of the constant quest for achieving tissue selectivity mainly so that the anabolic effects of these compounds on muscle and bone can be achieved without any risk to the prostate. This tissue selectivity is of tremendous importance in the elderly who have a higher incidence of prostate-related events. This was finally accomplished in 1998 when it was developed the first SARM. This compound was derived from androgen receptor antagonists like flutamide and bicalutamide. Over the years, four groups of SARMs have been synthesized. These include arylpropionamides, bicyclic hydantoins, quinolines, and tetrahydroquinolines. Based on their anabolic properties in musculoskeletal tissues, in January 2008, WADA added SARMs to its prohibited list. Because SARMs possess enormous structural heterogeneity, their detection remains a daunting task. However, most of the available SARMs can be detected either by liquid or gas chromatography tandem mass spectrometry [13003].
**Boosting in paraolympic**

“Boosting” is defined as the intentional induction of autonomic dysreflexia (AD) by athletes with a spinal cord injury (SCI) at or above the level of T6 for the purpose of improving sports performance. Boosting has been shown to confer up to a 10 percent improvement in race time. Additionally, to compete in a hazardous dysreflexic state, whether intentional or unintentional, would present an extreme health risk to the athlete. For these reasons, the International Paralympic Committee strictly bans the practice of boosting, and has developed a protocol to test for its presence. Testing was performed at three major international Paralympic events. Education regarding the dangers of AD was provided to athletes and team staff. Testing was conducted on athletes from the relevant sport classes: Athletics (wheelchair racing classes T51/T52/T53) and Handcycling (H1). Key parameters included the athlete’s demographics (gender, country of origin), classification and blood pressure measurements. An extremely elevated blood pressure was considered to be a proxy maker for AD, and a systolic blood pressure of ≥180 mm Hg was considered a positive test. A total of 78 tests for the presence of AD were performed during the three games combined. No athlete tested positive. The number of athletes tested, by classification, was: 6 in Athletics T51, 47 in Athletics T52, 9 in Athletics T53 and 16 in Handcycling H1. Of those tested, the average systolic and diastolic blood pressures were 135 mm Hg (range 98-178) and 82 mm Hg (range 44-112), respectively. All athletes were compliant with testing. No athletes were withdrawn from competition due to the presence of AD. Testing for the presence of AD in paralympic athletes with SCI prior to competition has been carried out for the first time at three major international paralympic competitions. There have been no positive tests thus far. Knowledge gained during these early testing experiences will be used to guide ongoing refinement of the testing protocol and the development of further educational initiatives.

**Possible lack of effects of doping**

Doping is a serious issue bedevilling the sporting arena. It has consequences for athletes’ careers, perception of sports in the society and funding of sports events and sporting organisations. There is a widespread perception that doping unfairly improves results of athletes. A statistical study of information on best lifetime results of top 100 m sprinters (males better than 9.98 s, females 11.00 s), over the period of 1980-2011 was conducted. Athletes were divided into categories of "doped" (n=17 males and 14 females), based on self admission, the confirmed detection of known doping agents in their bodies or doping conviction, and "non-doped" (n=46 males and 55 females). No significant differences (unpaired t-test) between dopers and non-dopers were found in their average results: male "dopers" 9.89 s identical with 'non-dopers' 9.89 s, females 10.84 s and 10.88 s respectively. Slopes of regressions of best results on dates for both 'dopers' and 'non dopers' were not significantly different from zero. This indicates that no general improvement as a group in 100 m sprint results over a quarter of a century occurred irrespective of doping being or not being used. Since there are no statistical differences between athletes found "doping" and the others, one of the following must be true: (1) "doping" as used by athletes so detected does not improve results, or (2) "doping" is widespread and only sometimes detected. Since there was no improvement in overall results during the last quarter of the century, the first conclusion is more likely. Objectively, various "doping" agents have obvious physiological or anatomical effects. These may not translate into better results due to the clandestine use of doping that prevents its scientific structuring. Perception of the effectiveness of doping should be reconsidered. Policy changes may be required to ensure the continued fairness and equity in testing, legislation and sports in general.
Cognitive doping

A growing concern in today's society is the consumption of substances to increase physical or cognitive performance. For example, the use of drugs such as anabolic steroids in professional sports has long been a concern. In order to combat physical doping in professional sports, the World Anti-Doping Agency (WADA) annually lists banned substances. Besides illicit or banned drugs, athletes also consume legal and freely available substances such as analgetics, caffeine, and other ergogenic aids (e.g. creatine, vitamins, minerals, carbohydrates, proteins), which may also improve physical performance. Cognitive doping can include illicit substances (e.g. cocaine) and prescription drugs (pharmacological neuroenhancement) such as stimulants (e.g. methylphenidate and amphetamines), antidepressants, beta-blockers, or modafinil, which are primarily designed and used for the treatment of diseases. Prevalences for the use of such cognitive-enhancing substances range from 1.2 to 35 percent among German and American students, and are estimated to be 20 percent among readers of the journal Nature, 20 percent among surgeons, and 5 percent among office workers in Germany. Besides illicit and prescription drugs, the use of legal and freely available substances such as ginkgo biloba or caffeinated drinks (e.g. coffee, energy drinks) are also a matter of debate although their ergogenic potential is still unknown. It is of particular concern that these nutritional supplements have been shown to fail tests of safety, purity, and quality of ingredients and may contain prohibited substances. One study assessed, for the first time, prevalence estimates for physical and cognitive doping within a single collective of athletes using the randomized response technique (RRT). Furthermore, associations between the use of legal and freely available substances to improve physical and cognitive performance (enhancement) and illicit or banned substances to improve physical and cognitive performance (doping) were examined. An anonymous questionnaire using the unrelated question RRT was used to survey 2,997 recreational triathletes in three sports events in Germany. Prior to the survey, statistical power analyses were performed to determine sample size. Logistic regression was used to predict physical and cognitive enhancement and the bootstrap method was used to evaluate differences between the estimated prevalences of physical and cognitive doping. 2,987 questionnaires were returned (99.7%). 12-month prevalences for physical and cognitive doping were 13 and 15 percent, respectively. The prevalence estimate for physical doping was significantly higher in athletes who also used physical enhancers, as well as in athletes who took part in the European Championship in Frankfurt compared to those who did not. The prevalence estimate for cognitive doping was significantly higher in athletes who also used physical and cognitive enhancers. Moreover, the use of physical and cognitive enhancers were significantly associated and also the use of physical and cognitive doping. The use of substances to improve physical and cognitive performance was associated on both levels of legality (enhancement vs doping) suggesting that athletes do not use substances for a specific goal but may have a general propensity to enhance. This finding is important for understanding why people use such substances. Concerning a potential gateway for cognitive doping, the prevalence estimate for cognitive doping was significantly higher in athletes who used cognitive enhancers than those who did not. Since we do not know which type of substance was used first by the athletes – legal and freely available or illicit substances – these data do not strongly support the gateway theory that the use of cognitive enhancers is the first step for cognitive doping. Consequently, more effective prevention programs against substance abuse and doping could be developed [13023].

Doping may be defined, broadly, as the use of unauthorised means to increase performance in sport. Doping is most commonly associated with the use of drugs. It was discussed some recent advances in neuroscience that suggest that the skills and abilities underpinning sports performance can be enhanced using technologies that change the activity of the brain. These factors may include motor learning, enhanced muscular strength or reduced fatigue,
or even changes to mental state or concentration. The devices needed to generate these
effects are already available, and are currently in use in laboratories or clinics to produce
short- or long-term changes in performance. It was argued that brain stimulation, or
neurodoping, will become a key technology for the future of sport and sports medicine. It will
be suggested that neurodoping may have different uses in different sports, and it was argued
that each sport must determine whether neurodoping should be considered as cheating, or
should be considered a legitimate aid to training or performance [13024].

Brain stimulation techniques

Two main brain stimulation techniques are available. Transcranial magnetic stimulation
(TMS) involves the discharge of brief magnetic pulses through a stimulating coil held against
the subject’s head. This rapidly-changing magnetic field induces electric currents in the brain
tissue near to the centre of the coil. The immediate effect of this is to generate action
potentials in those cells, followed by a refractory period as the cell recovers. The fire-
and-recover pattern is most visible when a TMS pulse is triggered over the hand area of
primary motor cortex: muscle activity of the contralateral hand, measured with electromyo-
graphy, shows a burst (called the motor-evoked potential) followed by relative quiescence
(called the silent period). Recent developments in the application of TMS have involved
temporally patterning the pulses delivered by the stimulator to induce both inhibitory and
excitatory effects in the target brain area (called thetaburst stimulation). These effects outlast
the stimulation phase by several tens of minutes, with the possibility of longer-term
reorganisation of brain activity if the stimulation is applied at regular intervals. Transcranial
current stimulation (tCS) comes in two common variants. Transcranial direct current
stimulation (tDCS) involves passing a weak electric current from a negative electrode
cathode) to a positive electrode (anode). The magnitude and polarity of the electric field at
the brain surface near the electrodes determines its effect: cells in the vicinity of the anode
will tend to increase in excitability, through a process thought to involve a modulation of the
resting membrane potential of the cells; conversely, cells near the cathode become less
active through the same process. Transcranial alternating current (tACS) uses a similar
principle, except that the current alternates at a specific frequency. Researchers typically
apply tACS with a frequency related to functionally-relevant oscillatory brain potentials, such
as might be seen with electroencephalography. tCS has a number of advantages over TMS.
The technology is cheaper and more portable. Indeed, wireless tCS stimulators are now
commercially available, and websites exist that give instructions for home-made tCS
stimulators. TMS is, however, a more focal technique, with a relatively small area of the brain
being affected by the sti13ulation, whereas the electric field induced by tCS spreads across
the whole brain surface [0024].

What can be done with brain stimulation?

It is foreseen two domains where neurodoping may potentially change performance in sport.
These divide into immediate gains from increasing cortical excitability versus longerterm
gains from stimulation during training. In the “acute” phase following stimulation, participants
have demonstrated enhanced motor skills including: improved time-to-fatigue, response time,
and tremor suppression. The effect of tDCS is maximal shortly after the end of stimulation
and declines over roughly a 20- to 60-min period, depending on the stimulation parameters.
The effects of theta-burst TMS last for a similar length of time, but with the peak of effect
some 5 min after the end of stimulation. So, it is possible to envisage a time when an athlete
might take a “hit” of stimulation before shooting a pistol or setting off on a ski slalom. A
second use of neurodoping might be in skill acquisition. Skills learned in the context of
anodal tDCS are acquired more rapidly, and reproduced more accurately, than those learned
without. Sports performance at the highest levels require good technique and good timing. These are skills learned during training, so enhancing the efficiency of learning during the training phase will be of greater benefit at competition time. It has been suggested that an athlete could use these techniques to make training more efficient and thereby gain an advantage. It is possible that neurodoping will add little to the performance of elite athletes. Most studies of brain stimulation recruit non-expert, healthy participants from the community of the laboratory (in practical terms, university students) and test in conditions where performance is likely to be changed but not reach its maximum. Elite athletes who are already performing close to the physical limits of the human body may not gain from the potential benefits of brain stimulation. Further research is needed to explore whether neurodoping and elite performance are compatible [13024].

Can it be detected? What are the risks?

There is no known way to detect reliably whether or not a person has recently experienced brain stimulation. A modern technique for analysing brain composition is magnetic resonance spectroscopy (MRS), which can detect changes in the concentration of neurotransmitters and related metabolites. Theta-burst TMS appears to affect inhibitory processing by gamma-amino butyric acid (GABA) while not affecting excitatory processing involving glutamate. Anodal and cathodal tDCS modulate GABAergic and glutamatergic processing differently. When looking specifically at the brain region targeted by TMS or tDCS, the changes in brain chemistry are of the order of 10 percent in metabolite concentration, and require carefully controlled conditions to pick out the signal from the noise. Several factors mean that MRS is unlikely to be of practical utility in detecting neurodoping [13024].

Ethical issues

There is an argument that human enhancement of any type is not wrong in sport or in any other context. It has been argued that enhancing performance with drugs is analogous to an act of creativity whose only limit should be the safety of the participant. So, by this argument, regulating pharmacological enhancers places an unnecessary constraint on the limits of human achievement. Allowing drug enhancement would simply add another option for athletes who wish to choose among all the available means of improving their overall performance. While the analogy with the kind of neuroenhancement is not perfect, nonetheless brain stimulation offers a potential adjunct for immediate performance or for training and should be considered as if it were another form of drug doping. A related question about the use of human enhancement is whether the performance shown by the person is “authentically” theirs. If a neuro-enhanced sprinter is faster in reacting to the starter’s pistol, is that advantage hers or should the stimulator be given the credit? It was recently argued that brain training with tDCS enhances the person’s own latent cognitive abilities and increases the efficiency of a training programme. Thus, brain stimulation mediates a person’s ability but does not enhance it in the strictest sense. A third argument is practical: as discussed above it is not possible, as far as is known, to determine whether a person has or has not had brain stimulation. Each sport determines its own rules. It would be suggested that each sport determines whether neurodoping poses a risk to its ethos. For example, performance in a sport such as pistol-shooting would be greatly improved by tremor reduction, so governing bodies should decide whether shooters should be prevented from using tACS during or immediately before competing to reduce tremor, just as beta-blockers are banned in many sports. Conversely, a tennis player’s performance in a match is heavily influenced by the probability of regularly getting the first service in, which is a skill learned in training and therefore potentially susceptible to neurodoping [13024].
Effect of international results during the anti-doping era

100 meter and 5000 meter

The introduction of doping substances and methods in sports triggers noticeable effects on physical performance in metric sports. Here, we use time series analysis to investigate the recent development in male and female elite sprinting performance. Time series displaying the average of the world's top 20 athletes were analyzed employing polynomial spline functions and moving averages. Outstanding changes in performance over time were statistically analyzed by Welch's t-test and by Cohen's measurements of effect. For validation we exemplarily show that our analysis is capable of indicating the effect of the introduction of in- and out-of-competition doping testing on women's shot put as well as the effects of the market introduction of erythropoietin (EPO) and the introduction of EPO and continuous erythropoiesis receptor activator (CERA) testing on 5000 m top 20 male performances. Time series analysis for 100 m men reveals a highly significant drop by more than 0.1 s from 2006 to 2011 with a large effect size of 0.952. This is roughly half of the effect size that can be found for the development of the 5000 m performance during the introduction of EPO between 1991 and 1996. While the men's 200 m sprinting performance shows a similar development, the women's 100 m and 200 m sprinting performances only show some minor abnormalities. It was discussed why the striking sex-specific improvement in sprinting performance is indicative for a novel, very effective doping procedure with insulin-like growth factor-1 (IGF-1) being the primary candidate explaining the observed effects [13068].

100 meter in Olympic Games

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However, so far no clinical trials have demonstrated a beneficial effect of IGF-1 administration on athletic performance. IGF-1 acts in endocrine, autocrine, and paracrine modes on skeletal muscle. Exogenous administration of IGF-1 causes powerful anabolic actions similar to the effects of hGH. Due to limited experience with long-term administration of exogenous IGF-1, only side-effects from short-term usage have been documented in clinical trials. Next to edema, arthralgia, headache, and jaw pain especially hypoglycemia should be mentioned. Furthermore, it is appealing to use IGF-1 in combination with other drugs like hGH, insulin, and anabolic steroids, as the combined application most likely provides an enormous potential to improve performance. Consequently, IGF-1 is included in the WADA list of banned substances, but its abuse by elite athletes is assumed to be lower than for hGH, because of its lower availability due to the lack of a natural resource from which IGF-1 could be harvested. Nevertheless, IGF-1 will be more attractive to use than hGH, since testing for hGH is improving. Preparations containing IGF-1 were first approved for the American market in August 2005 by the US Food and Drug Administration (FDA) for the treatment of growth failure in children. Therewith the possibilities to acquire and abuse IGF-1, despite the lack of a natural source, have been increasing even more. The results from the time series analysis disclose a time decreasing trend in male short-distance running that started around 2005/2006. Over 100 m, elite athletes underwent a large effect (0.952) in the last five years that is clearly displayed by both approaches: spline and moving average. The last six years over 200 m entailed an effect size of 0.481. In women's track and field sprinting, an effect is also detectable over 100 m. However, the moving average is at the same level in the beginning and the end of the regarded interval and over 200 m there is no effect noticeable after 2004. These explicit developments can be discussed to be associated with doping, but it is important to mention the existence of other possible or additional explanations. Simultaneously with the time decreasing trend over 100 m for men, we noted that more athletes from the West Indies entered the annual top 20 lists. Thus changing conditions concerning training for these populations of athletes could contribute to the stated developments. When taking doping into consideration as a plausible explanation, several different substances have a strong enough physiological effect and therefore could be related to this development. HGH may still be used as presently no robust long-term testing method for the detection of hGH administration exists. Unfortunately it is also possible that new synthetic ‘designer steroids’ have been developed that cannot be detected by the tests currently available. Additionally the list of other substances that could possibly improve sprinting performance is long. Next to hGH, IGF-1 and ‘designer steroids’ another example would be oestrogen receptor antagonists. However, it was argued in the following that among the substances that were recently introduced to the market, IGF-1 is a very important candidate that could explain the observed development in sprinting performance. The mechanism of IGF-1 with its beneficial actions concerning physical performance presupposes its abuse, alone and in combination with other agents, in track and field. 2008 is the year in which we first noted a significant change over 100 m, but IGF-1 was approved in 2005. Interestingly, we detected the first sign of an EPO effect in 1992, although it entered the American market in 1989. In one opinion, athletes first need to get acquainted with drugs they want to abuse. Its handling is usually only known for medical purposes. So the doping-related application of the drug and its incorporation in the training process take some time to be optimized. Additionally, examination of the number of athletes that ran the 20 fastest times of each year over 100 m shows a fall from ten in 2005 to four in 2006 and eleven athletes ran the fastest times in 2011. It was believed that this progression illustrates a slow integration: IGF-1 abuse started with a small amount of athletes and now is integrated in the world elite of track and field sprinters. Induced by the effect detected over the women's 100 m, it was suspected female athletes to be using IGF-1, too. Even so, it seems to be less beneficial for women, because there is no parallel effect over 200 m. Furthermore, athletes usually try to deliver top performances in Olympic Games years. Over the women's 100 m and 200 m, 2008 – the year of the Olympic Games in Beijing – is the minimum. In contrast,
the men have their minima in 2011 and there is a local maximum in 2008 over 200 m. A study showed that women responded with a smaller increase in IGF-1 levels, although they received a larger dose of hGH compared to men. So far it is not known whether treatment with IGF-1 leads to sex differences in the related physiological response. However, sex differences can be found for serum levels of IGF-1. IGF-1 would not be the only doping substance with different effects depending on sex. For example, our analysis of women’s and men’s shot put shows greater effects for female shot putters. Considering the whole period from the introduction of anabolic steroids to the beginning of out-of-competition controls, it was computed an effect size of 3.482 for men from the earlier effect onset in 1961 to 1988 and 5.154 for women from 1966 to 1988. Franke and Berendonk mentioned that enormous effects, especially in female athletes, were noticed in the 1970s during the systematically organized doping efforts of the German Democratic Republic. Anabolic steroids and EPO are the best-known doping substances and the portrayed examples account for enormous effect sizes. Anabolic steroids had an effect of 3.177 over ten years in the women’s shot put and EPO 1.925 over a five-year period in the men’s 5000 m. Both were abused in a variety of sports disciplines. Nowadays blood doping, including potential abuse of EPO, is widespread among athletes regardless of the progress in testing. This is well in line with our notion that the introduction of EPO testing had no profound effect. In comparison, out-of-competition testing for anabolic steroids led to a clear decrease in performances displayed through the arithmetical mean of the 20 best athletes of each year. The effect sizes that was computed for short-distance running from 2001 to 2011 are not as large, but still enormous enough to animate other athletes to misuse IGF-1. In our opinion, IGF-1 could definitely also be effective concerning track and field in the jumping disciplines, although analysis of long-jump and high-jump did not display any recent significant developments, but future examination might. Thus, the official introduction of the recently developed test procedure for IGF-1 could prevent the expansion of the abuse of IGF-1 in professional sports [13069].

Contrary to that statement, when analyzing men’s 100 m performance at the Olympic level, the winners (average 86.36 kg) have outweighed the rest of the finalists (77.72 kg) dating back thirty years. This phenomenon is also consistent when comparing the medal winners (80.45 kg) versus non-medal winners. Contrary to the claims made by the author’s there have been statistically significant improvements over the last quarter century in elite performance of the 100 m dash, both in the men’s and women’s division. A separate one-way ANOVAs compared the 100 m finals times for men and women from the Olympic Games over the past 20 years with Bonferroni post hoc analysis when appropriate. There were significant differences observed for men and women. Post hoc analysis revealed men’s times from 2012 were significant lower than 1992 and 2000; with 2008 also being lower than 1992. Women’s times from 2012 were lower compared to 2000. This holds true for not only the medal winners of the Olympic Games, but also of the participants in the finals as a whole [13070].

It is believed that the remarkable improvements observed in the 100 and 200 m are mostly due to the performance of one single athlete, Usain Bolt, who has repeatedly broken the world record of these sprint disciplines in 2008 and 2009, by lowering the limits by notable coefficients of 0.984 and 0.993, respectively. Since the dramatic drop of the polynomial lines is almost entirely due to the performance of one athlete, it cannot be attributed to an entire group of athletes. This phenomenon has been recently defined as the Usain Bolt effect, and has been attributed to stature and reduction in stiffness as a consequence of the increased contact time and lower step frequency, which both result in an advantage in relative power development and mechanical efficiency. This would not support the theory of improvement by doping but – rather – the well known possibility of “extreme outliers” that seldom occur in a normal distribution of athletes, and may remarkably account for an improvement in records. A second important aspect is that Usain Bolt, the athlete who has dramatically improved both
100 and 200 m world records, has never been found positive during anti-doping controls, either in- or out-of-competition, by whatever anti-doping authorities. Until opposite evidence can be provided, this is the only reliable proof that we have that the world records were broken by a fair athlete. Then, it is also questionable to assert that the use of hGH and/or IGF-1 explains the effect on 100 m performance between 2006 to 2011, since it has been clearly acknowledged that athletes have been abusing hGH for its anabolic effects since the early 1980s, whereas the first test was not introduced until 2004. Accordingly, it is much more likely that the abuse of hGH had been commonplace before 2006, and not afterwards. An identical consideration can be made on the potential abuse of IGF-1, since this substance appeared much earlier than 2006 on the black market [13071].

Is doping-free sport a Utopia?

The impressive legend of 7-time Tour de France winner Lance Armstrong has died, replaced by an equally impressive legacy of shameless lying and cheating on a grand scale, team doping orchestration and discipline on and off the road, and conspiracy to fool the world while earning tens of millions of dollars. But, the harsh reality is that the doping-control system did not catch arguably the biggest, boldest, and most brazen drug cheat in the history of sport. Hundreds of analytical doping tests performed over nearly a decade in state-of-the-art laboratories failed to reveal his charade. And the same system failed to catch any of his teammates as long as they were on his team. Riders have confessed under oath how easily the tests could be manipulated. Doping testing failed miserably. A federal investigation compiling 1000 pages of evidence and testimony from 26 different cycling teammates and support staff finally caught Armstrong. All of this evidence is now public. If one of the world’s most visible athletes evaded detection despite “500 doping tests” (probably the real number was about half that) over all those years while the whole world watched and the doping-control officials took blood and urine samples, why should we have any confidence that the situation is better today? In numbers, the power-to-weight ratio for the best cycling climbers in the world on their hardest, most decisive climbs rose from about 5.8 W/kg in the late 1980s and early 1990s quite rapidly to ≥6.3 W/kg by the mid-1990s and remained there to the mid-2000s, before beginning to fall again to 5.8 to 6.0 W/kg in the most recent tours. This anecdote suggests that EPO plus consumption/injection of anabolic agents facilitating accelerated recovery may have been “worth” as much as a about 10 percent increase in sustainable power over climbs of 30 to 40 minutes. The Festina doping scandal of 1998 seemed to scare the peloton straight in 1999, because climbing power (otherwise) inexplicably dropped about the same 10 percent, before rising again in 2000 and thereafter. That is a measurable, chronologically preciseblip that adds some contrast to the doping fingerprint. With the exception of doping-positive Alberto Contador, the fastest climbs in Tour history were performed during the 1996-2006 time window, with slower climbing times since by the best climbers and the Tour de France winners in the last 2 years [13028].

Armstrong’s doping violations were detected by antidoping laboratories on several occasions, but these results appear to have been systematically swept under the rug at a higher level. A broomshaped checkbook may have been used, as evidenced by large “financial contributions” by Armstrong to the International Cycling Union (UCI). This would mean not that doping testing systematically failed but that the system was corrupt. This is a second small piece of evidence that the drug labs can go a long way toward keeping cycling and all sports cleaner, provided that the sport-governing bodies they serve are 100 percent clean. A third source of encouragement amid all the gloom is that the culture of collective “tight lips” among athletes has collapsed. In addition to a substantial increase in positive tests since 2006, a large share of a generation of top cyclists has confessed their doping
practices. A long-standing code of silence has now been broken. Effective doping on the immense scale uncovered with Team US Postal (and subsequent versions of Team Armstrong) exemplifies how critical the cooperation of athletes, sport scientists, doctors, and coaches is to achieving the combined goals of extensive and effective doping on one hand and detection avoidance on the other. Good sport science and the open dissemination of research and best-practice methodology in all aspects of athlete preparation have probably contributed to making the race to the top of the international podium more expensive. The top-20 medal-winning countries are winning a smaller share of the total medals in the Olympics, from 90 percent in 1992 down to 75 percent in 2012. This is great for sport and in part a result of better sport-science support in a larger number of countries, all along the path from talent identification to physiological and technical development, to performance peaking, to race management. The difference between a gold medal and finishing out of the medals is smaller than ever before. Good sport science can make the tiny difference between gold and fourth place on a given day. At the world-class level, the performance-enhancing effects of doping clearly exceed what cutting-edge sport science can achieve with further optimization of training programs, recovery methods, etc. For the sake of sports, it must be hoped that good sport science, and not just good chemistry, matters more in the future [13028].
DOPING AND ANTI-DOPING HISTORY

Overviews

Though we may still sing today, as did Pindar in his eighth Olympian Victory Ode, "... of no contest greater than Olympia, Mother of Games, gold-wreathed Olympia...", we must sadly admit that today, besides blatant over-commercialization, there is no more ominous threat to the Olympic games than doping. Drug-use methods are steadily becoming more sophisticated and ever harder to detect, increasingly demanding the use of complex analytical procedures of biotechnology and molecular medicine [13001].

The use of drugs to enhance physical performance has occurred since the beginning of recorded time. Ancient Greeks at emushrooms and sesame seeds to enhance performance, and Roman gladiators used stimulants to increase endurance. In modern sports, documentation of the abuse of performance enhancing drugs appeared in the early1900s, when athletes ingested stimulants (caine, amphetamines, ephedrine, and strychnine) to alleviate fatigue and increase focus. Anabolic-androgenic steroids (AAS) are now the most common illicit drugs used to enhance performance at the modern Olympic Games along with stimulants, primarily by weight lifters and athletes in track-and-field. The AASs are a group of synthetic derivatives of testosterone with both androgenic, anabolic, and masculinizing (androgenic) effects. In 1889, physiologist Charles E. Brown Seuard reported improvement in a variety of his body functions (strength, intellect, and force of urine stream) following the injection of an extract of testicles from the dog and guinea-pig. The primary natural male hormone, testosterone was first isolated from the testis of bulls in 1935 by Davidet al. Butenandt and Hanisch and Ruzicka et al independently synthesized testosterone in the same year, and both chemists received the Nobel Prize in 1939 for their work. Most of the AASs were developed during the 1950s when chemists attempted unsuccessfully to separate the anabolic and androgenic properties of these testosterone derivatives. Nandrolone, the 19-nor analog of testosterone was the first anabolic steroid with sufficient dissociation of androgenic and anabolic properties to justify introduction into clinical practice during the 1950s. Dr. John Ziegler, an American physician- weight lifter, administered AASs to 3 future American weight lifting champions after learning of the success of AAS-using Russian weight lifters at the 1954 World Championships. In 1958, the US Food and Drug Administration (FDA) approved the use of methandrostenolone (Dianabol) for the treatment of hypogonadism, resulting in the increased availability of this steroid. By the mid-1960s, the use of AASs to enhance performance in sports spread, particularly among weight lifters and other strength athletes. An estimate done was that a third of the US track-and-field athletes in the 1968 pre-Olympic training camp were using AAS. From 1966 until the collapse of the German Democratic Republic in 1990, hundreds of East German physicians and scientist performed doping research and administered prescription drugs as well as unapproved experimental drug preparations to adult and adolescent athletes of both sexes. In 1963, the Council of Europe defined doping in sports as a result of the death of a Danish cyclist at the 1960 Olympics, the death of a UK cyclist at the Tour de France, and the prevalence of potentially life-threatening drugs in sports. In 1964, the International Olympic Committee (IOC) unanimously voted to ban doping in sports. By 1967, the IOC established a Medical Commission with responsibilities to prohibit doping, to develop the Olympic Movement Anti-Doping Code, and to formulate a list of prohibited substances. In 1974, the IOC banned the use of AASs, and testing for AASs by immunoassay screening and gas chromatography-mass spectrometry confirmation began in 1976. In 1984, the use of testosterone was also banned. From the 1960s through the 1980s, the German Democratic Republic established a systematic doping program for thousands of their athletes that included the use of
parenteral preparations of epitestosterone propionate to avoid detection of illicit AASs. In 1988, the IOC stripped Ben Johnson of his Olympic gold medal and world record in the 100-meter dash for using an AAS. In the same year, the distribution of possession of AAS with intent to distribute without a valid prescription became a felony, when US Federal Food, Drug, and Cosmetic Act (FFDCA) was amended as part of the Anti-Drug Abuse Act. In 1990, the Anabolic Steroids Control Act defined an AAS as any drug or hormonal substance chemically and pharmacologically related to testosterone (other than estrogens, progestins, and corticosteroids) that promotes muscle growth. These synthetic compounds became DEA schedule III drugs as defined by the US Controlled Substances Act. Later, this act was amended by the Anabolic Steroid Control Act of 2004; on January 20, 2005, the amended Controlled Substance Act added both anabolic steroids and prohormones to the list of controlled substances, making possession of the banned substances a federal crime. In response to continuing demand for illicit AASs, designer AAS appeared as a means to avoid detection of these illicit drugs. An example was the synthesis of tetrahydrogestrinone from the palladium-charcoal catalyzed hydrogenation of gestrinone by the Bay Area Laboratory Cooperative, an American nutritional supplement company. However, analyses and legal action resulted in the banning of several athletes as a result of the use of these synthetic steroids. Subsequently, major league baseball revamped their AAS policy calling for a 50-game ban for first-time offenders (up from 10 days), a 100-game penalty for second-time offenders (up from 30 days), and a life time ban for a third positive test. Previously, a baseball player could be suspended for life only after the fifth positive test [13002].

The earliest records of doping in sport come from the Ancient Olympics games when athletes are reported to have taken figs to improve their performance. With the advent of modern pharmacology in the 19th century, many athletes began to experiment with cocktails of drugs to improve strength and overcome fatigue. As this practice was not illegal, there are good records of the lengths athletes would go to in order to win. Alongside the benefits, came the dangers and following several fatalities, a code to ban performance enhancing drugs was gradually developed. Growth hormone was first isolated from the human pituitary gland in the 1950s. Its anabolic effects were soon recognised and athletes had begun to abuse it by the early 1980s, at least a decade before it was used therapeutically by adult endocrinologists. A number of high profile athletes have admitted using growth hormone. Detection of its abuse has been challenging and the lack of an effective test has undoubtedly encouraged its abuse. Only now are methodologies being developed that should stem this tide [09001].

Doping is as old as sport itself. The word itself is likely derived from the Dutch word, dop, the name of an alcoholic beverage made from grape skins used by Zulu warriors to enhance their prowess in battle. In the 20th and 21st centuries, various agents have been used: alcohol, caffeine, strychnine, amphetamines and then after World War II anabolic/androgenic steroids and more recently some of the peptide hormones including insulin, erythropoietin, human GH, and a host of other growth factors. Anabolic/androgenic steroids remain a mainstay in the performance enhancement drug arena given that they are really the only major class of steroids that are unequivocally anabolic with salutary effects on athletic performance. The arms race will continue as long as designer steroids are produced, tested in vitro, and then, for the more difficult parameter, that they and their metabolites will not lead to a positive test at the doping control laboratory [11554].

Sporting associations have stated that the fundamental aims of doping controls and anti-doping policies are to [06101]:

- uphold and preserve the ethics of sport
- safeguard the physical health and mental integrity of the players
- ensure that all competitors have an equal chance.
The word doping is probably derived from the old Dutch word dop, which was the name of an alcoholic beverage made of grape skins used by Zulu warriors to enhance their prowess in battle. Ancient Greek athletes are known to have used special diets and stimulating potions to fortify themselves. Strychnine, caffeine, cocaine, and alcohol were often used by cyclists and other endurance athletes in the nineteenth century. Thomas Hicks ran to victory in the Olympic marathon of 1904 in Saint Louis with the help of raw eggs, injections of strychnine, and doses of brandy administered to him during the race. The term “doping” progressed into mainstream use in the early twentieth century, originally referring to drugging of racehorses. The practice of enhancing performance through foreign substances or other artificial means, however, is as old as competitive sport itself. By the 1920s it had become evident that restrictions regarding drug use in sports were necessary. In 1928 the International Amateur Athletic Federation became the first international sport federation to ban the use of doping (use of stimulating substances). Many other international federations followed suit, but restrictions remained ineffective as no tests were performed. The death of Danish cyclist Knud Enemark Jensen during competition at the Olympic Games in Rome 1960 – the autopsy revealed traces of amphetamine and nicotinyl tartrate – increased the pressure for sports authorities to introduce drug tests. In 1966 the International Cycling Union and the Fédération Internationale de Football Association (FIFA) were among the first international sports federations to introduce doping tests in their respective world championships. In the following year the International Olympic Committee (IOC) instituted its Medical Commission and set up its first list of prohibited substances. Drug tests were first introduced at the Olympic Winter Games in Grenoble and at the Olympic Summer Games in Mexico in 1968 after the urgency of anti-doping work had been highlighted by another tragic death, that of cyclist Tom Simpson during the 1967 Tour de France. A reliable test method to detect anabolic steroids was finally introduced in 1974 and the IOC added anabolic steroids to its list of prohibited substances in 1976. This resulted in a marked increase in the number of drug disqualifications in the late 1970s, notably in strength related sports such as throwing events and weightlifting. Blood boosting or blood doping, which involves removal and subsequent reinfusion of the athlete’s blood in order to increase the level of oxygen-carrying haemoglobin, has been practised since the 1970s. The IOC banned blood doping as a method in 1986. Anti-doping work was complicated in the 1970s and 1980s by suspicions of state-sponsored doping practised in some countries. The most famous doping case of the 1980s concerned Ben Johnson, the 100 metre runner who tested positive for stanozolol (anabolic steroid) at the 1988 Olympic Games in Seoul. Johnson's case focused the world's attention to the doping problem to an unprecedented degree. In 1998 a large number of prohibited medical substances were found by the police in a raid during the Tour de France. The scandal led to a major reappraisal of the role of public authorities in anti-doping affairs. As early as 1963, France had been the first country to enact anti-doping legislation. Other countries followed suit, but international cooperation in anti-doping affairs was long restricted to the Council of Europe. In the 1980s there was a marked increase in cooperation between international sports authorities and various governmental agencies. Before 1998 debate was still taking place in several discrete forums (IOC, sports federations, individual governments), resulting in differing definitions, policies, and sanctions. One result of this confusion was that doping sanctions were often disputed and sometimes overruled in civil courts. The Tour de France scandal highlighted the need for an independent international agency, which would set unified standards for anti-doping work and coordinate the efforts of sports organizations and public authorities. The IOC took the initiative and convened the World Conference on Doping in Sport in Lausanne in February 1999. Following the proposal of the Conference, the World Anti-Doping Agency (WADA) was established on 10 November 1999. On 5 March 2003, at the second World Conference on Doping in Sport, some 1200 delegates representing 80 governments, the IOC, the International Paralympic Committee, all Olympic sports, national Olympic and Paralympic committees, athletes, national anti-doping
organisations, and international agencies supported the World Anti-Doping Code as the basis for the fight against doping in sport. The Code entered into force on 1 January 2004. On 19 October 2005, the World Anti-Doping Code was adopted at the 1st International Convention against Doping in Sport by the General Conference of UNESCO at its plenary session. Some 184 countries have signed the Copenhagen Declaration on Anti-Doping in Sport, the political document through which governments show their intention to implement the World Anti-Doping Code by the ratification of the UNESCO Convention [06002].

Drug use by athletes to improve performance is not a new practice. As early as BC 776, the Greek Olympians were reported to use substances such as dried figs, mushrooms, and strychnine to perform better [06003].

Anti-doping efforts started in earnest after the 1960 Olympic Games in Rome. During a team time trial, 23-year-old Danish cyclist Knud Enemark Jensen collapsed, fractured his skull and died. An autopsy reportedly found traces of amphetamine and a blood-vessel dilator in his system. Although the drugs might not have caused his death, the episode forced cycling officials to take a closer look at doping. The UCI banned some performance enhancers, and in 1967 the International Olympic Committee established a commission to ferret out doping in sport. The task is thankless: anti-doping agencies thwart one cheating strategy, only for another to emerge. The 1972 Olympic Games in Munich, Germany, ushered in testing for stimulants, but athletes had moved on to undetectable, naturally occurring hormones, such as testosterone. Anti-doping authorities now measure the ratio of testosterone in the blood to a related molecule called epitestosterone. In response, some athletes have reportedly found ways of regulating epitestosterone to keep the ratio in check. For cycling and other endurance sports, human recombinant EPO fuelled a doping revolution. EPO is a natural hormone that promotes production of oxygen-carrying red blood cells. The first synthetic, or recombinant, version was developed by the biotechnology company Amgen in Thousand Oaks, California, and in 1989 it was approved by the US Food and Drug Administration to treat anaemia. It also offered cyclists an easy endurance boost that helped them to excel in gruelling stage races. The drug is nearly identical to the hormone naturally churned out by the kidneys, so was impossible to detect. It is also easier to administer than blood transfusions, which had been used to the same effect. Typically, red blood cells account for 40-45 percent of the blood, but in the heyday of EPO doping, some riders were showing up at starting lines with haematocrits of more than 60 percent. The UCI instituted a “no-start” rule, disqualifying riders if their haematocrits on the morning of a race were above 50 percent for men and 47 percent for women. So cyclists began diluting their EPO-boosted blood with saline solution to keep their haematocrits below the threshold. The drug companies that produce EPO have helped anti-doping laboratories to develop direct tests based on subtle biochemical differences between the recombinant molecules and the natural form. The first of these was approved for use in 2000. But athletes increasingly obtain knock-off forms produced in China and India, and researchers have struggled to keep up [11415].

The use of doping agents, once restricted to professional athletes, has nowadays become a problem of public health, since it also concerns young people and non-competing amateurs in different sports. The use is also diffused in social life for improving physical appearance and enhancing performance and even dietary supplements assumed to improve performance often contain anabolic steroids. While decades ago the so-called “classical doping agents” (like stimulants and narcotics) were used, to-day anabolic steroids are more widely diffused. Anabolic steroids are synthetic substances prepared by introducing modifications in the molecular structure of testosterone, the main natural androgenic anabolic steroid that forms in testes interstitial cells. The first report concerning the use of anabolic
steroids by an athlete who searched for increased weight and power dates 1954. In 1974 the misuse of anabolic steroids in sports was banned by the International Olympic Committee and control tests were implemented in 1976 Montreal Olympic Games through radioimmunoassay analysis: the technique, however, only allows for unspecific detection of a limited number of exogenous steroids. Over the years, always new doping substances are synthesized and, as a consequence, the list of prohibited compounds is continuously updated and new suitable analytical methods for their detection and determination in biological matrices are continuously required. In doping control analysis the knowledge of steroid metabolism pathway in human body is of primary importance and the analytical methods must permit the simultaneous detection and determination not only of the forbidden precursor agents but also of their metabolites. In addition, the potential presence and amount in the biological samples of species that can interfere in the analysis should be evaluated. Also the several anabolic steroids, specifically designed to circumvent doping control, put on the market have been incorporated in the list of the prohibited substances of the World Anti-Doping Agency (WADA). In WADA list steroids figure in three main classes, namely anabolic steroids, corticosteroids and substances with anti-estrogenic properties. It must be strongly reminded that assumption of doping agents not only leads to athletes the possible failing of doping tests but causes important health risk and WADA prohibited list establishes criteria to highlight the alteration of the natural steroid profile caused by exogenous administration [12001].

**Specific dates**

Some important dates in the history of anti-doping [11416]:

1928  The IAAF becomes the first federation to ban doping

1966  The IAAF, the Union Cycliste Internationale (UCI), and the Fédération Internationale de Football Association (FIFA) introduce urine drug tests in their respective championships

1967  The International Olympic Committee (IOC) institutes its Medical Commission and sets up the first list of prohibited substances

1968  Drug tests introduced at the Olympic Games

1970s  Marked increase in the number of doping-related disqualifications after the introduction by the IOC of anabolic steroids to its list of prohibited substances

1980s  Introduction of out-of-competition testing

1986  Blood transfusion banned by IOC

1990s  rEPO included in the IOC’s list of prohibited substances

1990s  Introduction of blood tests

1999  WADA is established

2004  The World Anti-Doping Code is adopted worldwide

2005  United Nations Educational, Scientific and Cultural Organization (UNESCO)
adopts the International Convention against Doping in Sport

The drugs-in-sport problem first came to prominence in the 1960s with the use of amphetamines among professional European cyclists. At the same time, steroids were becoming widespread in the United States and Eastern Europe. As money flowed in commensurate with an unprecedented media interest, sport began to globalise in the 1980s, and its commercial value increased exponentially. A number of high-profile drugs scandals occurred in the 1980s, culminating in the Ben Johnson affair in 1988. The consequent media feeding-frenzies encouraged a number of sporting bodies to introduce anti-doping regulations. Plagued by constant allegations of drug use in international sport, along with the Tour de France drug crisis of 1998, the IOC led the push for the establishment of an agency with the responsibility for managing and enforcing global anti-doping policy. WADA was born in 1999 and has become a global force in the war on drugs-in-sport. WADA's success in establishing an international drug code has been underpinned by three developments. First, WADA is funded jointly by the IOC and a group of national governments. This has provided the agency with both capital and influence. Secondly, WADA has secured a series of international declarations that have commended and ratified the policy code it has developed. Thirdly, WADA policy has recently been approved by the United Nations Educational, Scientific and Cultural Organisation (UNESCO) as an international convention. These achievements have consolidated WADA’s position as the central international agency for regulating drug use in sport [10312].

Despite the fact that doping is not a new phenomenon in sport, enhancing performance through artificial means has only been banned since the 1960s. Doping as a potential danger to the modern Olympic movement was recognized in the '50s and officially acknowledged ten years later by the creation of a list of banned substances. After an agonizing period over athletes' amateur status, performance enhancing drugs have taken over as the major basis for tension and concern within the Olympic movement since 1972. Researchers seem to agree that doping is unwelcome in sport. However, opinions are divided between doping being a serious deviance one must fight against and doping as undesirable but unavoidable consequence of the institutionalized sport. Notably, the reason behind banning doping initially was the growing concern about athletes' health. Doping only became established as unethical after that point [07004].

The seriousness of the doping problem is reflected by the recent increase in organised effort to combat doping in sport. The first step toward a globalised effort was the creation of the Anti-Doping Code of the World Anti-Doping Agency (WADA) in 1999 as an organisational level response to the Festina Scandal at the Tour de France, parallel to the European Union's (EU) pledged support in the fight against doping. The first report (known as the HARDOP report) was commissioned in 1998 and published in 1999, followed by targeted research projects under the EU's Competitive and Sustainable Growth run under 5th Framework Programme. The globalised effort was recently manifested in the creation of the International Convention Against Doping in Sport by the United Nations Educational, Scientific and Cultural Organization (UNESCO). The UNESCO convention is the first legally binding international framework setting out the responsibilities of national governments and is currently signed either as ratification, acceptance, approval or accession by 65 countries [07004].

**Early (modern) history**
The use of drugs and ergogenic substances to augment athletic performance, commonly referred to as doping, has evolved along with sporting events. Ancient Olympic athletes consumed mushrooms, plants, and herbs in an attempt to gain a competitive edge. The modern Olympic Games made their debut in 1896, and mixtures of cocaine, ephedrine, and strychnine were used to enhance performance [08007].

Alfons Bukowski (1858-1921) is commonly regarded as the pioneer of anti-doping research. In 1910, he developed a method to detect alkaloids in horse saliva. One hundred years later, this is a good moment to remember Bukowski, an outstanding Polish pharmacist, often mistakenly represented in world literature as a Russian chemist. It is also an occasion to mention that the real driving force in the history of doping were events related to horse rivalry [10313].

In the 1904 Olympics, marathon runner Thomas Hicks used a mixture of brandy and strychnine and nearly died. Mixtures of strychnine, heroin, cocaine, and caffeine were used widely by athletes, and each coach or team developed its own unique secret formulae. This was common practice until heroin and cocaine became available only by prescription in the 1920s. During the 1930s, it was amphetamines that replaced strychnine as the stimulant of choice for athletes. In the 1950s, the Soviet Olympic team first used male hormones to increase strength and power [08006].

History of anabolic steroids

Almost half a century before the discovery of androgens, Brown-Sequard (the father of andrology) had recognized that the contents of testicular extracts could improve libido, energy, and muscle strength. After synthesis of testosterone, Boje was the first to suggest that sex hormones may enhance physical performance. Although the most well-known phase of AAS abuse in Olympic history is that of the Soviet weight-lifting team in the 1952 and 1956 Olympic Games, it is believed that some German athletes were given androgens even during the 1936 Berlin Olympics. The introduction of AAS among the American athletes is attributed to Dr. John Ziegler (a physician-member of the US Weight-Lifting Team) who learned about the use of AAS by the Russian team in 1954 during his trip to weight-lifting championships in Vienna. Upon his return, Dr. Ziegler experimented with testosterone on weight lifters in the York Babel Club in Pennsylvania. That is considered to be the beginning of AAS abuse in sports in the United States, which later spread from high-intensity strength-training games to sports such as field athletics, baseball, swimming, etc. The two common patterns of AAS abuse are “stacking” and “cycling.” Stacking involves the use of two or more androgens in progressively increasing doses over a short period of time. Cycling refers to the intermittent use of AAS where use of steroids is followed by a drug holiday. The practice of “cycling” is based on the notion that drug holidays prevent desensitization to large doses of androgen [13003].

The first report concerning the use of anabolic steroids by an athlete who searched for increased weight and power dates 1954. In 1974 the misuse of anabolic steroids in sports was banned by the International Olympic Committee and control tests were implemented in 1976 Montreal Olympic Games through radioimmunoassay analysis: the technique, however, only allows for unspecific detection of a limited number of exogenous steroids [13004].

Detection of testosterone
The history of AASs is a tale that has its roots in ancient "endocrinology." More than 6000 years ago, farmers noted an enhanced ability to domesticate animals after castration. Years later, the medical theories of "humoralism" developed. This doctrine was based on a theory that attempted to explain diseases based on imbalances among the four humors: sanguine, choleric, melancholic, and phlegmatic. In addition, ancient Egyptians and Romans believed that testicles and animal penises held special healing powers. Ancient Greek athletes used a wide variety of alleged performance-enhancing drugs, such as plant extracts and testicular extracts. These early theories and practices marked the beginning of future discoveries. John Hunter (1728-1793) was a Scottish surgeon who was later appointed as Surgeon General for the British army. He made many noteworthy contributions to science, including contributions to the understanding of digestion, fetal development, venereal diseases, dentistry, and lymphatics. He conducted the first testicular transplant in 1786 in which he removed a testicle from a rooster and implanted it into a hen. However, it was not until 1849 that Arnold Adolf Berthold (1803-1861) found evidence of a "bloodstream substance" from roosters that affected their appearance and behavior. His theory was correct, but it was not widely accepted by his contemporaries. Berthold was a professor at the University of Göttingen, and he performed experiments on roosters while he was a curator at a local zoo. He observed the impacts of castration and the reimplantation of testicular tissues on roosters. Once castrated, the roosters' combs decreased in size, they lost interest in the hens, and they lost their aggressive male behaviors. Those effects were reversed after reimplanting testicular tissues or extract, despite denervation. Despite these findings, other researchers did not cite Berthold's work for nearly 50 years [07007].

Perhaps the most well-known researcher of anatomy and physiology was Charles Edouard Brown-Sequard (1817-1894). Brown-Sequard, a prominent French physiologist and Harvard professor, was one of the founders of modern endocrinology. He had a strong interest in endocrinology, and he studied adrenal glands, testes, thyroid, pancreas, liver, spleen, and kidneys. He is probably most famous for his auto-experimentation with testicular substances (extracted from guinea pigs and dogs), the results of which were published in 1889. He reported increased strength, mental abilities, and appetite and even claimed that the process relieved constipation and increased the arc of his urine stream. Although no one is sure why he experienced these effects, his experiment caused others to investigate the testicular substance as a possible cure for various ailments, such as diabetes, tuberculosis, epilepsy, paralysis, gangrene, anemia, influenza, arteriosclerosis, Addison's disease, hysteria, and migraine headaches. He encouraged testing of his testosterone products by providing free samples to physicians. Unfortunately, with such widespread use, shoddy researchers subjected animals and humans alike to high risks for infection and inflammation. Austrian physiologist Oskar Zoth was the first person to propose injecting athletes with a hormonal substance, as published in his 1896 paper describing how the use of an "extract" improved muscular strength and the "neuromuscular apparatus," thus potentially improving athletic performance. He and his physician partner, Fritz Pregl (1869-1930), self-injected testosterone extracts from bulls and measured the strength of their middle fingers by plotting them on "fatigue curves". They won the Nobel Prize in chemistry in 1923 [07007].

Substances referred to as "chemical messengers" were discovered in 1902 by English physiologists and professors, William Maddock Bayliss (1860-1924) and Ernest Henry Starling (1866-1927), at University College London. Bayliss' research team was the focus of an animal rights controversy in 1903 – the Brown Dog Affair – in which Bayliss was alleged to have performed a live dissection of a brown dog in his laboratory. He, of course, denied the accusation and won a civil suit, donating the money to the University for further research; he even wrote articles promoting the humane treatment of animals. Other accomplishments included contributions on shock, digestive system, and endocrinology; being knighted in 1922; and authoring four editions of Principles of General Physiology. Starling officially
coined the term “hormone” in 1905 when giving a Croonian Lecture (prestigious lectureships) titled “The Chemical Control of the Functions of the Body” to the Royal College of Physicians. The term “hormone” means “to urge on” or “impulse or arouse” in the sense of “to set in motion” in Greek. Years later, reports suggested that a Cambridge physiologist, William B. Hardy, actually suggested the term “hormone” to Bayliss and Starling. In 1911, Andre Pezard first noted a direct relationship between the amount of testicular extract injected into a rooster and the size of his comb [07007].

An Austrian physician, Eugen Steinach (1861-1944), developed the “Steinach operation,” an “autoplastic” treatment for the “middle-aged and listless”. The 20-minute operation involved ligation of the vas deferens, often at the most proximal position to the testicle. This allegedly increased testosterone production. He believed that the incision produced a “back pressure” on the testicle, thus increasing testosterone production by the interstitial cells. He also implanted testicular tissue grafts between the peritoneal muscles. He reported that his patients were able to regrow hair, had better erections with less premature ejaculation, and had increased libido. Despite little clinical evidence of his claims on “rejuvenation,” the results of his operations, at best, likely were due to the power of suggestion; however, he performed this procedure on some famous patients, including Sigmund Freud and William Butler Yeats. He also discovered, by transplanting male sex glands into females and vice versa, that guinea pigs developed sexual behaviors of the opposite sex. Later research proved that sex hormone injections have no effect on sexual orientation but that high doses of testosterone may increase sexual desire. In 1913 in Chicago, Victor D Lespinasse (1878-1923), a urologist, claimed that he cured a patient who had sexual dysfunction by transplanting a testicle from a donor. He removed the organ, made three transverse slices, and inserted them into muscle tissue around the patient's scrotum. His most famous patient was Harry F. McCormick (husband of Edith Rockefeller), whose case was described in The New York Times. Five years later, the first journal of Endocrinology was published. In the 1920s, Sergio Voronoff, a Russian-French physician and surgeon, made a fortune from removing testes from animals (including the controversial monkey and chimpanzee gland transplants by way of vivisection, sparking campaigns from animal rights groups and satirical cartoons and books on the subject) and transplanting them into men. The chimpanzee tissue was not implanted inside the scrotum but instead in the tunica vaginalis. He concluded that his experiments with testicular transplants helped to relieve pain and provided a sense of well-being [07007].

It was apparent to researchers that some substance circulating in the blood was responsible for their findings; however, it was not until 1929, when a German chemist and professor, Adolf Butenandt (1903-1995), isolated the first sex hormone, that a new path of discovery was initiated. He isolated estrone from the urine of pregnant women and later isolated 15 mg of androsterone (“andro” = male, “ster” = sterol, “one” = ketone) from 15,000 L of urine from a local policemen. Over the next few years, researchers found that the hormones isolated from the testes were more androgenic than were those isolated from urine. Perhaps the most famous, and perhaps unethical, research of “organotherapy” occurred in the 1920s and 1930s at San Quentin prison in California where Leo Stanley transplanted the testicles from executed prisoners into impotent prisoners. He had a limited supply, so he turned to substituting a variety of animal gonads (from ram, sheep, goat, deer, and boar) to treat men who suffered from senility, epilepsy, and paranoia. Over the years he performed hundreds of operations. During the 1930s, three pharmaceutical companies each hired research teams to isolate the testicular hormone. The term testosterone (“testo” = testes, “ster” = sterol, “one” = ketone) was coined in 1935 by Karoly David and his research team. Ernst Laqueur isolated testosterone from bull testes. The research team was funded by the pharmaceutical company Organon in Oss, The Netherlands. Later that same year (on a team funded by Schering Corporation in Berlin, Germany), Butendant and Gunir Hanisch published “A
method for preparing testosterone from cholesterol" in a German journal. Only a week later, Leopold Ruzicka (who synthesized androsterone in 1934) and A Wettstein published “On the artificial preparation of the testicular hormone testosterone (andro-sten-3-one-17-ol)” in Helvetica Chimica Acta and applied for a patent. Butenandt and Ruzicka won the Nobel Prize for chemistry in 1939. Butenandt spent a large part of his career studying the sex hormones and their relationship with one another. His work laid the foundation for the production of cortisone [07007].

In the late 1930s, experimentation using humans involved testosterone propionate (slow-release derivative) and methyl testosterone (oral form that was slower to metabolize). Most of the research at that time was focused on treating hypogonadism in men (inducing and maintaining secondary sexual characteristics and treating impotence). Charles D Kochakian discovered an increase in protein anabolic processes, thus opening the door for the treatment of a variety of disorders by restoring tissue and stimulating growth. In 1939, it was reported that daily topical application of testosterone by females enlarged the clitoris and increased sexual desire. The use of synthetic testosterone skyrocketed after publication of the book “The Male Hormon”e by Paul de Kruif in 1945, which made claims of increasing libido and boosting athletic performance. Testosterone was a proposed treatment for menorrhagia, dysmenorrhea, estrogen-derived breast cancers, and other breast conditions. It was reported to help relieve pain, increase appetite, and promote a “sense of well-being.” Despite these claims, physicians remained reluctant to begin widespread use among women because of the virilizing side effects. Most of the profits from sale of this substance were obtained by way of the black market [07007].

In 1849, Arnold Berthauld, a curator of a zoo in Germany, observed that castrated roosters ceased to fight, crow and mate, that their combs and wattle regressed, and that these symptoms were reversed by re-implantation of their testes. In 1889, Charles-Edouard Brown-Sequard, a French physician and Harvard professor, announced that his vigor and sense of well-being were transiently but markedly restored after injecting himself with testicular extract from guinea pigs and dogs. These observations led to trials of animal and human clinical research. In 1935, testosterone was identified as 17beta-hydroxyandrost-4-en-3-one (C19H28O2), a solid polycyclic alcohol with a hydroxyl group at the 17th carbon atom [12008].

A landmark discovery was made in 1889 when Dr Brown-Sequard announced at a scientific meeting in Paris that he had found a substance that reversed his 72-year-old body’s ailments. He reported having injected himself with the extract of dog and guinea pig testicles under the assumption that these organs had “internal secretions that acted as physiologic regulators.” This bold statement was confirmed with the discovery of hormones in 1905 and the isolation of testosterone in 1935 [06003].

The first characterized androgen was androsterone, which was isolated from urine. Shortly thereafter, in 1935, the characterisation and synthesis of testosterone was done by Butenandt and Ruzicka [06004]. The characterization and synthesis of testosterone resulted in the 1939 Nobel Prize in chemistry for them [12005].

The era of anabolic steroids

In 1923 Bob Hoffman formed the famous York Barbell Company in the United States. A dominant figure in US weightlifting, he published the “Strength and Health magazine” and sold health and food supplements in his gym. As a weightlifting coach, his success led to him being named the head coach of the US Olympic weightlifting team. At the 1954 World Championships in Vienna, he met with a Soviet colleague who told him of a synthetic form of
testosterone developed by the Nazis which produced dramatic improvements in strength and power. He and his colleagues contacted Ciba Pharmaceuticals in pursuit of synthetic testosterone. Ciba had conducted a number of studies on the use of synthetic testosterone in pain patients and the physically disabled. This resulted in the development of danazol, which rapidly became a doping substance abused by weightlifters [08008].

Anabolic androgenic steroids (substances similar to the hormone testosterone) were used already directly after World War II by Soviet athletes to increase muscle mass and power in weightlifting and bodybuilding events. When the Berlin Wall fell, the East German government's program of performance enhancement by meticulous administration of steroids and other drugs to young athletes was exposed. These well-documented and controlled hormonal doping experiments on adolescent athletes by the East German Sports Medical Service at Kreischa and Leipzig yielded a crop of gold medalists (mostly young females as they responded more dramatically to male hormones). Some of these athletes later suffered severe medical abnormalities, including premature death [08009].

The first reports of athletes using anabolic steroids searching for an increase in weight and power appeared in 1954. After that there has been an increasing use of doping substances by athletes. Furthermore, it was found that not just stimulants were being used but also anabolic androgenic steroids (AAS). However, the banned list did not include those substances. Therefore, the IAAF banned them and developed an immunological method for their detection. It was used for the first time at the European Athletic Championships in Rome in 1974. No cases were found as the method was still immature, but the IAAF initiative paved the way for the IOC who banned steroids in time for the 1976 Games and found eight cases at the Montreal Games with an improved method. The IAAF experience soon showed the need for strict procedures to be applied at every stage of a doping control, including the laboratory analysis. Therefore, the Federation started to work out procedural guidelines for doping controls as well as specific requirements for laboratories that were used for the analysis of doping-control samples. Some heads of laboratories were not so happy since they felt that their competence was questioned, but in 1979 the IAAF decided to only recognize analytical results from laboratories that met the specific requirements. The “Accreditation of Doping Control Laboratories” was born. Subsequently, two years later, the IOC adopted the IAAF system, and for a couple of years laboratories were jointly accredited by the IAAF and IOC. In 1986 the IOC took over full responsibility for the accreditation program. Today, doping-control laboratories are accredited by WADA [12005].

The 1960s through 1980s were the golden age of anabolic steroid use in sports. After learning that the success of the Russian weightlifting team was in part due to their use of testosterone, Dr. John B Zeigler began experimenting with Dianabol (methandrostenolone) on weightlifters at the York Barbell Club in 1958. The weightlifters became strength and conditioning coaches in a variety of other sports in the United States and spread use of anabolic steroids to other sports, such as American football. The German Democratic Republic operated a state-supported anabolic steroid doping program that produced many medals in the 1970s and 1980s, especially for women in swimming and track and field. The doping program, and its health effects for the women, was the subject of an excellent review by Franke and Berendonk and a television special by the Public Broadcasting Service. Ben Johnson of Canada had anabolic steroids detected in his urine at the 1988 Olympic Games in Seoul, and he was stripped of his gold medal. While increased muscle mass was the goal of early doping with steroids, since the late 1990s steroids in Olympic sport have been primarily used to enhance recovery to allow more frequent and more intense workouts. Testosterone is also largely responsible for the larger red blood cell (RBC) mass in men as opposed to women, so it has benefits beyond its effect on muscle. Other anabolic steroids have a similar effect on RBC production [12006].
Soon thereafter in the 1950s, Russian weightlifters began to outpace American Olympians through performance-enhancing injections. Attempting to make up lost ground, the then US Olympic physician teamed with chemists to produce an anabolic steroid for the Americans, now known as Dianabol. In the decades that followed, steroids and stimulants spread throughout sports, and in 1959, the first reported case of a high school football player’s taking steroids surfaced. In the 1960s, the International Olympic Committee banned steroid use and began formal drug testing in the ensuing decade. During the 1980s, the reported positive test results ranged from 2 to 50 percent, depending on whether the tests were announced or conducted at random. At the 1988 Seoul Olympics, the first gold medal in track and field was stripped when the Canadian sprinter Ben Johnson lost his 100-m victory after failing drug tests. Then, in 1994, an often-referenced survey was conducted by Goldman when aspiring Olympians were asked 2 simple questions. The first was, “If you were offered a banned performance-enhancing substance that guaranteed that you would win an Olympic medal and you could not be caught, would you take it?” Remarkably, 195 of 198 athletes said yes. The second was, “Would you take a banned performance-enhancing drug with a guarantee that you will not be caught, you will win every competition for the next 5 years, but will then die from adverse effects of the substance?” Still, more 50 percent of the athletes said yes. This survey made it clear that modern athletes often approach their sports with a “win at all costs” mentality [06003].

In 1954, the first reports appeared of athletes using anabolic steroids searching for an increase in weight and power. As a result, the misuse of anabolic steroids in sports led to a ban by the International Olympic Committee of these substances in 1974 and testing was implemented on a large scale at the 1976 Montreal Olympic Games via radio-immunoassays. In 1994, the Drugs Supplement Health and Education Act (DSHEA) was approved in the United States and several new steroids were commercialized as nutritional supplements. Initially these new steroids were precursors of testosterone, commonly referred to as “prohormones”. Late 2004, the US Congress approved the Anabolic Steroid Control Act (ASCA), restricting the sale of anabolic steroids as nutritional supplements. However, by 2004 a range of prohormones derived from other steroids, including 19-nortestosterone, boldenone and even 17alpha-alkylated steroids were available as over-the-counter preparations [06004].

The evolution of the history of testosterone therapies is as interesting as the history of its development. Erectile dysfunction is one of the most researched ailments treated with testosterone, although any positive effects are questionable. In men with absent to low circulating levels of testosterone, treatment with testosterone increased libido, improved erectile function, and helped to maintain secondary sexual characteristics. In men with normal or mild hypotestosteronemia, studies have not shown consistent response to therapy. Those treated were reported to have increased sexual interest, increased arousal, increased frequency of intercourse, and nocturnal erections. In the early twentieth century, there was much interest in the hormonal influence of testosterone on sexuality and sexual preferences. It even was prescribed to “treat” homosexuals because it was theorized that male homosexuals had higher estrogen levels [07007].

Testosterone has even played an important role in various ailments affecting women, such as treatment for some metastatic breast cancers. Approximately one third of breast cancers are hormone dependent and respond to androgen therapies. Other uses for testosterone are as postmenopausal hormone replacement therapy, for sexual dysfunction (by increasing libido), and for increasing bone density. Some clinical case studies showed an increase in appetite, lean muscle mass, and strength and an improved overall sense of well-being. Before the use of erythropoietin and bone marrow transplants, testosterone was used to help
treat anemia (i.e. chronic renal failure/hemodialysis). Psychiatrists prescribed anabolic steroids from the 1930s to the 1980s to treat psychoses, depression, and melancholia. Testosterone has been used as an adjunct in people with growth hormone deficiency or in boys with pubertal delay [07007].

Body builders and athletes began using testosterone to increase muscle mass and to intensify training protocols on the West Coast of the United States in the late 1940s and early 1950s. The US Food and Drug Administration approved methandrostenolone in 1958. In the 1950s, Soviet Union and East German Olympic athletes were using AASs. They later found their way into the hands of Olympic competitors, including track and field athletes from many countries. Paul Niehans wrote the 1960 book "Introduction to Cellular Therapy", in which the main emphasis was on testicular secretions. He believed that testicle cell injections increased testosterone derivative excretion. Some of Paul Niehans' famous patients included Pope Pius XII, Bernard Baruch, and Aristotle Onassis. In 1974, the International Olympic Committee banned the use of testosterone and its derivatives. AASs were widely abused in a variety of sports, including volleyball, cycling, swimming, soccer, and bobsledding. Testosterone was studied using different forms. Scientists quickly learned it was ineffective, and even toxic (like 17 alpha-methyl testosterone), when taken orally; instead, it was synthesized into tiny pellets that were inserted subcutaneously. Longer-acting injectable forms of testosterone were synthesized in the 1950s (i.e. testosterone enanthate). Over the following decade, the hormone was modified into derivatives that possessed more anabolic qualities. In the 1970s, oral testosterone undecanoate was synthesized; however, it did not fare well in the oral form because of hepatic clearance and hepatotoxicity. Transdermal scrotal patches were derived in the 1990s. These allowed physiologic levels of testosterone to be acquired. Nonscrotal skin patches were developed, and testosterone gels were marketed. Today, there are short-acting buccal forms as well as the long-acting injectable testosterone undecanoate [07007].

By the early 1990s, several pharmaceutical companies had stopped producing AASs. It was about at this time that the black market sales of AASs and counterfeit products increased secondary to the ease of Internet shopping and availability. Authentic steroids, as well as placebos and unpurified forms, were sold and abused. The US Congress placed anabolic steroids into the schedule III category of the Controlled Substance Act (CSA) in the Anabolic Steroid Control Act of 1990. This act included testosterone and all related chemical or pharmacologic substances that promoted muscle growth. Corticosteroids, progestins, and estrogens were not included in this act. The Anabolic Steroid Act of 1994 was an amendment to the CSA. It placed anabolic steroids as well as their precursors on the controlled substance list. Possession of the drugs without a prescription was now a federal crime. Studies of the effects of supplemental testosterone on aging men in the 1990s suggested an increase in word memory, special cognition, increased libido, decreased bone resorption, and increased lean body mass and strength. McKinlay reported in the Journal of Urology that testosterone does not treat impotence. In theory, prostatic tissue, including cancer and benign prostatic hypertrophy, can be stimulated by testosterone, but no compelling evidence has been reported that suggests an increased risk [07007].

Androgenic-anabolic steroids (AAS) have been misused by athletes at the Olympic Games, both before and after they were prohibited in sport in 1974. Systematic doping with AAS occurred in the German Democratic Republic from 1965 to 1989 which assisted that country to win many medals at Olympic Games, especially in female events. Currently, androgenic-anabolic steroids are the most frequent category of prohibited substances detected in the urine of athletes both globally and at the last two Summer Olympic Games. Scientific confirmation that AAS are effective in enhancing sports performance was difficult because ethical approval was difficult for research involving male subjects taking massive doses of
androgens as some athletes and bodybuilders did. Methods to detect androgenic-anabolic steroids have evolved gradually over the past three decades and currently, despite an impressive array of sophisticated analytical equipment and methods, anti-doping authorities and analytical scientists continue to face challenges as have occurred from the use by athletes of designer AAS during the past few years. The future development and use of selective androgen receptor modulators can be anticipated to pose problems in the years ahead. Endocrinologists should be aware that on occasions, replacement testosterone therapy may be authorized in sport as a therapeutic use exemption (TUE) [08060].

Definitive proof of anabolic steroid abuse in sports was not possible prior to the introduction of combined gas chromatography/mass spectrometry (GC/MS). It was now given a report of the early history (1960-1980) of GC/MS and radioimmunoassay, and how these techniques were utilized in the first years of steroid doping control in athletics. There were several key individuals and research groups involved in the early technical developments, and their essential contributions have been acknowledged. The Oakland USA laboratory was the first IAAF (International Association of Athletic Federations) sanctioned site to do steroid GC/MS steroid analysis resulting in athletes being disqualified from competition. This gave notable successes, including the only East German female competitor ever suspended during the tenure of the DDR (Deutsche Demokratische Republik). By the early 1980s, in anticipation of the Los Angeles Olympic games, dedicated year-round sports testing facilities had been established and part-time amateurs could step aside [08125].

High levels of anabolic-androgenic steroids abuse have been attributed to professional football players, bodybuilders, weight lifters, and track and field throwers since the 1960s. The exceptional athletic performance of the East German female swimmers in the 1976 Montreal Olympics brought further public attention to AAS athletic use. It was not until the 1980s, however, that the medical community admitted that these substances were effective. Since that time, the pervasive use of AASs by professional athletes has garnered significant media attention, culminating most recently in the ongoing investigation of the use of illegal performance enhancing drugs by some of baseball's top players. “Juiced”, a book by Jose Canseco, details his steroid use and the widespread use of anabolic steroids in Major League Baseball [07008].

US professionals

Despite years of aggressive anti-doping testing by international sports federations such as those for cycling, athletics and soccer, steroid abuse scandals involving high profile athletes continue to be front page news across the globe. Professional sports in the United States were not subject to extensive anti-doping programs, as players’ unions and collective bargaining agreements prevented such extensive testing to be put into place. However, they did establish limited anti-doping programs, as the professional sports organizations recognized the potential of doping to harm athletes and their sport. In 1998, when Mark McGuire, an American baseball player, broke Roger Marris' home run record, it was revealed that he had been taking a supplement containing a precursor to nandrolone, a steroid. At that time Major League Baseball did not ban steroids and did not believe that steroids were a problem within the league. However, subsequent government investigations and former players revealed that steroid abuse was a problem in the League, which resulted in a limited steroid testing program [08006].

Anabolic steroid prodrugs in the US
The potential performance-enhancing benefits of testosterone precursors were brought to the attention of the public and athletic community in the US in 1998 when Major League Baseball player Mark McGwire set the home run record and openly admitted to using androstenedione. Sales skyrocketed by 500 percent, and many supplements containing prohormones became available in the United States market. Questions and concerns of contamination with other supplements arose but their purity was unknown because these supplements were not regulated by the FDA. Also, their popularity was fueled by the misperception that nutritional supplements are natural, and, therefore, safe. In 2004, after much controversy and debate, the US Department of Health and Human Services (HHS) and the FDA announced a crackdown on companies that manufacture, market, and distribute products containing androstenedione. They recognized the potential serious adverse health risks that were similar to those associated with AASs. As part of their concern about its safety, the FDA and HHS sent warning letters to 23 companies asking them to stop distributing dietary supplements that contained androstenedione and warned them that enforcement actions would be taken if they did not comply. As a result of this action, the Anabolic Steroid Control Act of 2004 was passed. This act added the steroid precursor androstenedione to the list of schedule III controlled substances in the United States. Schedule III substances have limited medicinal use, require a prescription from a licensed physician, and allegedly can threaten public health without government regulation. DHEA was not added to the controlled substance list; industry lobbyists contended that it had proven effective as an antiaging supplement and that its risks were minimal [07009].

**Designer drugs**

Synthetic organic chemistry can be traced back to 1865 when Friedrich August Kekule published two theoretical papers on the structure of aromatic organic molecules. Paul Ehrlich postulated in the early 1870s that differences in chemoreceptors between micro-organisms, parasites, and cancer cells from those in host cells could be exploited for therapeutic purposes. In the absence of current ligand-based and receptor-based molecular design techniques, there were limited approaches to identify minor structural changes in biologically active compounds that would enhance selectivity and/or potency of therapeutic molecules. Hamett made the first significant contribution relating structure to activity of small organic compounds with his study correlating electronic properties of organic acids and bases with reaction rates and equilibrium constants, focusing on benzoic acid derivatives. Moving beyond the linear free energy relationships provided by the Hammett equation, the next major development was the introduction of quantitative structure-activity relationships (QSAR) by Corwin Hansch et al. in two seminal papers in the early 1960s, providing a new tool to systematically relate molecular descriptors (electronic, steric, topological, and hydrophobic indices) to biological activity. These early efforts concentrated on naturally occurring plant hormone mimics, and relied on statistical analysis of published accounts of the biological activity of phenoxyacetic acid derivatives and other plant growth regulators. Electronic indices were found insufficient for QSAR of biological systems; rather, a measure of lipophilicity (classically measured as an octanol-water partition coefficient) was essential to predict targeting of compounds to specific tissues, cells or organelles, and subsequent biological activity. John Topliss developed a method to automate QSAR; however, it is of limited utility in many experimental systems and ignores possible interactions between multiple substituents. QSAR remains a dynamic tool for drug design and optimization. Approximately 15 years ago, pharmaceutical companies realized that existing screening libraries were inadequate for newly developed high-throughput efforts for lead drug design. The challenge has been to balance size and structural diversity of new libraries against screening cost, while maintaining affinity and selectivity against a portfolio of targets. Two major approaches have emerged: fragment-based screening (FBS) and diversity-oriented synthesis (DOS). Despite major advances in chemical screening and synthesis, discovery of...
new drugs is difficult, expensive, and the efficacy of target-based drug discovery has been questioned [11555].

**BALCO**

Although a general topic of interest in the 1990s, designer drugs first made international headlines in 2003 with the Bay Area Laboratory Co-operative (BALCO) scandal involving the widespread use by athletes of tetrahydrogestrinone (THG). This simple reaction created a potent agonist for androgen and progesterone receptors whose presence could not be detected by standard multiple reaction monitoring (MRM) methods used by anti-doping laboratories for steroid detection. Subsequent characterization of this steroid derivative led to US federal prosecution of many involved with BALCO, culminating in March 2011, with the most high profile case so far, involving the former San Francisco Giant (US Major League Baseball) Barry Bonds [11555].

**History of blood doping**

Blood transfusions as a means for improved endurance were researched as early as 1947 by Pace and coworkers. Transfusion of 500 ml of allogeneic erythrocytes on four consecutive days reduced the pulse rate during exercise in simulated hypoxia. However, even though the methods would not meet today’s standards; this was the first of many studies to confirm the possible performance enhancing effect of blood transfusions. Accordingly blood transfusions in sports were later banned by the International Olympic Committee (IOC) in 1986 [13005].

Lasse Viren, a Finnish long distance runner who won gold medals at the 1972 and 1976 Olympic Games in the 5,000 m and 10,000 m, is believed to be among the first athletes to have used blood transfusions to improve performance. It should be noted that this technique was not banned at the time and although the ethical debate on the topic was in full swing, it was only in 1986 that the International Olympic Committee banned blood transfusions. Other than the anecdotal evidence from the Nordic distance runners, there are other reports on more systematic use of transfusions in the context of major sporting events in the 1980s. Notably, it is well established that a large part of the US cycling team was involved in a systematic blood doping program that earned them unprecedented success at the 1984 Olympic Games in Los Angeles. There is also some evidence that blood transfusions were an integral part of the doping regime used for the enhancement of performance for athletes from the Eastern bloc (Soviet Union, East Germany) at that time. Nevertheless, it can be assumed that because of the logistic requirements of blood withdrawal and reinfusion, the technique was not widespread, as the technical necessities were only available to a small number of athletes, but nevertheless available to certain elite athletes [13006].

The blood doping situation changed dramatically with the commercial introduction of recombinant human erythropoietin (rhEPO), the human hormone that regulates the erythropoietic system in the organism. EPO was first isolated in the 1950s. The gene for human erythropoietin was successfully cloned in 1983, and the first recombinant erythropoietin (rhEPO) was approved by the FDA for treatment of anemia in renal failure in 1989. One year later the IOC banned the use of erythropoietin (EPO) [13005].

Studies investigating the effect of rhEPO on performance were soon published and demonstrated positive effects on maximal oxygen uptake of 6-12 percent. Although the authorities rapidly banned rhEPO, the easy access to the substance and the huge impact on performance resulted in widespread abuse of rhEPO during the 1990s/2000s. It is believed
that this substance had a considerable impact on the development of peak performances in all endurance sports during these years and there are even scientific attempts to prove this for several sports on the basis of performance analysis. The abuse was facilitated by the fact that no detection method was readily available at that time. From a practical point of view, the impact of rhEPO on performance in endurance sports is best illustrated by a quote from Greg Lemond, an American cyclist who won the Tour de France in 1986 and 1989, i.e., before rhEPO became available, recalling the 1991 race: “I was the fittest I had ever been, my split times in spring training rides were the fastest of my career, and I had assembled a great team around me. But something was different in the 1991 Tour. There were riders from the previous years who couldn’t stay on my wheel who were now dropping me even on modest climbs.” These words accurately describe how rhEPO changed the entire world of endurance sport in the following decades and divided the athletes’ performance primarily between rhEPO users and non-users [13006].

Logically, following widespread abuse from the 1990s onwards, doping scandals involving rhEPO or blood transfusions have shaken the world of sport on a regular basis, culminating recently with the investigation of Lance Armstrong, who subsequently admitted the use of both rhEPO and blood transfusions throughout his career. Although, it is therefore common belief that many recent doping cases were not unveiled by conventional anti-doping testing, but rather by police investigations or admissions from athletes or staff, thus non-analytical approaches, anti-doping laboratories were able to detect about 400 cases testing positive for rhEPO between 2003 and 2011 (World Anti-Doping Agency (WADA) statistics). Analytics have therefore come a long way in the detection of blood manipulation in sports and still outperform police investigations by 10 to 1 [13006].

Blood transfusions, as a method to enhance endurance performance, first gained attention after the Olympic games (OGs) in Mexico City in 1968. Before these OGs, evidence was presented that a lowered atmospheric pressure would decrease performance in all athletic disciplines dependent on a high level of sustained oxygen uptake. This was indeed confirmed in Mexico City, where all winning times in running races above 800 m were significantly worse than the world records at that time. This highlighted the impact of the oxygen delivery to the working muscles as a limiting factor during whole body endurance exercise. It also became evident that runners hailing from higher altitudes tended to be superior to competitors from lowlands because they had “thick blood” with high hemoglobin content. A relatively straightforward way to increase the hemoglobin concentration (Hb), and, hence, oxygen delivery to the muscles is, thus, by blood transfusions. This was documented in the classic study by Ekblom et al from 1972 where a high correlation between Hb and performance capacity after blood withdrawal and reinfusion was presented. An overnight increase in Hb by 13 percent caused by the reinfusion of 3 units of stored autologous blood resulted in an increase in maximal oxygen uptake and physical performance capacity of 9 percent and 23 percent, respectively. The method of transfusing blood in a sport setting was hereafter dubbed “blood doping” by the media, and its potent effect on athletic performance was quickly noted in the sports community. Blood doping was used already at the OG in 1972 by a Finnish steeplechaser, and during subsequent OGs, several athletes admitted having used blood doping. Not until after the OG in Los Angeles in 1984, where the US cycling team used blood doping and won 9 medals after not having won a medal in cycling for 72 years, the method (both homologous and autologous transfusions) was prohibited by the International Olympic Committee, although no method was available to detect its use. Because of its logistic advantages compared with blood transfusions, human erythropoietin (rhEPO) became the preferred blood boosting method by athletes after it had been available. At the OG in Sydney in 2000, two tests for rhEPO were introduced: a “direct test” that was able to distinguish rhEPO from endogenous molecules by isoelectric focusing and an “indirect test” based on changes in blood parameters caused by rhEPO administration.
Because of the introduction of these tests, old-fashioned blood doping reentered the scene. At the Salt Lake City Winter OG in 2002, discarded blood transfusion equipment was found at the headquarters of the Austrian cross-country skiers. After DNA testing, 2 skiers were disqualified. Although testing for homologous blood transfusions had been performed at the Lillehammer OG in 1994 by use of antigen testing cards, it was not until 2004 at the OG in Athens that a test had been validated and implemented. At this event, the gold medal winner of the men's time trial in cycling was first tested positive, but because the backup sample (B-sample) was frozen and, thereby, the red blood cells (RBCs) destroyed, no doping offense could be proven. After he failed further doping tests at the 2004 Vuelta a España, the rider was suspended for 2 years [12009].

The word “doping” was used in the 1860s to describe a drug used for horse racing that consisted of opium and narcotics. With human athletes, “blood doping” originally referred to a process whereby athletes increased their oxygen-carrying capacity by receiving blood transfusions from previously donated blood to increase their hematocrits a few days before competition. The first report of blood doping in a controlled experiment was reported in 1947. Today, “blood doping” is used more synonymously with cheating of any kind, including any of the various blood-boosting methods or classes of ergogenic aids that are available to athletes. Advances in genetic medicine have allowed athletes to raise their level of sophistication significantly by using PESs that are virtually undetectable. In June 1989, the first rHuEPO product was marketed in the United States. It was isolated and purified from Chinese hamster ovaries and reproduced using DNA recombinant techniques. The popularity and effectiveness of rHuEPO in elite endurance athletes is demonstrated by a long list of anecdotes associated with its misuse during international competition. When the average speed of the cyclists racing in the Tour de France began to increase suddenly during the 1990s, rumors of rHuEPO use began to circulate. The gene that produces EPO was cloned in 1985, and rHuEPO was available in Europe by 1987. Between 1987 and 1991, more than 20 Dutch and Belgian cyclists died at rest – some of them while sleeping – as a result of unexplainable cardiac arrest. Between 1997 and 2000, 18 more cyclists died from pulmonary embolisms, stroke, and myocardial infraction. Finally, suspicions of rHuEPO use in professional cyclists competing in Europe were confirmed during the 1998 Tour de France; boxes of ampules containing rHuEPO were found in team vehicles and the personal rooms of riders from many of the biggest and most successful teams. It became embarrassingly clear that rHuEPO use in elite professional cyclists was organized, widespread, and sophisticated. The International Olympic Committee (IOC) added rHuEPO to its list of banned substances in 1990, even though all forms of blood doping had been officially prohibited since 1984. Despite justified suspicions of rHuEPO use in cycling and the inability of current methods to detect its use, in 1997, the governing body of the International Cycling Union (UCI) enacted hematocrit cutoffs for male (50 %) and female (47%) cyclists while more reliable methods of detection could be developed. The hematocrit cutoffs were based on existing normative data on elite athletes, taking into consideration the expected effect of dehydration, in an attempt not to exclude athletes with normal variations but to protect athletes from danger. Anyone over that limit would be considered “unfit to race” and could not compete for 2 weeks, although they were not subjected to official sanctions. To circumvent this, an athlete could inject rHuEPO every 2 to 3 days over 3 to 4 weeks, along with some form of iron supplementation, to get a desired effect and then reduce the dose to match the basal rate of endogenous EPO production to maintain one’s hematocrit just below the “legal limit.” During the 2000 Sydney Olympics, the IOC approved the use of a test developed by the Australian Institute of Sport to detect rHuEPO users [07010].

Shortly after the discovery of blood circulation by the English physician William Harvey in 1628, the first empiric blood transfusion was attempted. There is therefore a long history of blood doping, conventionally originating with the anecdote of athletes being encouraged to
drink reindeer blood or something like that to achieve extraordinary performances. Although the earliest proof of improved sport performances after blood transfusions was provided already in 1947, the first evidence of blood doping came later, in 1972, when a controlled experiment clearly showed a considerable increase in performance of athletes undergoing autologous transfusion of packed RBCs from an earlier venesection. Since then, there are consistent records of athletes experimenting with blood transfusions who achieved incredible success in competitions. Besides the first anecdotal reports, this technique became fairly popular during the 1980s and was widely used by distance runners, cyclists, and skiers, particularly during the 1980 and 1984 Olympics. Although no reliable test had been devised for unequivocal detection, the International Olympic Committee (IOC) officially banned blood doping after the 1984 Olympics. In the same year, the USA Olympic Committee declared that seven cyclists, including four medallists, out of 24 athletes of the national team who participated in the Olympic Games, used transfusions. Years later, following the implementation of reliable strategies for detecting doping with recombinant erythropoietin and analogues, blood transfusions, which had fallen out of favor, made a strong comeback. In March 2002 at the Salt Lake City Olympics, the IOC investigated the discovery of discarded blood transfusion equipment at the quarters of the Austrian cross-country skiers. Following DNA testing, two Nordic skiers (who had been placed in the 40s, and not the Austrian team’s three medallists) were disqualified and had their results cancelled. For the same reason, some professional cyclists, one of whom nearly died after being injected with poorly stored blood, were found guilty and suspended in 2004 [06005].

The suspension of several professional road cyclists from the 2006 Tour de France could represent the tip of the iceberg, with more than 200 athletes in different sports disciplines implicated in an international doping probe including blood transfusions and exogenous hormone administration. In an apartment building in Madrid occupied by a doctor, Spanish police discovered clandestine equipment for international performance enhancement, seizing more than 200 450 mL blood bags, along with records and several other doping substances, which allowed investigators to finally match code names of athletes with their highly detailed doping records. This sophisticated pan-European doping ring either treated athletes locally or arranged the transport of stored blood through a system of couriers to athletes at race sites. Hence, based on the riders named in this one investigation, the problem is endemic [06005].

**Early testing strategies for blood doping**

Originally, the only means to test for doping by blood transfusion was the adoption of arbitrary thresholds for hematocrit and/or hemoglobin. Blood doping practices were suspected when blood tests showed hemoglobin values exceeding 175 g/L for men and 155 g/L for women (International Ski Federation), and hematocrit values above 0.50 for men and 0.47 for women, with reticulocytes < 2 percent (according to the International Cycling Union). Athletes with random values exceeding such limits were prevented from racing in official competitions. Nevertheless, such a questionable strategy involved several drawbacks, including the difficult interpretation of several hematological parameters because of wide inter-individual variability, the possible occurrence of false positive results that would have penalized clean athletes with naturally increased values, and the possibility to arbitrarily expand or titrate the RBC mass up to the allowable threshold [06005].

The obvious alternative to homologous blood transfusions is autologous blood transfusions (ABTs). In 2006, just before the Tour de France, a large Spanish doping scandal evolved, known as Operación Puerto. A doping ring involving several physicians was uncovered by the Spanish police, and more than 200 autologous blood units belonging to professional athletes were found in freezers and refrigerators for subsequent reinfusion. Detailed doping
calendars from individual athletes were published, and the modus operandi of involved athletes and their physicians was uncovered. From these calendars, it became evident that besides the massive abuse of a wide range of different performance-enhancing drugs and masking agents, ABTs were used during important competitions. The procedure of blood withdrawal and reinfusion was performed numerous times for each individual athlete during the year by using specialized equipment for phlebotomy and storage. Typically, blood was withdrawn after competitions and reinfused few days before 1-day races or before and during multiple-day competitions. Since then, athletes testing positive for other substances have confirmed the ongoing abuse of ABT today [12009].

Increasing delivery of oxygen to the active muscles and making energy efficiently from the oxygen is the most effective way to increase performance. Increasing the number of RBCs is the most effective way to increase aerobic performance. The US pursuit cycling team unexpectedly won gold at the XXIII Olympic Games in Los Angeles. It was later revealed that they transfused blood and that some of the cyclists had suffered severe transfusion reactions. The advent of recombinant protein therapeutics in the late 1980s ushered in a new era for dopers. The lay press speculated that the deaths of 18 European cyclists were related to the availability of recombinant human erythropoietin (rhEPO). rhEPO stimulates the production of red blood cells in the bone marrow, resulting in increased red blood cell mass. The development of a test for rhEPO caused the athletes to change the route of administration from subcutaneous to intravenous, decrease the dosage, and increase the frequency of administration in order to avoid detection. At the Salt Lake City Games in 2002, three winter endurance athletes had Aranesp (darbepoetin-alpha), a novel erythropoietin stimulating protein, detected in their urine samples – 7 months after approval in the European Union and 5 years before the FDA approved its medical use in the United States. Information gathered from investigations confirms that with the advent of tests for prohibited peptides and proteins like EPO, some cheating athletes changed to autologous blood transfusions to increase RBC mass [12006].

History of detection of recombinant erythropoietin and derivates

Direct detection of a forbidden substance in a biological matrix such as urine or blood obtained from an athlete is the classical forensic approach to prove doping. This approach has long been the sole strategy, only fine-tuned by improving the sensitivity of the analytical detection methods and by optimizing the timing of testing. The basic principle of the direct detection of forbidden substances relies on the fact that these substances are different from the normal constituents of the human organism. With the introduction of recombinant drugs such as rhEPO, this principle was not valid anymore, as the recombinant constituent was virtually identical to the endogenous version of the substance. Thus, at first, doping tests could not differentiate between the natural, endogenous and the artificial, exogenous recombinant version of the drug. This has, for a long time, been a major difficulty for the testing laboratories. In 1995, it was described a method to separate the natural from exogenous EPO through electrophoresis, but other laboratories could never replicate their results and the described method never reached the stage of validation. Only in 2000, thus more than 10 years after the estimated beginning of rhEPO abuse in sports, the first practicable and validated test to directly detect rhEPO in urine was published. This test relied on a difference in glycosylation between the endogenous and the exogenous EPO molecules, which resulted in different migration characteristics during isoelectric focusing (IEF). The recombinant EPO was industrially harvested from transfected hamster kidney or ovarian cells and, owing to the difference in cell organelles, a minor posttranslational difference in glycosylation between the rhEPO (made by the hamster cells) and the endogenous EPO (made by the human kidney cells) occurred, although the amino acid
sequence is identical. The rhEPO molecules are less negative and will thus move differently from endogenous EPO in an electric field, which can be demonstrated using the IEF. Further developing the approach of Wide, IEF is then followed by double blotting, which addresses the problem of non-specific binding of the EPO molecules. Although relatively cumbersome, the method soon identified the first athletes testing positive, namely Roland Meier and Bo Hamburger, both cyclists. Ironically, Hamburger was later acquitted by the court of arbitration of sports on formal grounds (i.e. a lack of harmonization of the positivity criteria for the EPO tests between laboratories was identified). This issue has since been addressed and strict positivity criteria apply, based on acceptance, identification, and stability principles. rhEPO positive samples, for example, have to show at least three acceptable, consecutive bands in the basic area and the two most intense bands measured by densitometry must be in the basic area. When the analysis is performed in blood (serum/plasma) the intensity of those bands must be approximately twice or more than any band in the endogenous area. Many laboratories nowadays use computer-based classification algorithms to guarantee objectivity in this context [13006].

At the 2000 Olympic Games in Sydney, the Australian WADA-certified laboratory first launched a sophisticated anti-doping test for erythropoietin that required both urine and a blood sample. Over 300 tests were performed for erythropoietin for the first time in Olympic history but no positives were reported. This could be due to the fact that the technology for the test was new and questions still existed about the assay [08006].

Hypotheses on erythropoietin doping practice
It has been noted that recombinant erythropoietin (rEPO) appeared in Europe in 1987, and the unusual deaths began soon thereafter. From 1987 to 1991, up to 20 competitive cyclists died suddenly and unexpectedly. Autopsy results were elusive, but cycling authorities said the deaths were from “heart attack,” “cardiac arrest,” or “cardiac failure.” However, about 15 of the deaths seemed to fit a profile that suggested another explanation: They were young and improving fast, “rising stars” who died not during a race but at rest before or after a race. The rationale for abusing rEPO in competitive cycling is that by raising the hematocrit without unduly raising blood viscosity, one can enhance aerobic performance by enhancing oxygen delivery to muscles. By 1990, the first experiment on the effects of rEPO on athletes had been done, and the lead researcher was quoted to the effect that rEPO might enable an elite athlete to shave 30 s off a 20-min racing time. The problem is that the higher the hematocrit, the greater the risk of clots. Blood clots are the proximate cause not only of pulmonary emboli but also of many strokes and heart attacks. Coagulability hinges partly on blood viscosity, which is set by the plasma fibrinogen level, the deformability of red cells, and the hematocrit. Hematocrit also influences platelet adhesion, the first step in arterial thrombosis. Hematocrit, then, modulates the flow, fluidity, and coagulability of blood. As hematocrit increases toward 60 percent as in mountaineering, for example, blood clots can become a menace [13066].

In 1991, a warning appeared in a prestigious medical journal on how fast hematocrit can rise with large doses of rEPO and how rEPO abuse by athletes could drive hematocrit to “dangerously high levels."By 2007, after esteemed studies in patients with renal disease, cancer, and other major illnesses tied higher dosing of rEPO to greater risk of death from thrombotic events (heart attack, stroke, or venous thromboembolism) or heart failure, the Food and Drug Administration issued a black box warning on these risks from rEPO. Even this did not end the abuse of rEPO by athletes or the deaths. Cyclists continued to dope, dupe, and die [13066].

Sudden deaths in Swedish orienteers
A spate of deaths in orienteers paralleled the cycling deaths. Suddenly, among young, elite orienteers (but in no other Swedish sports), the death rate spiked to 1 percent a year for 3 years in a row. From 1989 to 1992, seven elite-level orienteers, all from the same small area of central Sweden, died during or after competitions or training. They knew one another and occasionally trained together. All performed very well shortly before they died; some placed near the top in national competitions. The last, Melker Karlsson, 24, was a rising star who died after a training run and sauna. His death was the final straw that led to a meeting of Swedish health experts to probe potential causes and solutions. As in the cyclists, the deaths were considered “cardiac,” and a popular hypothesis was a transmissible myocarditis, ascribed first to Chlamydia and then to Bartonella. Their supporting evidence, however, is not compelling and does not dissuade skeptics, from speculating that a culprit in this spate of sudden deaths in top Swedish orienteers was abuse of rEPO [13066].

Cyclists
Evidence from many believable anecdotes, from sworn testimony to the United States Anti-Doping Agency (USADA), and from police raids at the 1998 Tour de France and before the 2006 Tour de France shows that cyclists continue to abuse rEPO. Indeed, top South African cyclist David George was caught on rEPO (and admitted it) in August 2012. The culture of competitive cycling dies hard; despite the deaths and the black box warning, rEPO abuse in cycling has endured for 25 years. In his affidavit to USADA, Stephen Swart, a teammate of Lance Armstrong in 1994 to 1995, said that their Motorola Team used rEPO for the 1995 Tour de France, and that most riders, including Lance Armstrong, had a hematocrit over 50 percent. It is widely reported that Marco Pantani abused rEPO and had a hematocrit of 60 percent in a 1995 race. As noted in Tyler Hamilton’s recent tell-all book, Bjarne Riis won the 1996 Tour de France on rEPO, and his peak hematocrit was an astonishing 64 percent. For cyclists who abuse rEPO, there may be a thin line between winning and dying. Alas, the deaths continue. From early 2003 to early 2004, eight more European cyclists died, and up to five fit the profile of a likely rEPO death. Notable were French cyclist Fabrice Salanson, 23, found dead in his hotel room just hours before he was to start the Tour of Germany, and Belgian cyclist Johan Sermon, 21, who went to bed early to rest up for a planned 8-h training ride the next day, but was found by his mother dead in bed at dawn. In early 2009, the promising Belgian cyclist Frederick Nolf, 21, died at night in his Ritz Carlton hotel room, after the fourth stage of the Tour of Qatar. He went to bed laughing and happy and never woke up. No autopsy was done. How the autopsy results on Salanson were described to the press may come closest to the truth in this long, sad saga. Dr Jan Dressler of the University of Dresden Medical Institute said the death was probably caused by the heart enlarging and the coronary vessels failing to pump enough blood [13066].

Background to the athlete biological passport
In an investigation of samples obtained as part of routine International Ski Federation blood-testing procedures in participants at the World Ski Championships, abnormal hematological profiles, defined as those deviating from the 1989 Nordic Ski World Championships and the IOC Erythropoietin 2000 project data set, were identified in 36 percent of the skiers tested and finishing within the top 50 places in the competitions. In addition, 50 percent of medal winners and 33 percent of those finishing from 4th to 10th place had highly abnormal hematological profiles. In contrast, only 3 percent of skiers finishing from 41st to 50th place had highly abnormal values. Although these data cannot be immediately associated with blood doping practices, including blood transfusions, and it is very unlikely that blood doping would be less common in other endurance sports, the present situation is highly suggestive of a phenomenon that is not being controlled by the ongoing antidoping testing program. In fact, it has been hypothesized that a combination of blood transfusion and recombinant human erythropoietin administration could also be used by such athletes [06005].

132
History of doping with growth hormone

In 1998 at the Tour de France that French customs arrested Willy Voet, a physiotherapist of the Festina cycling team, for the illegal possession of needles, syringes and over 400 bottles containing erythropoietin, human growth hormones, steroids, amphetamines, narcotics and stimulants [08006].

Ben Johnson also admitted to using human growth hormone along with steroids during investigations after his disqualification in Seoul. The abuse of growth hormone in sports seems to be escalating, with large caches of needles and vials of hGH being confiscated at sporting events worldwide. Six months prior to the 2000 Olympic Games, a pharmacy in Sydney was broken into and 1,575 multiple dose vials of growth hormone were taken while nothing else was touched. Also, on their way to Australia, the Chinese swimming team was detained, as needles, syringes, and vials of human growth hormone were found by customs officials in their baggage [08006].

hGH is a naturally occurring hormone produced by the anterior pituitary gland and is one of the major hormones influencing growth and development. Harvey Cushing discovered the hormone in 1912 and isolated it from human and monkey cadaver brains in 1956. Two years later it was used to treat dwarfism in children by injection. The unfortunate development of Creutzfeldt-Jakob disease, a degenerative brain disorder, in boys who were treated with cadaver growth hormone led to the discontinuation of all products derived from the human pituitary gland. Because of this ban, the abuse of hGH was rare in sport until the middle to the end of the 1980s. In 1985 Genentech received approval from the US FDA to market Protropin® for children with growth hormone deficiency. This was the first recombinant DNA form of growth hormone (rhGH) that was safer than cadaver extracts used in the past. Recombinant DNA technology made the production of pharmaceutical grade growth hormone easier and cheaper. Most human growth hormone used in medicine and diverted to sports doping is now obtained by recombinant technology, and is simply referred to as hGH (but it may also appear as rhGH or HGH) [08006].

Growth hormone (GH) is an important and powerful metabolic hormone that is secreted in a pulsatile pattern from cells in the anterior pituitary, influenced by several normal and pathophysiological conditions. Human GH was first isolated in the 1950s and human derived cadaveric GH was initially used to treat patients with GH deficiency. However, synthetic recombinant GH has been widely available since the mid-1980s and the advent of this recombinant GH boosted the abuse of GH as a doping agent. Doping with GH is a well-known problem among elite athletes and among people training at gyms, but is forbidden for both medical and ethical reasons. It is mainly the anabolic and, to some extent, the lipolytic effects of GH that is valued by its users. Even though GH's rumour as an effective ergogenic drug among athletes, the effectiveness of GH as a single doping agent has been questioned during the last few years. There is a lack of scientific evidence that GH in supraphysiological doses has additional effects on muscle exercise performance other than those obtained from optimised training and diet itself. However, there might be synergistic effects if GH is combined with, for example, anabolic steroids, and GH seems to have positive effect on collagen synthesis. Regardless of whether or not GH doping is effective, there is a need for a reliable test method to detect GH doping. Several issues have made the development of a method for detecting GH doping complicated but a method has been presented and used in the Olympics in Athens and Turin. A problem with the method used, is the short time span (24-36 hours) from the last GH administration during which the test effectively can reveal
doping. Therefore, out-of-competition testing will be crucial [08238].

Today, many medical interventions that begin as treatments for disease often expand into therapies that reduce disability, lessen disadvantage, or even confer advantage. Forces that propel profitable drugs, devices, and procedures dominate over considerations of efficient and equitable distribution of resources. This dominance is fueled by industry-physician collaborations often biased by prior assumptions, reliant on surrogate outcomes, and advantageous to marketing. Interventions are justified by "medicalization" of physiologic variations (e.g. short stature) as defects or disease, and nudged into "standard practice" by key opinion leaders. The story of recombinant human growth hormone (hGH) treatment of short stature is one vivid example, but others (e.g. expansion of drug treatment to "optimize" cholesterol profiles, bone health, psychological well-being) can be found throughout medicine. In the new obesity era, lessons learned from the hGH era will be needed to keep the field of pediatric endocrinology empowered to make the key clinical decisions, and free of unintended consequences for patients and runaway health care inflation for society [11469].

After testing over 1,000 samples, the first adverse analytical finding of growth hormone came in February 2010 when the British Rugby League player, Terry Newton, tested positive. Since then, several other positive tests have been reported including the announcement in September 2010 from the Canadian Center for Ethics in Sport that Matt Socholotiuk, a University of Waterloo football player, had tested positive for GH use on 31 March 2010. The following year, Colorado Sky Sox first baseman Mike Jacobs became the first baseball player to test positive for GH and was subsequently suspended for 50 games by Minor League Baseball. In 2011, Andrus Veerpalu, an Estonian Olympic gold medal winning skier, tested positive for GH. However, he pleaded his innocence and challenged the laboratory finding in the Court of Arbitration for Sport who subsequently acquitted Veerpalu on 25 March 2013 as the court was not convinced that the threshold for considering an adverse analytical finding was sufficiently reliable to uphold the doping conviction; nevertheless, the court stated "that there are many factors in this case which tend to indicate that the Athlete did in fact himself administer exogenous hGH" [13007].

History of doping with caffeine

From 1962 to 1972 and again from 1984 to 2003 caffeine was on the WADA banned list, with a concentration >12 microg/ml in the urine considered as doping. Caffeine has been demonstrated to be ergogenic at doses lower than those doses that result in a urine concentration of 12 microg/ml, and higher doses appear to exhibit no additional performance-enhancing effect. During the second banned period, many athletes tested positive for caffeine. The sanctions ranged from warnings up to 2 year suspensions (maximum penalty, usually only 2-6 months). Since 2004, caffeine has been removed from the prohibited list, however, it is still part of WADAs monitoring program (stimulants but in competition only) in order to monitor the possible potential of misuse in sport. According to WADA, one of the reasons caffeine was removed from the Prohibited List was that many experts believe it to be ubiquitous in beverages and food and that having a threshold might lead to athletes being sanctioned for social or dietary consumption of caffeine. Furthermore, caffeine is metabolized at very different rates in individuals and hence urinary concentrations can vary considerably and do not always correlate to the dose ingested. In addition, caffeine is added to a wide range of popular food products such as coffee, tea, energy drinks and bars, and chocolate [13008].
History of doping with ephedrine

Ephedra is a Chinese shrub which has been used in China for medicinal purposes for several thousand years. The pure alkaloid ephedrine was first isolated and characterised by Nagai in 1885. It was then forgotten until it was rediscovered by Chen and Schmidt in the early 1920s. Its actions on the adrenoceptors could be classified into separate alpha and beta effects – a defining moment in the history of autonomic pharmacology. Ephedrine became a highly popular and effective treatment for asthma, particularly because, unlike adrenaline (until then the standard therapy), it can be given by mouth. Ephedrine as a treatment for asthma reached its zenith in the late 1950s, since when there has been a gradual and inevitable decline in its therapeutic use. From mainstream medicine, ephedrine moved into the twilight zone of street drugs and nutritional supplements. Ephedra and ephedrine products are now banned in many countries, as they are a major source for the production of the addictive compound methamphetamine (crystal meth) [11001].

Ephedrine is not only efficacious in the treatment of numerous ailments, but also has a long history of misuse. Research was needed to examine ephedrine policy over time in order to determine potential regulatory flaws that allowed misuse to continue. One review is based on primary literature derived from systematic searches of historical and scientific archives, as well as grey literature. Ephedrine managed to pass through numerous regulatory loopholes within seventy years. Despite warnings of misuse over the latter half of the century, ephedrine, and its herbal source, ephedra, were regulated in a piecemeal fashion and remained easily available to the public. Health authorities have struggled to control ephedrine, as an amphetamine "look-alike," as a methamphetamine precursor, as a dietary supplement, and as a medication. Despite being a potentially dangerous stimulant, under-regulation was perhaps more problematic than the substance itself. Tighter control of all ephedrine products, drugs and dietary supplements alike, might have prevented adverse outcomes and allowed this substance to remain available in a safer manner. Stringent regulation of all ephedrine products is necessary to prevent misuse and to protect the public's health [11002].

History of doping with cannabis

The medical properties of cannabis have been known for many centuries. The first documented use of cannabinoids for medical purposes dates back to 2800 BC in the Chinese herbarium Pen-ts'ao, a herbal pharmacopoeia describing many drugs among which cannabis, which was referred to as "ma", meaning “chaotic”. Pen-ts'ao described the pain-relieving, stupefying and hallucinogenic properties of cannabis and recommended cannabis for constipation, malaria, gout, rheumatism, and menstrual anomalies. Cannabis therapeutic use was introduced in Western medicine during the first half of the nineteenth century by the Irish physician William Brooke O'Shaughnessy (1809–1889), who studied forensic toxicology and chemistry at the University of Edinburgh in Scotland. He conducted a number of experiments in animals and proved that cannabis was safe even at high doses; thus, he extended the use of cannabis to patients suffering from rheumatism, seizures, and tetanus [12006].

The International Olympic Committee included cannabis in the banned substance list beginning in 1989 and since 2004 the World Anti-Doping Agency has prohibited its use for all sports competition. Cannabinoids are substances prohibited in-competition only [0009].
The class of cannabinoids has been subject of much debate concerning its relevancy for sports drug testing, fuelled by the increase of the urinary threshold for the main cannabis metabolite 11-nor-delta9-tetrahydrocannabinol-carboxylic acid (THCCOOH) from 15 ng/mL to 150 ng/mL (being effective since 11 May 2013) while the MRPL for cannabimimetics remained at 1 ng/mL as well as prevalence studies demonstrating the widespread availability and misuse of cannabis and its synthetic analogs. Since the raise of the urinary threshold for THCCOOH came unexpected, studies from early 2013 concerning improved/accelerated quantification approaches have become obsolete, even though the principle is certainly still valid 130009].

History of doping with alcohol

Alcohol is prohibited in-competition only and it is prohibited in the following sports: aeronautic, archery, automobile, karate, motorcycling and powerboating. Until 2010, modern pentathlon was also included in this list. The limit (blood tests) eligible for a doping violation is 0.10 g/L [13008].

History of doping with gamma-Hydroxybutyric acid (GHB)

Occurring naturally in many parts of our body from the brain to heart, to most muscles, kidneys and brown fat, gamma-hydroxybutyric acid or GHB for short was first synthesized in 1960s by Laborit in an attempt to study the effects of GHB and GABA, producing a compound that would interfere with beta oxidation and cross blood–brain barrier. It was later discovered that GHB was an endogenous compound and an endogenous metabolite of GABA. GHB was thus discovered in search for therapeutic GABA analogs. Since its discovery, GHB has played many roles in the laboratory. It was used to create an absence seizure model. GHB was also shown to have tissue-protective effects in the setting of myocardial infarction, stroke, sepsis, small bowel ischemia, hypovolemic shock, ionizing radiation and oxygen free radicals. Despite promising beneficial effects, GHB has not found widespread clinical use. In the 1960, GHB was used as a general anesthetic agent but fell out of favor due to an association with abnormal electroencephalographic (EEG) patterns in animals. In the year 1980, GHB could be bought in health food stores and the use began to rise amongst body builders as it was believed that taking this drug could improve muscle mass or improve exercise performance. While GHB has been present in laboratories and therapeutic trials for years, it has recently become a public health issue as a drug of abuse. Hence in the year 1990, FDA imposed ban over the counter sale of the drug throughout the United States. Simultaneously from 1997 to 1999, several states and countries passed laws to control the sale and consumption of GHB and finally designated it as a Schedule 1 substance in the United States in the year 2000 [13010].

History of biomarker approach regarding doping

In the late 1990s, as a first step, “no start” rules were introduced with the official objective to protect the health of the athletes when certain blood markers exceeded definite limits (e.g. hematocrit (Hct) above 50 percent or hemoglobin (Hb) above 17 g/dL (International Cycling Union, UCI) or Hb above 17.5 g/dL in men and 16.0 g/dL in women (International Ski Federation, FIS). In this time, the widespread use of rhEPO can be assumed on the basis of indirect evidence; e.g. in elite cross-country skiers extreme Hb values up to 20 g/dL were common between 1994 to 1996 but disappeared after the “no start” rule was introduced in 1997. Yet, mean Hb values continued to rise, suggesting the further use of artificial methods
with fewer extremes. It became obvious that the use of upper limits of definite blood values may result in athletes who would titrate rhEPO to approach the target Hb or Hct without exceeding it [13006].

Plasma volume fluctuations resulting from changes in posture, exercise, and training, altitude exposure, season as well as storage conditions influence concentration-based blood values such as Hct and Hb and thus represent a major limitation of their use with absolute limits. Additionally, cheating athletes may manipulate abnormally elevated Hb and Hct values by intravenous infusions of normal saline leading to hemodilution. On the other hand, even clean athletes may be declared unfit as Hb and Hct in a normal distribution may exceed the given limits. The panel of indirect markers was extended and more evidence was gathered on the effect on blood values of rhEPO administration in training athletes. It was suggested the use of a combination of indirect markers of altered erythropoiesis (reticulocyte Hct, serum EPO, soluble transferring receptor, Hct, %macrocytes) in a multivariate statistical model for detection of rhEPO during a possible administration phase (ON models) and after recent cessation of rhEPO use (OFF models). The sensitivity of these models was improved with larger numbers of subjects and resulted in the introduction of the so-called second-generation blood tests of which the OFF-hr model, a score combining Hb and %retics, is part of the current ABP according to the WADA ABP operating guidelines. Although OFF-hr was originally described for the detection of rhEPO use, it is also sensitive to other forms of blood doping such as blood transfusion [13006].

The application of these models by sports authorities and anti-doping organizations was problematic despite their scientific impact. The OFF-hr model was used by certain sports federations as another ‘no start’ criterion. Yet, infringements of the “no start” rule were equal to failing a “health test” but not considered a violation against WADA’s anti-doping code and therefore only yielded short mandatory interruptions of competition, e.g. 2 weeks. As even these improved biomarkers were only compared with a population-based reference range in a cross-sectional setting (e.g. universal limit of Hct above 50 %, OFF score greater than 122), it already seemed likely in 2000 that a longitudinal, individual hematologic profile, the so-called hematologic passport, could be advantageous to prevent and perhaps detect blood doping. Various attempts were made to define the natural within-subject and between-subject as well as analytical variability to use longitudinal measurements as an instrument against blood doping [13006].

**History of gene doping**

Gene therapist Ted Friedmann and multiple Olympic gold medallist Johann-Olav Koss were the first to describe the possibility of misusing the techniques and experiences of gene therapy in the athletic arena. In 2006, before the Turin Winter Olympic games, the president of the World Anti-Doping Agency (WADA), Dick Pound, called gene doping “the new threat that is now a reality.” Although Pound did not expect gene doping to pose a problem in Turin, he indicated that it could be a problem at the Summer Games, 2 years hence in Beijing. In fact, the problem did not materialize in China, in 2008, nor at the London 2012 Olympics, as far as the then available detection measures could determine [13011].

**History of athlete biological passport**

To detect cellular components of blood and those pharmacological agents that are too large to be excreted in urine, blood collection and analysis was begun in 2008. The Hematological
Module of the WADA Athlete Biological Passport uses the predictive model of Pottgiesser et al to monitor hematological marker changes within an individual. The fact that intraindividual variations in a number of blood (and urine) parameters are lower than interindividual variations has been used in the clinical chemistry laboratory since the 1970s. Blood is also analyzed for recombinant proteins, such as GH variants and biomarkers [12006].

**Doping in the Olympics**

*The ancient Olympic games*

The first instance of an athlete doping in competition is unclear, although there are examples of sportmen from the Greek era using natural substances to gain an advantage. The Olympic games, founded in 776 B.C. (date of the earliest recorded Olympic competition) in Olympia as a tribute to the gods but also to celebrate the virtues of athletic competition, peaceful coexistence and the magnificence of athletics, constitute Olympia’s perennial contribution to the world, this symbolized by the eternally burning Olympic flame. We may still sing today, as did Pindar in his eighth Olympian Victory Ode, “... of no contest greater than Olympia, Mother of Games, gold-wreathed Olympia...” [12002].

The ancient Olympic games were (almost) men only affairs. Successful athletes were highly honoured, and, perhaps for this reason, skulduggery was not unknown. For example, it is red in Wikipedia that “Sotades at the ninety-ninth Festival was victorious in the long race and proclaimed a Cretan, as in fact he was. But at the next Festival he made himself an Ephesian, being bribed to do so by the Ephesian people. For this act he was banished by the Cretans” [12003].

*Olympic nationalism*

In July 1912, the Boston Medical and Surgical Journal celebrated “American Supremacy,” noting that the “overwhelming success of the American athletes at the current Olympic games in Stockholm is as interesting physiologically as it is nationally gratifying. Although Sweden won the most medals, athletes from the United States won the gold medal count by the thinnest margin, 25 to 24.” The large population of the United States “is more mixed of all races, and we should therefore be able to select the best strains and breed the best mixtures.” American athletes were “better nourished and conditioned” than their competition, “which again should conduce to racial physical superiority.” To top it off, “the intensity of our national disposition leads our athletes to train much more eagerly and consistently, and with a keener professional intentness for winning.” Though eugenic undertones have faded, physicians have maintained their interest in the Olympics. The strobe of the quadrennial competitions illuminates dramatic changes in medicine and sport [12004].

*The first Olympic tests*

The first Olympic drug testing took place at the 1968 Games in Grenoble and Mexico City, but it was the Munich Games in 1972 that marked the introduction of a comprehensive testing program. Approximately 7000 athletes participated and just over 2000 samples were collected and analyzed for various types of stimulants. Since then the testing program has expanded for each (summer) Games, both in number and percentage-wise, and at the 2008 Beijing Games where 10,500 athletes took part, 4770 samples were collected. By then, the number of banned substances to be analytically identified had increased significantly. The IOC, however, had limited possibilities to conduct an efficient anti-doping activity as they
Doping during the modern Olympics

Athletes have always sought to outperform their competitors and regrettably some have resorted to misuse of drugs or doping to achieve this. Stimulants were taken by the first Olympic athletes to be disqualified in 1972. Although undetectable until 1975, from the 1950s androgenic anabolic steroids were administered for increased strength and power followed in the 1990s by erythropoietin for enhanced endurance. Both are highly effective doping agents. As analytical science validated improved techniques to identify these drugs, Olympic athletes, including many medallists were caught and disqualified. When the International Olympic Committee (IOC) prohibited beta blockers (beneficial in shooting), diuretics (assist weight classified athletes) and glucocorticosteroids, some athletes with genuine medical conditions were denied legitimate medical therapy. To overcome this, in 1992 the IOC introduced a system known now as Therapeutic Use Exemption (TUE). One paper discussed Olympic athletes who have been known to dope at past Games and some medical indications and pitfalls in the TUE process [12007].

The resolut fight during the Olympic games against doping in sports commenced as a result of the death of a Danish cyclist during the Rome Olympic Games in 1960 - directly seen by millions of people viewing TV. The International Olympic Committee (IOC) established a Medical Commission (IOC-MC) which had the task of designing a strategy to combat the misuse of drugs in Olympic Sport. It's today a far cry from the horror that ensued when drug testing was first introduced for the 1968 Winter and Summer Olympic Games and an athlete was busted, for of all things, drinking beer: the Swede modern pentathlete Hans-Gunnar Liljenwall was stripped of a bronze medal for dipping into the local cerveza at the Mexico City Summer Games [08012].

At the 2000 Olympics, 10 athletes were caught doping, including 6 medalists, while a record 27 athletes were caught doping at the 2004 Olympics. In total, 84 athletes, including 28 medal winners, have been caught doping at Summer Olympics, 37 of whom were weightlifters, the most notorious of sports amongst dopers. The Winter Olympics have generally witnessed fewer tests and fewer doping busts. Since 1968, only 13 athletes, including 6 medal winners, have been caught doping. Seven of them were cross-country skiers and 4 were hockey players. According to the International Olympic Committee there have been 84 infractions for doping since 1984, including such bizarre incidents as one in 2004 in which an Irish equestrian administered an antipsychotic drug to his horse. By Olympic Games, the failed, missed, refused or falsified test (and medals forfeited) have been [08012]:

<table>
<thead>
<tr>
<th>Year</th>
<th>City</th>
<th>Failed (Missed, Refused, Falsified)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1968</td>
<td>Mexico City</td>
<td>1 (1)</td>
</tr>
<tr>
<td>1972</td>
<td>Munich</td>
<td>7 (4)</td>
</tr>
<tr>
<td>1976</td>
<td>Montreal</td>
<td>10 (3)</td>
</tr>
<tr>
<td>1980</td>
<td>Moscow</td>
<td>0</td>
</tr>
</tbody>
</table>
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- Bulgaria 7
- USA 6 (3 gold and 2 bronze from Marion Jones)
- Hungary 3
- Germany 2
- Sweden 2
- Canada, Russia, Poland, Ireland, Romania, Finland, Mongolia, Greece, Spain, Armenia, the Netherlands & Ukraine 1

By sport 12 medals have been forfeited in weightlifting, 5 in athletics, 2 each in equestrian, wrestling and cycling, and 1 each in judo, modern pentathlon and rowing.

The increased number of positive tests is in part a function of the increased number of tests administered at each game. At the 2000 Olympics, about 2000 doping tests were administered. That number grew to 3700 by the 2004 Olympics. The increasing number is largely a result of the expansion of the rules governing who gets tested. In the past, the top 4 finalists in an event and 1 other athlete chosen randomly were subjected to tests. However, in Beijing, the top 5 athletes were tested in addition to 2 chosen at random in each final. As well, random tests will be conducted throughout earlier stages of competition. Beginning in 2000, Olympic athletes were also subject to pre-Olympic, out-of-competition testing to detect substances consumed prior to competition that wouldn't later appear on a test. Blood testing was introduced on a limited basis at the 1994 Winter Olympics and at the 2000 Summer Olympics. It is the International Olympic Committee itself that administered and monitored athlete testing in Beijing, not the World Anti-Doping Agency. The latter focuses on policies, regulations and monitoring the 33 facilities worldwide that have been approved for testing athletes' samples [08012].

Some International Sport Federations (IF) and National Sports Federations followed suit when the anti-doping process started, but progress was modest until the world's best male sprinter (Ben Johnson, Canada) was found doped with anabolic steroids at the Olympic Games in Seoul in 1988. Further progress was made following the cessation of the cold war in 1989 and in 1999 public authorities around the world joined the Olympic Movement in a unique partnership by creating WADA, the World Anti-Doping Agency, which has doubtless been the start of a new anti-doping era [08013].

The Olympics in medical journals

Even before Pierre de Coubertin revived the Olympics in 1896, lore from the ancient games circulated in the medical literature. An 1851 essay in the Boston Medical and Surgical
Journal about the power of mind over body described “the old Greek who died on the spot from excess of joy on seeing his three sons crowned with laurel at the Olympic games” (1851). Oliver Wendell Holmes invoked this same episode in his valedictory to Harvard medical graduates (1858). Other authors drew competing lessons from the Olympic legacy. One warned that excessive athletic training diverted energy from mental development, leaving adolescents “listless and stupid”: “It was especially remarked by the Greeks that no one who in boyhood won the prize at the Olympic games ever distinguished himself afterwards” (1867). An 1891 review, in contrast, expressed the hope that educators would learn from the ancient techniques and improve athletic training in U.S. schools [12004].

When the modern games began in Athens in 1896, physicians only slowly became interested – and mostly in marathons. Heat and humidity tormented marathoners in St. Louis in 1904: only 14 of 27 finished. The winner Thomas Hicks, who sustained himself during the race with strychnine sulfate, five eggs, and brandy, required the care of four physicians in the aftermath. Heat caused problems again in London and Stockholm. When the games resumed in Antwerp in 1920, athletes were subjected to physical examinations. The United States sent its first team physician – one who had fenced in Stockholm – to the Paris games in 1924. Medical scrutiny has continued ever since [12004].

Physicians have been interested in the Olympics for many reasons. In the 1920s, they probed the limits of human physiology. One group studied the Yale heavyweight rowers who won gold in Paris. An ingenious contraption revealed that at their racing speed – 12 mph – the eight men produced four horsepower, a 20-fold increase over resting metabolism (1925). A 1937 study published in the Journal showed that athletes at the 1936 Berlin games consumed 7300 calories each day. Sometimes the venue itself became the issue. The United States threatened to boycott the Berlin games until Hitler relented and allowed black and Jewish athletes to compete. Ignoring these tensions, the Journal, which had published a favorable review of Nazi health insurance in 1935, advertised the exhibits and lectures on “Medical Theory and Practice in the New Germany” that had been organized for physicians who visited the Olympics (1936). Boycott politics surfaced again when the Journal’s editor, Arnold Relman, visited the Soviet Union in 1980. Relations had been strained by tensions over the Soviet invasion of Afghanistan and the United States’ threat to boycott the Moscow games. Other venues created medical concerns. Roger Bannister, who eventually became a neurologist after being the first person to run a mile in under 4 minutes, “thoroughly disapproved” of holding the 1968 games at high altitude in Mexico City. And indeed, several hundred athletes collapsed at those Olympics, from migraine, shock, syncope, or emotional excitement. Fears of local pathogens emerged before the Olympics in Seoul (Japanese encephalitis) and Barcelona (multidrug-resistant strep). Each warning met with vehement rebuttal. Olympic events now attract millions of visitors and require careful medical and public health planning [12004].

The safety of Olympic sports has remained an enduring concern. The French Academy of Medicine appointed a committee before the 1924 Paris games “to study the effects of modern athletics on the human system.”. The resulting tests “revealed an alarming number of cases of athletic heart.” Subsequent studies from the 1920s (on athletes from the 1928 Amsterdam games) through the 1990s (on 310 Italian Olympians) have produced conflicting evidence on the question of whether intense physical training can cause cardiac hypertrophy. Some sports received special scrutiny. On the eve of the Atlanta games in 1996, a scathing review likened women’s gymnastics to child abuse, arguing that although “elite gymnastics can provide a profoundly meaningful experience for the athletes,” it could also “result in serious, life-endangering physical and psychological disabilities.” Citing injuries, eating disorders, and social problems, the authors warned that “talented youngsters at every competitive level should be supported rather than crippled by their sport as they enter...
adulthood” [12004].

A different kind of medical scrutiny emerged in the 1950s. Commenting on a symposium about “Pheidippidian physiology,” a 1957 editorial highlighted the recent dramatic improvements in performance at track and field events. What explained “these epidemics of broken records”? The editorial considered possible contributions from training, diet, antibiotics, and motivation but focused on “a speculative explanation”: “that amphetamine is being used by some athletes to help them break otherwise unassailable records.” Such practices, if they were in fact occurring, were both dangerous and “ethically undesirable.” Each passing decade brought new scandals about performance-enhancing drugs. After the U.S. Olympic Committee admitted that seven cyclists (including four medalists) had received blood transfusions at the Los Angeles games, a Sounding Board article in the Journal in 1985 condemned this practice. Not only was the practice dangerous, especially in light of the emerging AIDS epidemic, but also it “represents an attempt to use a medical therapy to provide athletes with an unfair competitive advantage.” When Ben Johnson was stripped of his gold medal at the Seoul Olympics, the Journal reviewed the medical risks and legal consequences of anabolic steroids in 1989. Erythropoietin came next. A Dutch physiologist wrote in the Journal that “the next Olympic Games have already been nicknamed the ‘Hematocrit Olympics,’” and physicians' obligation seemed clear: “the medical profession has a responsibility to consider carefully these untoward consequences of scientific progress.” [12004].

Performance-enhancing drugs have cast a long shadow on the modern Olympics. Whether the agents are the strychnine, heroin, cocaine, and morphine that athletes used in Athens in 1896 or the amphetamines, steroids, and erythropoietin that some use today, the dilemma remains the same. As a sports medicine specialist noted in 2004, the “attraction of performance-enhancing drugs is simply that they permit the fulfillment of the mythical promise of boundless athletic performance – the hubristic “faster, higher, stronger” motto of the Olympic Games.” The ensuing systems of medical surveillance have led, inevitably, to “a new type of competition,” in which some athletes try to stay one step ahead of the authorities [12004].

There are several historical journal article in the Boston Medical and Surgical Journal (1851-1925) and in the follower New England Journal of Medicine after that [12004]:

Boston Medical and Surgical Journal
1858. Holmes OW. Valedictory address. 58:149-59.
1867. The abuse of physical exercise. 77:425.
1891. Hartwell EM. The principal types of physical training compared. 125:641-4.
1924. French Academy of Medicine names committee to study effects on the human system of modern athletics. 190:397.

New England Journal of Medicine
1935. Davis MM, Kroeger G. Recent changes in German health insurance under the Hitler government. 212:1037-42.
1936. Information for doctors during the Olympiad in Berlin. 215:211.

Canada

It is now a long time since the Ben Johnson scandal at the Olympics in Seoul, Korea, drew attention to the issue of doping. In the scandal’s aftermath, an independent organization now known as the Canadian Centre for Ethics in Sport was established to develop a national antidoping program. It has served as a model for other national antidoping organizations. Still, 55 Canadian athletes violated antidoping rules in the past 3 years: 27 used “recreational” drugs such as marijuana, and 28 took performance-enhancing drugs, most often anabolic steroids or stimulants [08014].

Formation of the IOC Medical Commission

In 1961 the International Olympic Committee (IOC) created a Medical Commission (IOC–MC) at its 59th Session in Athens, Greece. The decision was triggered by the death of the Danish cyclist Knud Enemark Jensen during the road race for teams at the Rome Olympic Games the year before. He was said to have taken some stimulating drug, but was also reported to have suffered from heat exhaustion and dehydration. Probably a combination of all this caused his death, but this has never been officially confirmed. At any rate, the IOC could no longer ignore the use of stimulants that had obviously been in place in certain sports for quite some time. The creation of the IOC-MC marked the start of the modern era of the anti-doping campaign. The Commission was requested to propose a strategy for combating the use of performance-enhancing drugs in Olympic sports. It took quite a while to
analyze the situation and recruit the necessary competence. Not until the 1967 IOC session were some concrete proposals presented such as a list of forbidden drugs (stimulants) in Olympic sport and drug testing at the coming Games. Therefore, 1967 is often referred to as the start of the IOC-MC [12005].

**Formation of the World Anti-Doping Agency**

Probably the most serious challenge for the anti-doping fight during the 1970s and 1980s was the unwillingness of most countries and international federations to join the fight. There were several reasons, namely: the costs; the lack of competence; the negative image should a top athlete in their own country or sport test positive; and, the Cold War. The East Germans used success in sport as a political weapon and other countries had followed suit, although in a less sophisticated way. Officially, sports leaders were against doping, but far too many only paid lip service and some were even sabotaging the fight. When the Cold War faded following the political events in 1989 and the years that followed, the anti-doping fight gained increased support. In 1999 the public authorities actually joined the fight by accepting the invitation of the IOC to form WADA together [12005].

In 1999, the IOC recognized that an effective fight against doping required cooperation between sport and government. The First World Conference on Doping in Sport resulted in the formation of the WADA in 2000, which was charged with harmonizing the international antidoping efforts. WADA has developed a World Anti-Doping Program, which has been adopted by all Olympic sports. In order for governments to ratify an equivalent to the World Anti-Doping Program, it was necessary to develop the International Convention Against Doping in Sport through the UN Educational, Scientific, and Cultural Organization. To date, over 160 countries, including the United States, have approved the convention [12006].

**Formation of Court of Arbitration (CAS)**

During the late 1970s and early 1980s, one athlete after another who tested positive challenged the IAAF before national courts and the Federation spent a lot of money on court trials. In an attempt to take care of the problem of escalating legal costs the IAAF included in its constitution an “Arbitration panel” as the highest authority to settle disputes within athletics. This could not prevent athletes from bringing their cases before national courts, but the Arbitration panel served as deterrent. This happened in 1982 and a year later the IOC created the Court of Arbitration for Sport (CAS), which today is included in the WADA Code as the final appellate body on doping matters. There are examples of enormous legal costs following an athlete’s positive sample. The IAAF was ordered by a district court in USA to pay USD 27.4 million in damage compensation to the 400 m world-record holder Harry “Butch” Reynolds after their panel had disqualified him for steroid doping in 1990. The decision was overruled by the Circuit court, and the Supreme Court refused to hear the case. Thus, after several years of legal battle Reynolds lost the case (and money) but the IAAF also lost a lot of money. The winners were the lawyers on each side. In recent years, WADA successfully defended the testosterone analysis before CAS after it had been challenged by the Tour de France winner Floyd Landis following his positive test. The defense cost WADA about USD 2 million. The ever-increasing legal costs are a major challenge for sports organizations and a true threat to the anti-doping fight [12005].

**Out-of-competition testing**
The next challenge was to convince the world of sport that doping controls at competition only is not sufficient. Since AAS are taken during training periods in order to promote the building of muscles and strength, doping controls will also have to be conducted during training – so-called out-of-competition controls. Such controls had been in place in Norway and Sweden since the early 1980s but when the idea was brought to a broader international sports community it was met with great resistance. A majority saw it as an unacceptable intrusion into the athlete’s private life. However, the Ben Johnson scandal at the Seoul Games in 1988 made the skepticism fade and in 1990 the IAAF, as the first international federation, started testing out-of-competition after the necessary rules had been passed by the 1989 IAAF Congress. Today, out-of-competition testing is compulsory in the WADA Code, and any national anti-doping organization or international federation that does not conduct such testing will be declared noncompliant with the Code and possibly face serious consequences. The IAAF ambition to clean up its sport resulted in the identification of doped athletes, some very famous [12005].

**History of therapeutic use exemptions (TUE)**

The introduction of what today is known as therapeutic use exemption (TUE) was far from smooth. It started in the mid-1980s when a Swedish athlete asked the nation’s anti-doping organization for permission to use testosterone as replacement therapy following the removal of both his testes (unilateral cryptorchism as a newborn followed by cancer in his remaining testis as a teenager). It was granted with the remark that it was only valid within Sweden. Soon thereafter a similar case (bilateral testicular torsion) occurred in Australia. At the 1988 Olympic Games two athletes were allowed by the IOC-MC to use banned substances (corticosteroids for inflammatory bowel disease and diuretics for nephrotic syndrome). The permissions were granted although no clear rules existed. Some Commission members, therefore, suggested that such rules be worked out. Other members were against, arguing that athletes should not be allowed to use prohibited substances. If they were in such need they should quit sport. In 1992, however, a proposal was presented to the IOC-MC with strict criteria for “permitted use of prohibited substances” and the formation of a group that should evaluate applications for such use of IOC-MC. The proposal was accepted, and a “Medications Advisory Committee (MAC)” started its operation at the Barcelona Games in 1992. Although MAC’s authority was limited to the Olympic Games it became an advisory body for international federations and national anti-doping organizations. MAC worked under strict anonymity and, actually, in secret. The fear was that if MAC became generally known it would result in a flood of requests and abuse of the system. When the IOC Juridical Commission reviewed the 1999 edition of the IOC Medical Code they first refused to include the concept of “permitted use”. Only after pressure from the IOC-MC was it added as a brief ‘addendum’. When WADA was about to be formed, however, the IOC-MC requested that the concept of permitted use be accepted and clearly explained in the coming rules. Today TUE is an important part of the WADA Code [12005].

When the IOC undertook the first extensive doping controls (>2000) at the 1972 Munich Olympics, the only drugs that were prohibited were stimulants and narcotics. Androgenic-anabolic steroids (AAS) were prohibited prior to the 1976 Olympics, although endogenous steroids such as testosterone could not be identified. During this period, there was no discussion about athletes needing a Therapeutic use exemptions (TUE). However, after diuretics, beta-blockers and systemic glucocorticosteroids (GCS) were added to the IOC’s Prohibited List in the 1980s, significant interference occurred in the availability of essential medications to manage medical conditions in elite athletes. Around 1987-1988, two male
athletes without testes, one in Sweden and the other in Australia, sought to continue their testosterone replacement therapy and participate in their sport. Both were granted national approval. At the 1988 Calgary Games, an ice hockey player was approved to take oral GCS for inflammatory bowel disease (IBD) and later that year in Seoul, a rower with nephrotic syndrome was approved to continue her diuretic therapy and participate. Both were ad hoc decisions taken by all members of the IOC-MC, some of whom were not medically qualified. In February 1991, it was presented a paper “Permission for athletes to use drugs contained in the IOC List of Banned Classes” to the IOC-MC. This detailed the following criteria that should be met to approve any such application by an Olympic athlete [13013]:

- The athlete would experience significant impairment of health if the prohibited medication was withheld
- No enhancement of performance could result from the administration of the prohibited substance as medically prescribed
- The person would not be denied the prohibited substance if he/she was not a competing athlete
- No available permitted or practical alternative can be substituted for the prohibited substance
- Retrospective approval would not be granted

Independently, and at the same IOC-MC meeting, professor Manfred Donike presented a paper on “Replacement therapy” which focused on testosterone in anorchia. An interim Medications Advisory Committee (MAC) was appointed (D Catlin, A Ljungqvist and K Fitch, Convenor) and requested to develop guidelines to implement the proposal. This was undertaken and included [13013]:

- The complete medical details including the history, clinical findings and investigation must be submitted
- The necessity to administer the prohibited medication including the dosage, route and frequency of administration must be certified by a suitably qualified medical specialist
- The medical necessity to administer the prohibited substance cannot be the result, wholly or partially, of prior use of a drug from the banned classes or banned methods
- Additional investigations requested by the MAC will be undertaken at the athlete’s or his/her National Olympic Committee’s (NOC) expense
- Any doctor who provides the MAC with false information will be ineligible to be accredited as an Olympic team doctor or official
- Under no circumstances will permission be given to use any synthetic anabolic steroid

However, the concept had its doubters and it would be another year before the IOC-MC agreed to allow the MAC to start operating prior to the Barcelona Games. However, the IOC-MC Chairman, supported by some Commission members, would not permit any publicity for “fear of abuse of the system”. Hence, only two applications were received for Barcelona 1992. Both were for oral GCS for well-documented IBD and were approved. Each athlete won a medal, although neither was in an individual event [13013].

**Anorchia**

During the early years, MAC members were conscious that they were embarking on a new and potentially controversial undertaking and that their decisions must be able to withstand the closest scrutiny. In the mid-1990s, the MAC compiled the first document that established some circumstances in which certain prohibited substances and methods might be approved to manage specific medical conditions encountered in athletes. A number of conditions and
circumstances for which permission would not be granted were also documented. The classes of prohibited drugs under consideration were diuretics, corticosteroids, beta-blockers and AAS (only testosterone). No exemptions were contemplated for stimulants or narcotics. In 1994, two world-class sailors applied to continue to administer testosterone at the 1996 Olympic Games if selected. Both had anorchia, one a congenital condition and the other surgical due to bilateral malignancies. Having considered these applications, the MAC sought the advice of independent expert endocrinologists who were “blinded” as to the identity of each athlete, his NOC and his medical advisors. Both were approved and although neither athlete qualified at the 1996 Olympic yachting trials, one did compete in Athens 2004 [13013].

**Aplastic anemia**

One unusual request was for erythropoietin in a Winter Olympic athlete prior to the 1994 Lillehammer Games because she had recently been a bone marrow donor for her brother with aplastic anaemia. It was the usual policy of the oncology centre concerned to withdraw and store a unit of blood prior to harvesting bone marrow and reinfusing this postharvest. (This would have been a Prohibited Method.) Owing to not wanting to interrupt her Olympic training programme and the urgency of her brother’s need for a bone marrow donation, this was not undertaken and her haemoglobin fell by 2 g/dl. However, despite the compelling circumstances, the application was rejected [13013].

**Congenital adrenal hyperplasia (CAH) and hypogonadism**

Some early requests included an archer with 21-hydroxylase deficiency (salt losing) congenital adrenal hyperplasia (CAH) seeking oral GCS, which was approved. However, shortly after this, a shooter with 17-hydroxylase deficiency CAH who had been granted national approval was denied permission by her International Federation (IF) to administer GCS and compete internationally. Fortunately, intervention by the MAC reversed this decision and later, she competed successfully at the 2000 Olympic Games. Prior to Atlanta 1996, a female soccer footballer with C1-esterase deficiency hereditary angioneurotic oedema was approved to use danazol daily and participated. In contrast, an aging canoeist who had been prescribed testosterone for alleged hypogonadism and had the backing of his national antidoping agency sought permission to continue this therapy at Atlanta. The MAC advised that this athlete did not have a valid justification and rejected the application [13013].

**Stimulation medication**

A well-publicised case that regrettably reflected that the MAC was compelled to remain “clandestine” was that of an elite 22-year-old 3 m diver whose severe narcolepsy was diagnosed in 1996. Immediately, she sought permission from her NOC and then from FINA to take stimulant medication but was rejected by both bodies. She continued to compete nationally and internationally and won national titles but seemingly was not required to take a doping control until a Grand Prix event in 1999 when she admitted the use of dexamphetamine on the doping control form and tested positive. Despite being advised that her narcolepsy was so severe that she was at major risk of falling asleep on the 3 m diving platform and thus of potential injury, FINA’s Doping Control Panel imposed a 12 month suspension. Intervention by the FINA Medical Commission resulted in the sanction being reduced to 6 months, but on appeal to CAS, this was further reduced to 2 months. Ironically, less than a month after her positive test, FINA modified their doping regulations to permit the use of a prohibited medication in special circumstances. The opinion of the MAC was not sought until shortly prior to her initial FINA hearing and then at the request of her NOC. The MAC had no hesitation in advising that she was entitled to a TUE. This may or may not have
influenced both the FINA Medical Commission and the CAS. Had the IOC informed the stakeholders of the MAC’s existence, this diver would have applied and been approved to take her medication in 1996. Soon after this decision by the MAC, an application was received from a track and field athlete from the same NOC whose narcolepsy had been proven conclusively by sleep studies. On this occasion, the opinion of an independent expert in sleep medicine was sought. This request was granted by the MAC for the recently released but prohibited modafinil – a wakefulness drug [13013].

Danazol

Between 1992 and 2003, the IOC’s MAC (later TUEC) received numerous applications and its recommendations were believed to have been accepted on all occasions. In addition to functioning at the Olympic Games without publicity, the MAC provided advice to as many as 15 IFs and 11 NOCs who sought assistance. Few applications were received that could be deemed to be “opportunistic”, but one from a 33-year-old elite female weightlifter appeared to be. On the advice of a “Longevity Institute” doctor, she sought permission to administer transdermal testosterone following her hysterectomy and bilateral oophorectomies performed for endometriosis. This was in addition to her replacement permitted hormone therapy. The application was rejected because no woman should ever be granted approval to take any AAS except danazol for very strict criteria. From its early days, the MAC decided that no AAS, except testosterone, could be approved for any male athlete and only then with a conclusive diagnosis and if the necessity was confirmed by an independent expert. This policy remains today [13013].

Glucocorticoids and diuretics

In late 1998, approval for oral GCS treatment was granted to a young athlete in the sport of curling who had a successful renal transplant. Earlier that year, he had competed at the 1998 Nagano Olympics with an MAC-approved TUE for furosemide to assist in managing his chronic renal failure. At that time, a condition of approval was that the specific gravity of urine collected in doping control must be 1005 or greater by refractometer. However, improved analytical hardware has made this requirement no longer necessary [13013].

Beta-blocker

A 56-year-old elite international shooter with a history of coronary artery bypass surgery was denied permission to take a cardioselective beta-blocker and compete. Although his medical indication was not questioned, beta-blockers had been demonstrated to produce a 13 percent improvement in shooting performance and this was deemed to contravene the criterion of ‘not enhancing sports performance’. Currently, the status of beta-blockers in shooting remains unchanged, that is, TUEs should not be approved. Prior to Salt Lake City 2002, a bobsledder with low-serum testosterone after a unilateral radical orchiectomy for seminoma was denied permission to administer depotestosterone because of the presence of one intact testicle [13013].

Recognising the concept of TUEs

Many NOCs and IFs did not accept the policy of therapeutic use, first because it was not in the Medical Code of the Olympic Movement, and second, because of ignorance on the part of many sports authorities as to the concepts of therapeutic use. Thus, recognition of the concept of TUE continued to be difficult. In 1998, the IOC’s Juridical (Legal) Committee was incredulous at being advised by the author that TUEs had been approved for 6 years and
criticised the IOC-MC Chairman for denying the MAC and its operation any publicity. But it would be another 2 years before the Juridical Committee would finally permit a one sentence mention of the concept as an addendum to the 2000 edition of the Medical Code of the Olympic Movement. This permitted a small breakthrough when the Anti-Doping policy for the Sydney 2000 Olympics made mention of the principle. Australia is believed to be the first country to have a committee with legislative authority to approve TUEs from August 1999. During the Sydney 2000 Olympic Games, Australia's TUEC organised a meeting of NOCs, IFs and interested persons which recommended that templates of established and proposed criteria for specific TUEs be prepared. This task was undertaken by Australia's TUEC and the templates circulated to attendees for feedback in 2001 [13013].

It was the World Anti-Doping Agency (WADA), not the IOC, that provided global recognition and acceptance of TUE. Strenuous attempts to sell the idea of TUE occurred at the 1999 World Conference on Doping in Sport in Lausanne from which WADA had its origins. Fortunately, two members of the MAC had significant WADA roles from its start and were able to ensure that the principle of TUE was accepted in the World Anti-Doping Code. An International Standard of TUE (ISTUE) was prepared between 2001 and 2003 and became operative when WADA finally assumed global responsibility for doping in January 2004. Significantly, the TUE criteria and guidelines developed by the MAC in 1991-1992 were incorporated virtually unaltered in the initial ISTUE. WADA established a TUE Expert Group in 2004 and members reviewed the Australian TUE templates that had been updated at least annually. These were offered to WADA in 2005 but the offer was rejected. During the last 6 years, WADA has developed advice termed “Medical Information for TUE Committees”, which had a rocky start, but by involving experts in each field, it has become a valuable reference document for TUECs [13013].

Recent Olympic Games

It is difficult to compare TUEs at Olympic Games as changes to the Prohibited List have necessitated TUEs for different substances and methods. Insulin was prohibited prior to Sydney 2000 and 5/8 TUEs approved were for insulin-dependent diabetes mellitus (IDDM). But the number of applications increased significantly after WADA assumed global responsibility for doping from the IOC. At the Games in Athens 2004, 24 TUEs for athletes from 19 NOCs and 15 sports were approved with 9/24 (38 %) being for insulin. An appeal against a rejected application for systemic GCS was heard by WADA and the verdict confirmed that the IOC's MAC, now termed TUEC, had acted correctly. Intravenous infusions were prohibited prior to Torino 2006, although no athlete sought a TUE and 2/4 approved TUEs were for insulin for IDDM. Two applications were considered not to meet the criteria for approval and were discussed with each athlete's Chief Medical Officer (CMO) who accepted the TUEC's rationale and both were withdrawn. In Beijing, the TUEC recognised or approved 39 TUEs, of which nine were for IDDM. These 39 athletes were from 19 NOCs and 19 sports. For the first time, five intravenous infusions were approved. Interestingly, there were two Olympic athletes with well-documented Addison's disease approved for oral GCS. One application for stimulant medication for adult onset attention deficit hyperactive disorder was approved and a second withdrawn when a second opinion was sought, which was IOC's policy and later WADA's [213013].

Between 2002 and 2008, inhaled beta2-agonists (IBA) were managed by an IOC Independent Asthma Panel but not considered to be a TUE. However, in 2009, when WADA included IBAs in the prohibited list, the requirements to approve IBA use were identical to those of the IOC's Asthma Panel. This decision was partially rescinded just prior to Vancouver 2010 but two IBAs remained prohibited, formoterol and terbutaline. These accounted for 94 of the large number of 107 TUEs approved or recognised by the IOC's
TUEC at Vancouver. Of the remaining 13, there were three approvals for insulin for IDDM, two for ADHD and one for an IF infusion. One IF-approved TUE for a female athlete to take dehydroepiandrosterone was a fundamental error since no female athlete should ever be approved to take any AAS except danazol. An appeal to WADA was successful, but WADA rules permitted the athlete 14 days to cease taking this prohibited medication, which allowed her to continue until after the Games. This is unsatisfactory and should be changed.

A number of difficulties were encountered at the recently successful London Olympic Games. From 2004, the IOC’s TUEC had agreed to recognise TUEs for participating Olympic athletes approved by both IF and NADO TUECs after being subjected to close scrutiny. For Vancouver, the IOC was required to use WADA’s Anti-Doping Administration and Management System (ADAMS). This posed a number of problems which were augmented in London with almost four times the number of participating athletes and IFs. First, the IOC TUEC’s access to ADAMS was restricted since members were denied the opportunity to open uploaded medical files to allow them to confirm that an approved TUE had been granted according to established criteria. Second, more than half of the participating 205 NOCs do not upload TUEs onto ADAMS. Third, few NOCs heeded the request to provide the IOC’s TUEC with a list of valid TUEs for their athletes. Finally, much time was spent in fruitlessly examining ADAMS to identify athletes believed to be accredited for London 2012 who may have had current a TUE. Almost all TUEs identified on ADAMS for athletes presumed to be competing in London were either for substances that were currently not prohibited (mainly IBA) or short-term TUEs that were no longer necessary or valid. Clearly, the IOC needs WADA to provide superior access and a more efficient way of ascertaining valid TUEs if ADAMS is to be used at future Olympic Games.

Prior to the opening of the London Olympic Village, CMOs were requested to provide the IOC’s TUEC with details of all valid TUEs for their NOC athletes who would participate at the Games. Only two complied totally and 21 of the 31 preapproved TUEs that were recognised were from these two NOCs. During the Games, two athletes tested positive for prohibited substances and when advised, the NOCs of both athletes confirmed that each had a valid TUE that had not been disclosed. Hence, one must question how many other athletes had TUEs and did not advise the IOC. Although at each Games between 2002 and 2010, between 1/1000 and 1/1500 athletes were known to have IDDM, in London, only three athletes were reported to have a TUE for insulin (1/3500) which begs the question: were there more insulin-dependent diabetic athletes about whom the IOC was never advised? In London, the TUEC approved another 26 TUEs, with as customary most (15) being for systemic GCS and there were six intravenous infusions. One application was considered inappropriate and the CMO was contacted and the reasons provided. The NOC agreed to withdraw the application, institute an alternative permitted treatment and this athlete won a gold medal.

Legislation

In the early 1900s, endurance events lasted for days without rest. Open-water swimming, cycling, and long-distance running and walking athletes used stimulants such as strychnine, heroin, and amphetamine to alter the perception of fatigue. Only later did governments and sport recognize the serious health risks associated with the use of stimulants. The International Amateur Athletics Federation (IAAF; now the International Association of Athletics Federations) banned the use of stimulants in 1928. The amphetamine-related deaths of Danish cyclist Knud Enemark Jensen during competition at the 1960 Olympic Games and British cyclist Tommy Simpson during the 1967 Tour de France illustrated the
seriousness of the problem. In 1966, the cycling, soccer, and track and field international federations began testing for stimulants. The International Olympic Committee (IOC) formed its Medical Commission, which included a Subcommission on Biochemistry and Doping in Sport, in 1967 and tested for stimulants at the 1968 Olympic Games in Mexico City. France adopted antidoping legislation in 1963; the Council of Europe adopted the first international Anti-Doping Convention in 1968 [12006].

Concern regarding the effects of anabolic steroids on athletes resulted in US Congressional hearings in 1988. Anabolic steroids were scheduled under Class III of the Controlled Substance Act in 1990. That same year, the Dubin Commission report, commissioned by the Canadian government because of concerns regarding the use of public money in sport, documented widespread abuse of performance-enhancing drugs and poor testing by Canadian sporting authorities. In 1990, two governmental agencies, the Canadian Sport Anti-Doping Agency and the Australian Sport Drug Agency, were formed to deal with drugs in sports. The second Council of Europe Anti-Doping Convention was signed. A multilateral intergovernmental agreement, the International Anti-Doping Arrangement (IADA) was formed to promote more effective antidoping practices. The IADA group developed an International Organization for Standardization (ISO) Publicly Available Specification (ISO/PAS 188730) for collection of urine samples. This document eventually became the basis for WADA's International Standard for Testing [12006].

USA

The USADA was formed in 2000 by the US Olympic Committee in part to avoid the perception of the “fox guarding the hen house.” In 2001, Congress designated USADA “the official antidoping agency for Olympic, Pan American and Paralympic sport in the United States.” USADA was given authority to develop a comprehensive national antidoping program including testing, adjudication, education, and research. USADA and WADA have jointly worked to advance the science (analytical chemistry, biochemistry, endocrinology, hematology, laboratory medicine, pharmacology, physiology, sports medicine, and toxicology) of detection of doping [12006].

The Bay Area Laboratory Cooperative (BALCO) scandal was one of the early examples of information sharing between law-enforcement and antidoping agencies. BALCO was providing synthetic anabolic steroids not approved by the Food and Drug Administration and designed to avoid detection to a number of athletes including Kelli White, Marion Jones, and allegedly Barry Bonds. Sharing of information between the Internal Revenue Service Criminal Investigations, local law enforcement, and USADA enabled effective prosecution of the cases in criminal and sport venues, as appropriate. Prior to 2004, detection of a prohibited substance or its metabolites or markers was required to be prosecuted for a violation of the antidoping rules. The 2004 edition of the Code recognized other means for proving a case of doping, including any reliable information. USADA's prosecution of the first “nonanalytical positive” case that same year resulted in suspension of the athlete [12006].

Industrialized doping

In 2003, another significant event in the understanding of the institutional nature of doping occurred. A syringe was anonymously sent to a WADA-accredited laboratory in Los Angeles that contained tetrahydrogestrinone (THG), a “designer” steroid that was not known and not on the current WADA prohibited list, made specifically to avoid detection by modern antidoping technologies. This led to a series of investigations resulting in the indictment and
subsequent conviction of individuals running a performance-enhancing program for professional athletes at the BALCO pharmacy in San Francisco [08006].

In May 2006, Spanish police arrested five people and seized a variety of banned performance-enhancing drugs and blood-doping supplies at a Madrid doping clinic. Here, professional athletes would receive medically-supervised injections of hormones and other performance-enhancing drug regimes. The 40-page police report included a clear paper trail of doping procedures on at least 50 professional cyclists. The report was given to the International Cycling Union, which led to the disqualification of 23 professional cyclists, virtually all the top contenders from the 2006 Tour de France. The final of the 2006 Tour was also tarnished, as the champion, Floyd Landis, was found to have a positive anti-doping test for steroids. Landis was stripped of the championship and discharged from his team. At this writing the result is being challenged by Landis and his legal and medical experts, claiming that the test was invalid since several errors were made in the collection, analysis and reporting of the results [006].

In a separate investigation in Paris in 2006, 23 individuals were sentenced to 4 years in jail for trafficking a cocktail of amphetamines and other performance-enhancing drugs known as "Belgium Pot" to professional cyclists. In October of that same year, the cricket world was shocked to learn that two Pakistani fast bowlers, Shoaib Akhtar and Mohammad Asif, tested positive for the steroid nandrolone [08006].

Laboratory testing

Mass spectrometry has played a decisive role in doping analysis and doping control in human sport for almost 40 years. The standard of qualitative and quantitative determinations in body fluids has always attracted maximum attention from scientists. With its unique sensitivity and selectivity properties, mass spectrometry provides state-of-the-art technology in analytical chemistry. Both anti-doping organizations and the athletes concerned expect the utmost endeavours to prevent false-positive and false-negative results of the analytical evidence. The Olympic Games play an important role in international sport today and are milestones for technical development in doping analysis. Mass spectrometry has played an important role in doping control from Munich 1972 to Beijing 2008 Olympics [08015].

One brief note gave also a general overview on the activity of the antidoping laboratories accredited by the World Anti-Doping Agency outlining the evolution, over the last four decades, of the analytical methods and techniques in the detection of prohibited substances and methods. Special emphasis was given to the future trends of the fight against doping in sports, as seen from the perspective of a laboratory scientist, in the wider context of fair play, health protection, and perception of the activity of the antidoping laboratories by the general public [08016].

There were 35 WADA accredited laboratories in 2009. Approximately 3,300 additional samples were analyzed in 2009 compared to 2008, with a slight increase in Adverse Analytical Findings and Atypical Finding, from 1.84 percent (2008) to 2.02 percent (2009).

Anabolic steroids

The initial test for testosterone in urine was developed by Donike and coworkers, who showed that administered testosterone appeared in the urine as testosterone glucuronide. They also showed that for a population of athletes, the ratio of testosterone to
epitestosterone (T/E) had a positively skewed distribution, with a modal ratio of about 1:1. Initially, an athlete sample having a T/E ratio > 6:1 was considered a doping violation. The concept of intraindividual reference ranges (as opposed to population-based reference ranges) was introduced into the T/E test in the early 1990s. Computer programs are now used to compare an athlete's current sample result to their previous sample results. Results that are inconsistent with previous results are investigated and could result in targeted testing or an antidoping rule violation. The measurement of $^{13}$C/$^{12}$C ratios in testosterone and its metabolites has allowed the differentiation of pharmaceutical testosterone from natural testosterone. Donike's group also began the concept of the urinary “steroid profile,” which used a combination of other urinary steroids to increase the sensitivity of the test. Other antidoping research has identified a del/del genotype of UGT2B17 as the cause of a subpopulation of individuals who have low (<0.5) T/E ratios in urine, the use of 11 steroids in urine to improve test sensitivity, new metabolites of testosterone (e.g. testosterone cysteinate) in the urine, and several substances that affect the metabolism and excretion of testosterone [12006].

To measure testosterone in the urine to detect doping is not adequate because of large interindividual and intraindividual differences in urinary steroid concentration. However, the nearly constant ratio of urinary testosterone glucuronide to epitestosterone glucuronide became the basis of a better test. Epitestosterone is the 17alpha epimer of testosterone and has no known physiological function. It is not a metabolite of testosterone [08010]. An upper normal limit of six was calculated for the testosterone/epitestosterone ratio based upon population studies. In 1983 the Medical Commission of the International Olympic Committee (IOC) introduced this value as a criterion for testosterone abuse. Ratios above six should be considered suspicious, and the person concerned should be subjected to further testing. In 2004 the approved upper limit was set at four [08011].

The 2011 WADA list

International anti-doping efforts are harmonized and regulated under the umbrella of the World Anti-Doping Code and the corresponding Prohibited List, issued annually by the World Anti-Doping Agency (WADA). The necessity for a frequent and timely update of the Prohibited List (as the result of a comprehensive consultation process and subsequent consensual agreement by expert panels regarding substances and methods of performance manipulation in sports) is due to the constantly growing market of emerging therapeutics and thus new options for cheating athletes to illicitly enhance performance. In addition, “tailor-made” substances arguably designed to undermine sports drug testing procedures are considered and the potential of established drugs to represent a doping substance is revisited in light of recently generated information. The list that was published and has been authoritative from 1 January 2011 comprising a total of 10 different classes of banned substances (S0–S9), three different groups of prohibited methods (M1–M3), and two classes of drugs (P1 and P2) being banned from selected sports only. In comparison to the 2010 edition of the Prohibited List, few but significant modifications were made. A major novelty has been the installation of the S0 section, which interdicts the use of any pharmacological substance that has not (yet) received approval by governmental health authorities (or where development has discontinued) as a human therapeutic agent. This addendum is particularly important in the light of new drug entities that are not covered by any of the established classes of banned substances, either by their chemical nature or their biological effects. Ryanodine receptor-calstabin complex stabilizers, which have been proven to enhance performance in the laboratory setting are currently undergoing advanced clinical trials but do not represent compounds of S1–S9. These might exemplify such a new category of substances. The section S2 (peptide hormones, growth factors, and related substances) was
modified concerning the examples of erythropoiesis-stimulating agents (ESAs) by explicitly listing hypoxia-inducible factor (HIF) stabilizers, which also represent a considerably heterogeneous emerging class of substances targeted for clinical approval. In contrast to these additions to S2, the use of platelet-derived preparations has been legitimized and the paragraph removed in the 2011 Prohibited List accordingly. The category M2 (chemical and physical manipulation) was extended by a new paragraph (M2.3) that particularly emphasizes the illicit nature of the “sequential withdrawal, manipulation and reinfusion of whole blood into the circulatory system”, a strategy that includes, for example, the so-called UV-activated autohemotherapy (commonly regarded as alternative medicine). M3 (gene doping) was split into three sub-groups that define (1) the transfer of nucleic acids or sequences of these; (2) the use of normal or genetically altered cells; (3) the use of drugs manipulating gene expression with impact on athletic performance as prohibited methods [12016].

**The 2012 WADA list**

As of 1 January, the 2012 Prohibited List International Standard has come into effect, exhibiting minor but relevant alterations from to the previous 2011 version. In agreement with its predecessor, the List comprises a total of 10 different classes of banned substances (S0–S9), three different groups of prohibited methods (M1–M3), and two classes of drugs (P1 and P2). The latter are banned from selected sports only. The major modifications can be observed in the sections: S3 (beta₂-agonists), S4 (hormone and metabolic modulators), and M3 (gene doping). In the S3 group, quantitative consideration of formoterol has been considered with the allowance of a maximum daily therapeutic dose of 36 microg of inhaled formoterol and a urinary threshold of 30 ng/mL. If the determined quantity in urine exceeds this level, an adverse analytical finding is reported followed by penalty, unless the athlete can prove (e.g. by means of a pharmacokinetic study) that the concentrations were reached by the admissible route and daily dosage. The category S4 has been complemented by a new subsection named “metabolic modulators”. These host peroxisome proliferator activated receptor (PPAR)delta agonists such as GW1516 and PPARdelta-AMP-activated protein kinase (AMPK) axis agonists, such as 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR). These were formerly listed among gene doping (M3.3) in the previous list. Following a re-evaluation of the impact of the use of alcohol (P1) and beta-receptor blocking agents (beta-blockers, P2) on the athletes' performance in selected sport disciplines, the interdiction of alcohol was lifted for Ninepin and Tenpin Bowling (in agreement/on request of the Federation Internationale des Quilleurs) and so was the ban of beta-blockers for bobsleigh, skeleton, curling, modern pentathlon, motorcycling, sailing, and wrestling. In order to probe for potential patterns of abuse concerning selected substances that are currently not (or not at all times or at any concentration) prohibited, the established WADA monitoring programme has been expanded. Besides the stimulants bupropion, caffeine, phenylephrine, phenylpropanolamine, pipradrol, pseudoephedrine (< 150 microg/ml), and synephrine and the ratio of morphine over codeine, the prevalence of nicotine, hydrocodone, and tramadol was to be monitored in-competition. Moreover, the (ab)use of corticosteroids in out-of-competition periods is acquiring concern and appears as a new item on the 2012 monitoring programme. Concerning nicotine and its metabolites, a comprehensive compilation of monitoring data was published outlining an alarmingly high prevalence of nicotine use in selected sports disciplines. Further to these explicitly stated drugs, alternative medicine has necessitated greater attention in order to protect both the spirit of sport and the athletes themselves from inadvertent anti-doping rule violations. In continuation of the endeavor to keep pace with the changing trends of doping, manipulation, and innovations and improvements in analytical chemistry, anti-doping laboratories are urged to enhance their
procedures in terms of comprehensiveness, speed, and/or sensitivity. This, in combination with the fact that the International Standard for Laboratories allows for the long-term storage and re-analysis of doping control samples, is considered one of the main aspects causing deterrence to cheating athletes [13012].

The 2013 WADA list

Compared to its predecessor, the 2013 Prohibited List exhibited only few major modifications such as the re-categorization of insulins from S2 (peptide hormones, growth factors and related substances) to S4.5 (hormone and metabolic modulators) and the inclusion of M2.3 (chemical and physical manipulation) into M1.1 (manipulation of blood and blood components) by respective re-wording of the paragraph. In addition, the maximum daily therapeutic dose of 36 microg of inhaled formoterol (S3) was increased to 54 microg, resulting in a permissible urinary concentration of 40 ng/mL (formerly 30 ng/mL). In agreement with prior protocols, an adverse analytical finding is reported (followed by penalty) if the determined quantity in urine, including the measurement uncertainty, exceeds the threshold limit. The finding will be processed as an anti-doping rule violation unless the athlete can prove (e.g. by means of a pharmacokinetic study) that the concentrations were reached by the admissible route and daily dosage. In agreement with or on request of international federations, the interdiction of beta-receptor blocking agents (beta-blockers, P2) was lifted in selected sport disciplines including ninepin and tenpin bowling, aeronautic, boules, bridge, and powerboating. This is a continuation of the process initiated in 2012, where another 7 international federations removed the ban of beta-blockers from their sport [13009].

Category 0

The category S0 of the Prohibited List does not explicitly mention any specific substance; here, any pharmacological compound not covered by the other classes of prohibited substances and methods and without “current approval by a governmental regulatory health authority for human therapeutic use” is considered illicit. Potential candidates for this category are sirtuin-1 (SIRT1) activating drugs such as SRT1720 the characterization, metabolism. In case of the proposed routine doping control application, the mass spectrometer was a QqQ instrument with ESI source operated in positive mode and MRM, while compound characterization was conducted on a quadrupole-time-of-flight (Q-TOF) system [13009].

Monitoring program

In addition to the Prohibited List, WADA has established a monitoring program in order to probe for potential patterns of abuse concerning selected substances that are currently not (or not at all times or at any concentration) interdicted. The “in-competition” monitoring program, which included the stimulants bupropion, caffeine, phenylephrine, phenylpropanolamine, pipradrol, pseudoephedrine (< 150 microg/mL), synephrine, and nicotine as well as the ratio of morphine over codeine, hydrocodone, and tramadol in 2012, was complemented by the analgesic tapentadol in 2013. Further, as in 2012, the (mis)use of corticosteroids in out-of-competition periods has been investigated [13009].

Arne Ljungqvist
Professor emeritus Arne Ljungqvist from Karolinska Institutet, Stockholm, who has served in various high positions in the IOC, International Association of Athletics Federations (IAAF), Swedish Sports Confederation, to mention some of them, has dedicated a great deal of his life to service in sports and sports medicine. Arne Ljungqvist has especially dedicated his career to the fight against doping and to the protection of the health of the athletes. It all started over 60 years ago when Arne became a Swedish senior champion in high jump in 1951, jumping as high as 201 cm. Arne was multi-talented and won also the Swedish junior championships in pole vault and javelin. He was one of the favourites for the Gold medal in the high jump competition in the Olympic Games in Helsinki 1952, but unfortunately could not compete because of an injury that he sustained during a medical student carnival in the autumn of 1951 when a group of us were jumping for the general public in the streets with numerous hard landings on asphalt. Arne had to end his jumping career as his injury could not be cured, and it was not until the 1960s that the diagnosis of patellar tendinopathy could be made. Arne, however, continued his medical studies and soon became very successful. Arne was appointed professor at the Karolinska Institutet in 1972 because of his excellent medical research in the fields of renal and cardiovascular diseases and, later, oncology. He held several high professional positions such as Vice Dean of Medical Faculty, Karolinska Institutet, 1972–1977; Pro-Rector, Karolinska Institutet, 1977–1983; Chairperson, Department of Pathology and Cytology, Karolinska Hospital, 1983–1992; President, Swedish Council of Sports Research, 1980–1992; Dean Swedish School of Sport and Physical Education, 1992–1996; and President of the Swedish Cancer Society, 1992–2001. Interestingly enough, he took time to serve Chamberlain to His Majesty the King of Sweden, 1977-1986 and has since then been Lord in Waiting to His Majesty the King of Sweden. In 1971, Arne returned to sport as he was elected to the Board of the Swedish Athletics Association, where in 1973 he became chairperson. In 1975, he became a member of the Swedish Sports Confederation and was part of the initiation of the Swedish Commission against doping in 1977 with its own doping rules by 1979. During 1989–2001, Arne became the President of the Swedish Sports Confederation and in 1989 a member of the Swedish Olympic Committee. Arne has been a Member of the IOC Medical Commission and in 1994, Arne was elected Member of the IOC. When WADA was founded in 1999, Arne became the Member of WADA’s Foundation Board and Chairperson of WADA’s Health, Medical and Research Committee. Since 2003, Arne has been a Member of WADA’s Executive Committee and since 2008 has been WADA’s Vice President. Arne has been the front-line fighter against doping and his name is today one of the world’s most respected within international sports. Arne’s contributions in the fight against doping are second to none. He has been an athlete himself, which makes him understand the special language that is present in the athletic situation and also in the locker rooms. In his autobiography “Doping’s Nemesis”, Arne gives an unrivalled insider’s view of the biggest dope scandals over the years, including the Ben Johnson and Balco affairs and the history of the Greek sprinters at the Athens Olympics in 2004. Arne’s actions together with the Italian police during the Torino Olympics 2006 against the Austrian team are classic. Doping seems to be steadily on the decline, and there is no doubt that Arne has played a key role in this successful work. Arne’s legacy in the fight against doping is lasting [13014].
ORGANISATION OF ANTI-DOPING

The fight against the use of performance-enhancing drugs in sports has been in effect for nearly 90 years. The formation of the World Anti-Doping Agency in 1999 was a major event because an independent agency was entrusted with harmonization of the antidoping program. In addition to sports governing bodies, governments have endorsed WADA and its programs by signing a United Nations Education, Science, and Cultural Organization Convention on Doping. The first step in the harmonization process was the development of the World Anti-Doping Program. This program consisted of five documents – the Code, the International Standard for Testing, the International Standard for Laboratories, the Prohibited List, and the International Standard for Therapeutic Use Exemptions – which unified the approach of the international federations and national antidoping agencies in applying antidoping rules. For laboratory testing, the International Standard for Laboratories establishes the performance expectations for and competence of laboratories recognized by WADA, including accreditation under ISO/IEC 17025. The antidoping rules are adjudicated by arbitration using the internationally recognized Court of Arbitration for Sport [10006].

An international background to the anti-doping movement

Besides blatant over-commercialization, there is no more ominous threat to sports than doping. Drug-use methods are steadily becoming more sophisticated and ever harder to detect, increasingly demanding the use of complex analytical procedures of biotechnology and molecular medicine. It should also be mentioned in passing that doping is not limited to human sport competitions but is also practiced in animal sports, e.g. equestrian sports. Underlying this distressing phenomenon is regrettably a worldwide pervasive attitude that represents the exact contrary of the original and declared aims of the event as promulgated by de Coubertin in 1903, namely, the aspiration “not to have conquered but to have fought well”. It is important to note that there is frequently differentiation between the official methods employed for anti-doping detection in WADA/IOC laboratories and strategies applied experimentally in various other laboratories. However, laboratories that are not accredited by WADA may apply methods and develop strategies, such as WADA laboratories, in support of the Athlete Biological Passport Program, where all data produced by laboratories can be collated. Over the past few decades, doping has become ever more complex and widespread, increasingly involving exploitation of the fields of endocrine-pharmacology and molecular biology. It thus currently represents not only an individual health hazard but also a menace to society itself, undermining the principles and significance of all the great sports events, and in particular the Olympic games. Faced with this problem, the scientific world is today striving to confront the challenge, in particular in regard to the recent development of hormones abuse, ever more complex methods and new technology being deployed for the task. It is evident that anti-doping policy should proceed to implementation of newly developed analytical methodology and advanced instrumentation as part of a strategy to clearly distinguish between the use of legitimate medication and the use of illicit substances. Furthermore, a multidimensional strategy to counter the scourge needs to be developed combining strict “prohibitionist” measures with preventive-educational programs. Meanwhile, the effort needs also to entail an individualized approach, incorporating counselling on a personal basis, which takes strongly into account the social and psychological background of each athlete. Meanwhile, with regard to athletics, there must be full support of the effort exerted by the WADA to detect banned substances and
compounds so as to eradicate doping, accompanied by legislative changes and longer disqualifications. By continuing to upgrade collaboration with national Anti-Doping Agencies, WADA will be enabled to considerably improve efficacy, thereby gaining ever better control of doping, consequently reducing fraud while, vitally, lessening the health risks incurred by athletes. The prevention of harm to the athlete and the guarantee of fair play should be the target [12011].

The international sports community has recognized for many years the dangers of all forms of doping, but it has been only in the recent past that serious and increasingly effective regulatory mechanisms have been put into place for the detection and control of drug-based doping in sports. It has been clear that, given the opportunity, athletes and their trainers and handlers will resort to many illicit techniques and substances to provide a competitive advantage in sports. One should only remember the pervasive and officially sanctioned and operated doping programs established in East Germany between 1970s and 1980s; how effective these were in the short term, and how harmful these were to the athletes in the long term. It seems very likely that the world of sports will continue to seek out new drugs and stealthy drug delivery methods and even gene-based enhancement to ensure victory in competition. Athletics represents one of the provinces of human activity, most susceptible to the application of existing and future advances in the field of human gene therapy, for the enhancement of nondisease human traits. Modern athletics is as much an entertainment as it is a sport and is sodden with huge amounts of money to assure the victories and records that the public demands. Athletic events are also some of the most powerful instruments for international politics. The prestige, nationalism, and jingoism compel our political institutions to demand victory. Finally, athletes are by nature risk-takers who are driven to compete, excel, and win, even at the cost of injury and other harm to themselves. But even worse, athletes are highly vulnerable to potentially harmful manipulation by dishonest and venal rogue trainers, sports technicians, and sports associations and federations who disregard the ideals of sports and the welfare of the athletes in the interests of victory at all cost. The fact that a sport is already filled with many pervasive drug-based forms of doping should convince even the most skeptical that all current and future advances in pharmacology, sports physiology, and sports medicine, whether based on ever more sophisticated drugs, gene transfer technology, or other still unrecognized technologies, will be applied to the world of sports and will almost certainly occur before the underlying technology is known to be effective and truly safe [06006].

Taking as an example the Tour de France, it can be posited that in the last century, in virtually each year the winner and/or runner-ups were either known to have used doping or strongly suspected of having done so. Aspiring to organize a Tour without doping can therefore be seen as trying to invent a Tour that has never existed before. The difference between earlier and more recent Tours, admittedly important, is the kind of performance enhancing technology that has been made available by the biomedical revolution over the last decades. If in the early days of sports the arsenal of performance enhancing compounds was quite limited, today’s advances of biomedical science have indeed opened up Pandora's box with unlimited possibilities but also increased health risks. Since a doping culture has always been part of cycling it is quite understandable that these new possibilities from biomedical research were, and still are being exploited for performance enhancement practices by cyclists and their entourage. WADA’s claim that a culture of doping-free sport will develop and help attain the eradication of doping in sports remains to be proven; for competitive road-cycling, recent publications suggest that although doping practices certainly have changed, a culture of doping in professional cycling still prevails. A provocative editorial in the journal Nature in 2007 proposed that perhaps the Tour de France should be the first competition to accept pharmacological performance enhancement [12012].
World Anti-Doping Agency (WADA)

To improve the fight against this new potential kind of abuse, the International Olympic Committee (IOC) and national sports federations collaborated in 1998 to establish the World Anti-Doping Agency (WADA), an agency jointly funded by the IOC and cooperating nations and committed to develop programs for detection and control of athletic doping. It carries out its tasks by compiling and constantly updating a list of substances and methods that are inconsistent with the ideals of sports and that should be banned from athletic competition. It is also responsible for developing and validating new, scientifically sound detection assays and implementing effective international programs for in-competition and out-of-competition screening of athletes. The WADA has implemented its program on drug control in sports by issuing and continually updating the world Anti-Doping Code, including a list of banned substances and methods, the latest of which is presented as an appendix to this volume [06006].

One article provided a review of the leading role of the World Anti-Doping Agency (WADA) in the context of the global fight against doping in sport and the harmonization of anti-doping rules worldwide through the implementation of the World Anti-Doping Program. Particular emphasis is given to the WADA-laboratory accreditation program, which is coordinated by the Science Department of WADA in conjunction with the Laboratory Expert Group, and the cooperation with the international accreditation community through International Laboratory Accreditation Cooperation and other organizations, all of which contribute to constant improvement of laboratory performance in the global fight against doping in sport. A perspective is provided of the means to refine the existing anti-doping rules and programs to ensure continuous improvement in order to face growing sophisticated challenges. A viewpoint on WADA’s desire to embrace cooperation with other international organizations whose knowledge can contribute to the fight against doping in sport is acknowledged [12010].

Background

Athletes have a long history of using substances in an attempt to gain an advantage in sporting competitions. The ancient Greeks and Romans used herbs, fungi, poppy seeds and stimulants such as strychnine in order to boost performance. In the modern era, this practice continued mostly with the use of stimulants and narcotics. Sports federations took notice and in 1928 the International Association of Athletics Federations (IAAF) became the first federation to prohibit the use of performance-enhancing drugs (PEDs), although there would be no testing in sport for another 40 years. Amphetamine use was involved in the deaths of cyclists Knud Jensen and Tommy Simpson in the 1960 Olympic Games and the 1967 Tour de France respectively; this spurred the development of the International Olympic Commissions (IOC) Medical Commission, which published the first IOC Prohibited List in 1967. This became the de facto Prohibited List for Olympic Sport Federations. The “Festina affair” (1998 Tour de France), where a team trainer’s car was found to contain a panoply of PEDs, was the catalyst to create a new organisation to harmonise, coordinate and promote the fight against doping in sport in all its forms. The IOC convened the first World Conference in Doping in Sport in 1999, which resulted in the formation of the World Anti-Doping Agency (WADA) [13015].

Pre-WADA history
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The code

WADA is a unique, independent body representing equally sport and the governments of the world. The World Anti-Doping Code is the core document on which anti-doping programmes are modelled. The first version of the Code came into effect in January 2004. There are presently over 600 signatories, including almost all the world's sport federations. The Code applies to Athletes, as defined by their national anti-doping organisations (NADOs) or international federations. Who is considered an athlete for anti-doping purposes may vary widely and a NADO may still test recreational athletes but not apply all elements of the Code, for example, the requirement for whereabouts or advanced therapeutic use exemptions. Athletes may be subjected to sanctions based on possession or trafficking of prohibited substances and not simply due to a positive doping test. However, it is important to be aware that criminal legislation exists in certain countries (e.g. for narcotics) which may be in addition to, or completely separate from, anti-doping sanctions [13015].

The prohibited list

WADA also took over the role of publishing the Prohibited List (List), revised annually since 2004. The List has expanded considerably from the original IOC Prohibited List of the 1960s and contains numerous classes of substances as well as prohibited methods such as blood manipulation. A substance (or method) is considered for inclusion if it meets any two of the following criteria:

- potential for performance enhancement
- detrimental to the athlete's health
- contrary to the spirit of sport

The deliberations on whether to include substances in the List are a highly interactive and consultative process which includes stakeholders and experts. It is impractical to list all known and possible compounds; thus, most of the prohibited classes contain an important clause stating: "...and other substances with similar chemical structure or similar biological effect(s)" [13015].

Some substances have permitted routes of administration, (e.g. glucocorticosteroids are allowed by inhalation or topically). A few substances are permitted but only to a certain threshold level (e.g. pseudoephedrine). The List is divided into substances prohibited in competition only (e.g. stimulants), and those prohibited at all times (e.g. anabolic steroids and erythropoietin). It is irrelevant whether the prohibited substance is synthetic or from botanical
sources or whether it is considered a pharmaceutical product or a dietary supplement. “Strict Liability” means that every athlete is responsible for the substances found in their bodily specimen during a doping control sample analysis. The first line of defence for many cheating athletes has been to claim that the positive test resulted from a tainted dietary supplement. Many of these same athletes later confessed to deliberate ingestion of a prohibited substance. The athlete’s responsibility to explain how a prohibited substance entered his/her body (Strict Liability) has existed for many years, being initially implemented by the IOC. It has withstood the scrutiny of the Court of Arbitration in Sport and civil courts, and is a balance between protecting all athletes by ensuring fair, clean sport and the rights of individual athletes [13010].

**Codes since 2004**

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**Not only doping testing**

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**Sanctions of violations**

For an athlete confronted with an anti-doping rule violation, section 10.5 of the Code allows for no sanction, or reduced sanctions, if the athlete can demonstrate no fault or no significant fault. As far as supplements are concerned, simply stating the unknowing ingestion of a tainted dietary supplement is not sufficient – an athlete would have to demonstrate clearly
that every reasonable precaution was taken to avoid ingestion of a prohibited substance [13015].

**Indirect evidence of doping effects: world records**

Progression of world records in athletics is a reliable mean to assess the potentiality of the human body, which also reflects how society has evolved over time and will continue to evolve. It was conducted a quantitative analysis of world records in measurable Olympic events from nine representative disciplines (100, 400, 1500, 10,000 m, marathon, long jump, high jump, shot put and javelin throw) in order to identify progression and trends for the years 1900-2007 from the database of the International Olympic Committee. Overall, the relative improvement of athletic performance was higher in women than in men, being nearly doubled across the different specialities. The biggest increases were observed for javelin throw and shot put, in both men and women, respectively. Conversely, the improvement in race time was directly related to the race distance. It was also observed a consistent significant linear model of world record progression in time, although the improvement has substantially stopped or reached a plateau in several specialities. The observed trend might be explained by a variety of factors, including social and environmental changes, natural selection, advances in training and sport physiology, ergogenic aids and, possibly, doping [08017].

The performance-enhancing effects of doping and the introduction of intensive doping surveillance are especially reflected in the decline in performance in several women's track and field events [08018].

The introduction of doping substances and methods in sports triggers noticeable effects on physical performance in metric sports. It was used time series analysis to investigate the recent development in male and female elite sprinting performance. Time series displaying the average of the world's top 20 athletes were analyzed employing polynomial spline functions and moving averages. Outstanding changes in performance over time were statistically analyzed by Welch's t-test and by Cohen's measurements of effect. For validation we exemplarily show that our analysis is capable of indicating the effect of the introduction of in- and out-of-competition doping testing on women's shot put as well as the effects of the market introduction of erythropoietin (EPO) and the introduction of EPO and continuous erythropoiesis receptor activator (CERA) testing on 5000 m top 20 male performances. Time series analysis for 100 m men reveals a highly significant drop by more than 0.1 s from 2006 to 2011 with a large effect size of 0.952. This is roughly half of the effect size that can be found for the development of the 5000 m performance during the introduction of EPO between 1991 and 1996. While the men's 200 m sprinting performance shows a similar development, the women's 100 m and 200 m sprinting performances only show some minor abnormalities. It was discussed why the striking sex-specific improvement in sprinting performance is indicative for a novel, very effective doping procedure with insulin-like growth factor-1 (IGF-1) being the primary candidate explaining the observed effects [12013].

Doping is a very serious issue bedevilling the sporting arena. It has consequences for athletes' careers, perception of sports in the society and funding of sports events and sporting organisations. There is a widespread perception that doping unfairly improves results of athletes. A statistical study of information on best lifetime results of top 100 m sprinters (males better than 9.98 s, females 11.00 s), over the period of 1980-2011 was conducted. Athletes were divided into categories of “doped” (n=17 males and 14 females), based on self admission, the confirmed detection of known doping agents in their bodies or
doping conviction, and “non-doped” (n=46 males and 55 females). No significant differences (unpaired t-test) between dopers and non-dopers were found in their average results: male “dopers” 9.89 s identical with “non-dopers” 9.89 s, females 10.84 s and 10.88 s respectively. Slopes of regressions of best results on dates for both “dopers” and “non dopers” were not significantly different from zero. This indicates that no general improvement as a group in 100 m sprint results over a quarter of a century occurred irrespective of doping being or not being used. It was concluded that since there are no statistical differences between athletes found "doping" and the others, one of the following must be true: (1) "doping" as used by athletes so detected does not improve results, or (2) "doping" is widespread and only sometimes detected. Since there was no improvement in overall results during the last quarter of the century, the first conclusion is more likely. Objectively, various "doping" agents have obvious physiological or anatomical effects. These may not translate into better results due to the clandestine use of doping that prevents its scientific structuring. Perception of the effectiveness of doping should be reconsidered. Policy changes may be required to ensure the continued fairness and equity in testing, legislation and sports in general [12014].

Anti-doping rules

The ramifications of doping are not limited to topclass athletes who may feel compelled to risk their health for fame and money, but also extend to amateur athletes eager to exhibit superiority in the athletic field. Owing to difficulties in actually proving the intent to cheat, the World Anti-Doping Agency (WADA) enforces a principle of strict liability for positive test results for banned substances. Antidoping laws encompass a broad, continuously updated panel of laboratory tests for the most recent list of banned substances, which includes traditional as well as promising new drugs and techniques that may find actual applications in doping athletes [06005].

The rules

FIFA introduced an anti-doping programme in 1966 at the World Championship, being one of the first international sports federations to do so. The fundamental aims stipulated in the FIFA doping control regulations in 2006 are quite similar to the purpose of the World Anti-Doping Code programme. According to the definition of doping in the World Anti-Doping Code, doping is defined as the occurrence of one or more of the following violations [06002]:

- the presence of a Prohibited Substance or its Metabolites or Markers in an Athlete's bodily Specimen (strict liability rule).
- possession by an athlete at any time or place of a Substance that is prohibited in out-of-competition testing or a Prohibited Method, unless the athlete establishes that possession is pursuant to a therapeutic use exemption granted in accordance with the FIFA Doping Control Regulations regarding the therapeutical use of forbidden substances or other acceptable justification.
- possession of a Substance that is prohibited in out-of-competition testing or Prohibited Method by athlete Support personnel in connection with an athlete, competition or training, unless the athlete support personnel establishes that the possession is pursuant to a therapeutic use exemption as described previously
- trafficking in any Prohibited Substance or Prohibited Method is still a violation of the anti-doping regulations and in most of the law systems an illegal act against the medical preparations law

163
administration or the attempted administration of a Prohibited Method to any athlete, or assisting, encouraging, aiding, abetting or covering up as well as any other type of complicity involving an anti-doping rule violation or any attempted violation.

As set forth in the preamble of the World Anti-Doping Code, the purposes of the World Anti-Doping Program are:

- to protect the athletes’ fundamental right to participate in doping-free sport and thus promote health, fairness, and equality for Athletes worldwide
- to ensure harmonized, coordinated, and effective anti-doping programs at the international and national level with regard to detection, deterrence, and prevention of doping.

Prohibited substances in the context of these regulations are regularly published in the WADA list of prohibited substances (www.wada-ama.org) [06002].

**Strict liability rule**

The reason for the strict liability rule has been comprehensively stated by the Court of Arbitration for Sport, Lausanne in some cases. "It is true that a strict liability test is likely in some sense to be unfair in an individual case, where the athlete may have taken medication as the result of mislabelling or faulty advice for which he or she is not responsible – particularly in the circumstances of sudden illness in a foreign country. But it is also in some sense unfair for an athlete to get food poisoned on the eve of an important competition be altered to undo unfairness. Just as the competition will not be postponed to await the athlete’s recovery, so the prohibition of banned substances will not be lifted in recognition of its accidental absorption. The vicissitudes of competition, like those of life generally, may create many types of unfairness, whether by accident or the negligence of unaccountable persons, which the law cannot repair. Furthermore, it appears to be a laudable policy objective not to repair an accidental unfairness to an individual by creating an intentional unfairness to the whole body of other competitors. This is what would happen if banned performance-enhancing substances were tolerated when absorbed inadvertently. Moreover, it is likely that even intentional abuse would in many cases escape sanction for lack of proof of guilty intent. And it is certain that a requirement if intent would invite costly litigation that may well cripple federations – particularly those run on modest budgets in their fight against doping" [06002].

It is irrelevant whether the prohibited substance is synthetic or from botanical sources or whether it is considered a pharmaceutical product or a dietary supplement. **Strict Liability** means that every athlete is responsible for the substances found in their bodily specimen during a doping control sample analysis. The first line of defence for many cheating athletes has been to claim that the positive test resulted from a tainted dietary supplement. The athlete’s responsibility to explain how a prohibited substance entered his/her body (“Strict Liability”) has existed for many years, being initially implemented by the IOC. It has withstood the scrutiny of the Court of Arbitration in Sport and civil courts, and is a balance between protecting all athletes by ensuring fair, clean sport and the rights of individual athletes [13015].

For an athlete confronted with an anti-doping rule violation, section 10.5 of the Code allows for no sanction, or reduced sanctions, if the athlete can demonstrate no fault or no significant fault. As far as supplements are concerned, simply stating the unknowing ingestion of a
tainted dietary supplement is not sufficient – an athlete would have to demonstrate clearly that every reasonable precaution was taken to avoid ingestion of a prohibited substance. An athlete taking a spiked supplement with intent to dope may claim that he/she did not realise the product contained a prohibited substance. It is difficult to know the intent: nevertheless, the athlete will benefit from the ergogenic effect of the prohibited substance and have an unfair advantage over their competitor. There are many cautionary tales of athletes taking energy boosting supplements before or during the games and subsequently being sanctioned. Many dietary supplements that promise to enhance performance either contain a prohibited substance or are an example of false advertising [13015].

The reality is that a significant percentage (5-20 %) of supplements contains prohibited substances, either by inadvertent contamination or deliberate adulteration, during the production process. This phenomenon has been demonstrated repeatedly, and sporting federations as well as anti-doping organisations continue to impress this warning upon athletes. For example, several athletes have been recently sanctioned over the stimulant methylhexaneamine (MHA), explicitly prohibited since 2009. This was considered to be a dietary supplement from geranium oil, despite the fact that several studies demonstrated that its presence in supplements was not from geranium oil but due to the addition of synthetic MHA. Whether natural or synthetic, athletes need to avoid these types of products [13015].

**The whereabouts rule**

Apart from such special cases, effective doping controls are bonded to out-of-competition tests. Without accurate athlete location information such controls may be inefficient and sometimes impossible. This so-called “whereabouts rule” requires athletes and/or teams that have been identified for out-of-competition control to be responsible for providing and updating information on their whereabouts so that they can be located for No Advance Notice out-of-competition control. The applicable requirements are set by the responsible sport federation or national anti-doping organisation to allow some flexibility based upon varying circumstances encountered in different sports and countries. A violation of this rule may be based on either intentional or negligent conduct by the athlete, but it is known that the whereabouts rule may not be realistic in international team sports, in which players are normally playing for a club far from their home nation [06002].

**Separation of power**

An important legal principle is the separation of power between the anti-doping executive authorities and the disciplinary committee responsible for the administration of anti-doping sanctions. This is to minimise any accusations of bias or conflict of interest in the application of the Code. This principle is applied in a practical sense by having the Doping Control Sub-Committee (representing medical, pharmacological, and medicolegal expertise) dealing with the medical and biochemical aspects of the alleged doping event and, once this issue has been determined, a separate Disciplinary Committee which awards the appropriate sanction in view of the individual circumstances of the athlete concerned [06002].

**Non-approved substances**

Since 2011, this category (S0) of banned substances has been a part of WADA’s prohibited list and encompasses a virtually infinite number of compounds currently not covered by any of the other sections (e.g. anabolic agents, peptide hormones, growth factors and related substances). New representatives of this class of compounds are low molecular weight luteinizing hormone (LMWLH) receptor agonists. Focusing on two series of drug candidates
based on either pyrazole or thienopyrimidine core structures, two model substances were synthesized and used to establish a targeted/non-targeted screening method employing both diagnostic precursor-product ion pair detection and precursor ion scanning. In the absence of metabolism study data, the presence of the intact drug or at least a conserved nucleus must be present to allow the detection using the proposed strategy [12017].

This newly established category of banned substances encompasses a virtually infinite number of compounds with corresponding physicochemical and pharmacological properties; however, only those agents currently not covered by any of the other sections (e.g. anabolic agents, peptide hormones, growth factors, and related substances) are considered relevant for S0. These compounds [016]

- have not received approval for human therapeutic use
- comprise structures not related to any other listed group of banned substances
- exhibit biological effects that are different from all other drugs included in the Prohibited List.

**Therapeutic use exemption (TUE)**

The need for therapeutic use exemptions (TUEs) or the permitted use of Prohibited Substances and Prohibited Methods by athletes to treat significant medical conditions arose when several classes of drugs used commonly in medicine were prohibited in sport by the International Olympic Committee (IOC) during the 1980s. However, although the IOC Medical Commission (IOC-MC) gave qualified support for the concept to formally start at the 1992 Barcelona Olympics, the Commission’s fears that athletes might abuse the mechanism resulted in minimal publicity and its non-inclusion in the Medical Code of the Olympic Movement for 8 years. TUEs would not be widely publicised until the advent of the World Anti-Doping Agency which not only approved the principles of TUEs as developed by the IOC's Medications Advisory Committee (MAC) in 1991, but also introduced the name of TUE. Several changes to the Prohibited List have resulted in TUEs being necessary for substances that were permitted 20 years ago as disclosed in a review of TUEs approved at the 11 Olympic Games that the IOC’s MAC, later the TUE Committee (TUEC), has operated. The IOC and its TUEC played a pivotal role in developing the concept of TUE which is now globally accepted [13013].

Athletes can be allowed to use substances from the prohibited list (the doping list) if they have a medical condition. If so, a Therapeutic Use Exemption (TUE) is required. The boundaries between the use of pharmacological substances due to a medical need and doping are sometimes blurred. Although manipulating the system of TUE granting potentially represents an entry stage for doping, few studies examine how athletes perceive TUE management and relate this to current anti-doping policy. 645 Danish elite athletes (mean age 22) representing 40 sports completed a web-based questionnaire about their experience and perception of TUE (response rate: 43 %). Nineteen percent of the respondents had been granted a TUE. Eighty-five percent of athletes granted a TUE regarded their use of the TUE system as necessary to compete on equal terms with other athletes. Administrative hurdles for TUE prevented 7 percent of athletes from applying. Fifty-three percent of the athletes considered that being "allowed" to dope by means of a TUE was of importance for their (hypothetical) wish to try out doping. Fifty-one percent believed that athletes in their sport received TUEs without a medical need. Athletes granted TUEs had more than twice as high odds to distrust the efficacy of the system than athletes never granted a TUE. The belief that TUEs were misused was especially common among endurance athletes, regardless of them having experience with TUEs or not. Four percent believed it would be okay to receive a TUE without a medical need. The results confirm that TUE is a problem in anti-doping policy. The
fact that distrust in TUE administration increases once an athlete has experience of TUEs represents a challenge for anti-doping policy. We suggest more critical research on TUEs be carried out in order to improve harmonization and increase transparency in the regulations [13020].

Athletes who have either physical symptoms or disease after injury may need to be treated with specific medicines that are on the list of prohibited substances. Therapeutic use exemption may be granted to such players, in accordance with strictly defined criteria-these are presented in this article. Procedures of how to request for an abbreviated or a standard therapeutic use exemption are explained, and data on therapeutic use exemptions are also presented. A therapeutic use exemption (TUE) permitting the use of such a substance or method that is on the prohibited list, may be granted to the player, depending to the clinical situation. An exemption will be granted only in strict accordance with the following criteria [06007]:

- the athlete shall submit an application for TUE no less than 21 days before participating in an event
- the athlete would experience a significant impairment to health if the prohibited substance or method were to be withheld in the course of treating an acute or chronic medical condition
- the therapeutic use of the prohibited substance or method would produce no additional enhancement of performance other than that which might be anticipated by a return to a state of normal health following the treatment of a legitimate medical condition. The use of any prohibited substance or method to increase “low-normal” levels of any endogenous hormone is not considered an acceptable therapeutic intervention
- there is no reasonable therapeutic alternative to the use of the otherwise prohibited substance or method
- the necessity for the otherwise prohibited substance or method cannot be a consequence, wholly or in part, or prior non-therapeutic use of any substance from the prohibited list
- an application for a TUE will not be considered for retroactive approval except in cases where:
  - emergency treatment or treatment of an acute medical condition was necessary
  - due to exceptional circumstances, there was insufficient time or opportunity for an applicant to submit, or the granting body to consider, an application prior to doping control
- confidentiality of information: The applicant shall provide written consent for the transmission of all information pertaining to the application to members of the granting body and, as required, other independent medical or relevant scientific experts. If the assistance of external, independent experts is required, all details of the application will be circulated without identifying the player involved in the doctor's care. The applicant shall also give written consent to the decisions of the granting body to be distributed to the involved medical personnel of other relevant anti-doping organisations under the provisions of the Doping Control Regulations. The members of the granting body involved will conduct all of their activities in strict confidence according to the Hippocratic Oath and the medico-legal and ethical rules of confidentiality.

**Abbreviated TUE**
Abbreviated TUE requests are valid as soon as an approved anti-doping organisation has received the request. Treatment may start immediately after the receipt is confirmed. However, the anti-doping organisation has the right to ask for additional information should the applied indication for glucocorticosteroids or beta-2-agonists appear doubtful. WADA has also decided that for a beta-2-agonist TUE, following a clinical diagnosis of exercise induced or allergic asthma the results of lung function tests have to be submitted to substantiate the clinical diagnosis. This decision of the WADA Medical Committee clearly stresses the importance of sound clinical diagnosis following state of the art assessment to avoid misuse of beta agonists in the absence of clear clinical diagnosis [06007].

**Standard TUE**

In the case of a standard TUE application for which specialist expertise is required, the TUE committee appoints external independent experts for a second opinion to justify the decision. Standard TUE requests are valid as soon as an appropriate anti-doping organisation has sent the player a certificate of approval, except in rare cases of an acute life threatening condition, for which retroactive approval may be considered. TUEs are indispensable for improving medical cover of athletes with health impairments while avoiding anti-doping violations [06007].

**Therapeutic use exemptions (TUEs) at the Olympic Games**

That Olympic athletes, indeed all athletes with documented and significant medical conditions, may seek and be approved to administer a Prohibited Substance or a Prohibited Method and train for and compete in sports is now widely acknowledged and accepted. This is termed a Therapeutic Use Exemption (TUE), although some were granted to Olympic and non-Olympic athletes before this name was introduced in 2001. The International Olympic Committee (IOC), through its Medical Commission (IOC-MC), had a pivotal role in the development of TUE and its TUE Committee (TUEC) has functioned since prior to the 1992 Olympic Games in Barcelona. London 2012 was the 11th Olympic Games at which it had operated. But in its early years, acceptance of the concept of TUEs had many difficulties. However, although the IOC Medical Commission (IOC-MC) gave qualified support for the concept to formally start at the 1992 Barcelona Olympics, the Commission's fears that athletes might abuse the mechanism resulted in minimal publicity and its non-inclusion in the Medical Code of the Olympic Movement for 8 years. TUEs would not be widely publicised until the advent of the World Anti-Doping Agency which not only approved the principles of TUEs as developed by the IOC's Medications Advisory Committee (MAC) in 1991, but also introduced the name of TUE. Several changes to the Prohibited List have resulted in TUEs being necessary for substances that were permitted 20 years ago as disclosed in a review of TUEs approved at the 11 Olympic Games that the IOC’s MAC, later the TUE Committee (TUEC), has operated. The IOC and its TUEC played a pivotal role in developing the concept of TUE which is now globally accepted [13013].

Two decades of experience of TUEs at the Olympic Games have demonstrated that from a semiclandestine procedure in 1992, it has evolved so that currently there are established principles, a robust mechanism to apply and approve or reject applications, globally recognised medical indications and a mutual recognition of appropriately approved TUEs, at least at the Olympic Games. It is gratifying that the IOC’s 1991–1992 pioneering criteria and guidelines have undergone only minor modifications to those of WADA that operated at London 2012. However, despite widespread understanding of the TUE principles and processes, it is essential to continue to strive for superior knowledge of the topic, better
qualified and more experienced TUECs and hopefully a mutual recognition of correctly approved TUEs from experienced TUECs [13013].

Prevalence of use of TUEs in asthmatics

The prevalence of asthma and the use of anti-asthmatic medication is high among elite athletes. Elite athletes require a TUE certificate (Therapeutic Use Exemption) if they require anti-asthmatic medication which is on the prohibited list. The aim of one study was to determine the distribution of Danish TUE certificates and to examine the use of anti-asthmatic medication among Danish elite athletes. A cross-sectional study of all applications for TUE certificates in 2005 was carried out. It was focused on applications including anti-asthmatic medication. All applications resulted in certificates being issued. A total of 694 TUE certificates were issued. Of these, 445 (64 %) concerned anti-asthmatic medication. Short-acting beta-2-agonists (SABA) were the most frequent medication (79 %). Only 2 percent received long-acting beta-2-agonists (LABA) as single therapy. Inhaled steroids were used by 69 percent. Swimmers received significantly higher doses of inhaled steroids compared to all others athletes (1031 microg/day; n=148). The applications for TUE certificates were generally handled by general practitioners (78 %) [08039].

Prioritation in anti-doping

There is debate concerning whether the guiding paradigm for anti-doping policy should be the current legalistic approach or a “harm minimisation” approach prioritising athlete health. This study sought to determine whether a representative sample of Australians prioritises health above other concerns using the World Anti-Doping Code's Spirit of Sport statement which lists the 11 attributes that define the moral basis for anti-doping. A Best-Worst Scaling (BWS) Balanced Incomplete Block Design experiment using 11 choice sets of five Spirit attributes from the set of 11, with the attributes within each choice set in a random order. A representative sample of 168 Australians responded to an on-line survey. The BWS scores defined the relative ranking of each attribute to define an aggregate model and demographically defined models (gender, education, sports participation and sports following). Health was ranked as 7/11 in the aggregate model. Only those who did not follow sport prioritised health (2/11), with other demographic models failing to show a meaningful departure from the aggregate model. It was concluded that Australians ranked health below other attributes in the Spirit of Sport, appearing to prioritise "rule following" consistent with the legalistic approach. This challenges the harm minimisation approach to managing the role of drugs in sport and suggests that rule-following and legalistic approaches to drug use should take precedence over health messages [12031].

Strategy to reduce illicit drug

Australian football

The World Anti-Doping Agency (WADA) prescribes that drug testing is conducted in sports competitions to detect drug use in athletes. This testing includes performance-enhancing drugs as well as illicit substances such as marijuana, amphetamines and cocaine. Illicit drugs are tested for on match days but not on non-match days. Some athletes are known to use illicit substances for recreational purposes, away from competition times and this poses a serious health and welfare issue not addressed by the usual sport drug testing regimes. This paper reports the results of the first 7 years of an illicit drug-testing programme that included
non-match day testing in the elite Australian Football competition, the Australian Football League (AFL). Players in the AFL were tested for illicit drugs both in-competition and out-of-competition. Players were selected for illicit substance tests either randomly or targeted based on previous test history or time since previous test. The number of tests conducted was increased each year from 2005 to 2011 and testing was focused on high-risk times during non-competition periods. There were no positive match day tests. There was a significant reduction in positive tests (19-6) for illicit drugs during non-competition periods over the 7 years. The reduction in positive tests may be related to player education, the greater number of tests conducted and the harm minimisation approach of the illicit drug policy [12032].

**Agreements with the pharmaceutical industry**

With a steady stream of new therapeutic agents – from stimulants to steroids to protein hormones – with potential for abuse in sport entering the marketplace, antidoping scientists and collaborators are continually developing new approaches for detection of prohibited substances and methods. WADA has signed a declaration with the International Federation of Pharmaceutical Manufacturers and Associations, whose members will voluntarily assist in identifying products with doping potential in advance of their introduction into the marketplace [12006].

The misuse of medicines for performance enhancement in sport (doping) is not approved by regulatory agencies, and is illegal in many countries. In addition to the ‘traditional’ doping agents such as steroids, beta-blockers and blood transfusions, the list of agents and techniques used in doping is increasing and now includes newer medicines such as erythropoiesis-stimulating agents and growth hormones. Innovative new medicines are of particular interest as would-be dopers may believe them to be undetectable by current methods. Close collaboration between the biopharmaceutical industry and anti-doping agencies such as the World Anti-Doping Agency is critical to a successful anti-doping strategy. Industry is ideally placed to identify the doping potential of new medicines at early stages and to support early development of detection assays. A strong, united front between the biopharmaceutical industry and anti-doping agencies is essential to counter the misuse of medicines for performance enhancement, as well as to promote fair play and clean sport [12033].

WADA is making significant progress in building relationships within the pharmaceutical and biotech industries. Cooperation, similar to that which now exists with Roche, GlaxoSmithKline and the International Federation of Pharmaceutical Manufacturers Association (IFPMA), will give WADA a head start by working on pipeline compounds that have the potential for abuse in sport [12034].

David A Cowan and Campbell P Barker spoke to Ryan De Vooght-Johnson at Bioanalysis in May 2012 about the partnership between industry and academia for the setup and running of the doping-control laboratory for the London 2012 Olympic Games. David A Cowan is Director of the London 2012 anti-doping laboratory as well as Director of the King's College London Drug Control Centre, the UK's only WADA-accredited anti-doping laboratory, and Head of the Department of Forensic Science and Drug Monitoring. Cowan co-founded the Drug Control Centre in 1978 and became its Director in 1990. He has published extensively in the field of pharmaceutical analysis, especially as it relates to detecting drug administration in sport, and was awarded a personal chair in pharmaceutical toxicology in 1996. Cowan became Head of the Department of Forensic Science and Drug Monitoring at King's College London in 2002. He has served on a number of national and international committees,
including the Council of Europe Working Party Investigating Drug Abuse in Sport that led to the first World Anti-Doping Convention, the Laboratory Representative on the International Olympic Committee's Medical Commission, and WADA's Laboratory Accreditation Subcommittee. He is a member of the Crippen Club for Distinguished Toxicologists. In 1998 he was awarded the IOC Trophy for Sport Ethics by the BOA. He was a founding member of the World Association of Anti-Doping Scientists and became its first President serving on its Executive Board between 2001 and 2004. He was a Visiting Laboratory Director at the Salt Lake City Winter Olympic Games 2002, where the first novel erythropoiesis-stimulating protein (NESP) positive was discovered. He was also a senior advisory scientist at both the Turin Winter Olympic Games in 2006 and the Beijing Olympic Games in 2008. He was also a member of the IOC Medical Commission for the Sydney Olympic Games in 2000 and the Vancouver Winter Olympic Games in 2010. The Drug Control Centre undertook the sample analysis during the 2002 Manchester Commonwealth Games and Cowan was Co-Director of the laboratory for the Commonwealth Games in Kuala Lumpur in 1998. Cowan, who has directed the laboratory at King's College London for many years, was a member of the bid team making the presentation to the International Olympic Committee in February 2005. Campbell P Barker has been leading GlaxoSmithKline's delivery of the London 2012 laboratory services since September 2009. Prior to that time he was Director of Global Strategic Projects in GlaxoSmithKline's Consumer Healthcare business from 2006, and from 1994 to 2006 he worked in R&D for Procter & Gamble. Barker holds a PhD in chemistry from the University of Durham [12035].

Doping controls in practice

Drug testing is now ubiquitous in sport, and it often falls to the team physician to perform a variety of roles including interpreting test results, designing drug-testing programs, acting as medical review officer, and providing therapeutic use exemptions, education, and counseling. Proper understanding of current testing methods for drugs such as anabolic-androgenic steroids, erythropoietin, and growth hormone is essential if the team physician is going to assume these positions. One article outlined the basics of athletic drug testing from the collection process through the interpretation of results to assist the team physician in this field [06008].

International Sport Federations in the protection of the athlete’s health

To determine the priorities and activities of International Sport Federations (IFs) with respect to the promotion of health in their sport and for the general population all 35 IFs participating in Olympic Games in 2014 or in 2016 were asked to rate the importance of 10 indicated topics, and to report their programmes, guidelines or research activities on 16 health-related topics using an online questionnaire (response rate 97 %). On average, the “fight against doping” had the highest priority followed by “health of their elite athlete” and “image as a safe sport”. The topics with the lowest importance ratings were “health of their recreational athlete”, “increasing the number of recreational athletes” and “health of the general population”. All except one IF reported to have health-related programmes/guidelines/research activities; most IFs had 7 or 8 of the listed activities. Eight IFs (24 %) stated to have activities for “prevention of chronic diseases in the general population” but only FIFA and FINA reported related projects. It was concluded that IFs aimed to protect the health of their elite athletes through a variety of activities, however the health and number of their recreational athletes was of low importance for them. Thus, IFs are missing an important opportunity to increase the popularity of their sport, and to contribute to the health of the

171
general population by encouraging physical activity through their sport. FIFA's “Football for Health” and FINA's “Swim for All” projects could serve as role models [13025].

During the Olympics

One article outlines the process of preparation of an anti-doping laboratory in view of the activities to be performed on the occasion of the Olympic Games, focusing in particular on the accreditation requirements of the World Anti-Doping Agency (WADA) and ISO/IEC 17025, as well as on the additional obligations required by the International Olympic Committee, which is the testing authority responsible for the anti-doping activities at the Olympics. Due to the elevated workload expected on the occasion of the Olympic Games, the designated anti-doping laboratory needs to increase its analytical capacity (samples processed/time) and capability by increasing the laboratory's resources in terms of space, instrumentation and personnel. Two representative cases, one related to the Winter Olympic Games (Torino 2006) and one related to the Summer Olympic Games (Beijing 2008), are presented in detail, in order to discuss the main aspects of compliance with both the WADA and ISO/IEC 17025 accreditation requirements [12054].

A brand new 2012 London Olympics anti-doping lab made the headlines worldwide, accompanied by strong anti-doping messages for prospective Olympians and illustrated by an iconic photograph depicting the laboratory's head showing a blood sample to the Minister for Sports and the Olympics. The 4'400 m² laboratory, sponsored by a multinational pharmaceutical company, was operating 24 h/day during the Games, analysing urine and blood samples of one out of two participating athletes while a part of the samples will be stored for eight years, using the threat of future testing technology as a further deterrent [12012].

Biomarkers

Biomarker monitoring can be considered a new era in the effort against doping. Opposed to the old concept in doping control of direct detection of a prohibited substance in a biological sample such as urine or blood, the new paradigm allows a personalized longitudinal monitoring of biomarkers that indicate non-physiological responses independently of the used doping technique or substance, and may cause sanctioning of illicit practices. This review presents the development of biomarker monitoring in sports doping control and focuses on the implementation of the Athlete Biological Passport as the current concept of the World Anti Doping Agency for the detection of blood doping (hematological module). The scope of one article extends to the description of novel biomarkers and future concepts of application [12055].

"Exercisenomics"

Laboratory medicine is complex and contributes to the diagnosis, therapeutic monitoring and follow-up of acquired and inherited human disorders. The regular practice of physical exercise provides important benefits in heath and disease and sports medicine is thereby receiving growing focus from almost each and every clinical discipline, including laboratory medicine. Sport-laboratory medicine is a relatively innovative branch of laboratory science, which can provide valuable contributions to the diagnosis and follow-up of athletic injuries, and which is acquiring a growing clinical significance to support biomechanics and identify novel genomics and "exercisenomics" patterns that can help identify specific athlete's tendency towards certain types of sport traumas and injuries. Laboratory medicine can also provide sport physicians and coaches with valuable clues about personal inclination towards
a certain sport, health status, fitness and nutritional deficiencies of professional, elite and recreational athletes in order to enable a better and earlier prediction of sport injuries, overreaching and overtraining. Finally, the wide armamentarium of laboratory tests represents the milestone for identifying cheating athletes in the strenuous fight against doping in sports [12056].

**Multi-class and multi-analyte test methods**

Traditionally, doping control analytical assays have been drug-class dedicated and tailored to address requirements concerning sample preparation and chromatography/mass spectrometry resulting from specific physicochemical properties of target compounds. Improved analytical instrumentation (particularly based on liquid chromatography-(tandem) mass spectrometry have enabled the development of numerous cost-effective and rapid alternatives, allowing for multi-class/multi-analyte test methods. The trend towards comprehensive and preferably combined targeted/non-targeted screening procedures has been motivated in part in the requirement for analytical approaches to meet the minimum required performance levels (MRPLs) stipulated by WADA. Within the last year, several LC-MS/(MS)-based approaches were published representing options to complement or expand the currently employed methodologies of doping control laboratories. For example, employing targeted multiple-reaction monitoring (MRM), the detection of a total of 61 analytes (plus two internal standards) from urine covering seven classes of prohibited substances (S1–S7) and one agent categorized under M1 was reported. While these assays are all designed to specifically measure amplitudes of target compounds with dedicated precursor-/product-ion pairs and thus gate out all other information (for the advantage of sensitivity and speed), a trend towards combined targeted/nontargeted analytical methods has been recognized over the last few years. Here, particularly LC-MS/(MS) approaches with high resolution/high accuracy mass analyzers such as time-of-flight (TOF) and orbitrap as well as hybrids consisting of quadrupole or ion trap mass selective devices and TOF or orbitraps have been used. The benefit of analytical information being recorded in utmost extent (limited essentially only by sample preparation and/or ionization capability) has been especially recognized and appreciated [12017].

**Statistics**

**London Olympics**

More than 1000 samples were analyzed within a few days after each event for stimulants, steroids, masking agents, recombinant proteins like erythropoietin and growth hormone (GH), and other substances on the World Anti-Doping Agency (WADA) Prohibited List in the London Olympics in 2012 [12006].

**FIFA**

The fight against doping in sport receives considerable media interest and results in much speculation regarding the ability of athletes to compete on a level playing field. Football was one of the sports that took early leadership in this fight when the Fédération Internationale de Football Association (FIFA) introduced doping controls in football in 1970 as part of a wider strategy to ensure that the results of representative matches were a fair reflection of the ability of those taking part. As a result of the collaborative effort between FIFA and regional confederations and their member associations in conjunction with national anti-doping
organisations, more than 20,000 doping controls are performed annually on football players. The overall incidence of positive doping samples for prohibited substances accounts for 0.4 percent of all tests. Most of the positive drug tests are due to cannabis and cocaine, the so-called social drugs. Only a few individual cases (0.07% of the positive tests in 2004) were positive for anabolic steroids, such as nandrolone and testosterone. The majority of doping controls have been carried out in competition. FIFA, the Union of European Football Associations (UEFA), and some of the national anti-doping organisations also perform unannounced, out-of-competition controls at training venues during the football season. Prior to the 2006 FIFA World Cup being held in Germany, unannounced doping controls have been performed in the friendly matches between nations. Doping controls have also been performed during the training camps prior to the opening match on June 2006. All tests to date have proved negative. UEFA has also performed unannounced testing in the 2005–06 football season in all of the teams participating in the UEFA Champions League and UEFA Cup. Ten players were randomly selected from each of the 38 European top professional teams and were subjected to testing. No prohibited substances were found in any of the 380 samples tested. Since 1994, FIFA has followed a similar strategy in international competitions for both men and women. In these tests, two randomly selected players per team are tested after each finals match and a total of 3327 tests have been performed in 32 tournaments to date. Only three samples have tested positive since testing commenced: one for ephedrine, one for cannabis, and one for nandrolone. One sample tested positive for ephedrine during the qualifying matches for the 2006 FIFA World Cup being held in Germany. The incidence of positive tests in FIFA competitions over the past 12 years is 0.1 percent. During the 2000 Olympic Games in Sydney and the 2004 Olympic Games in Athens, none of the football players tested positive for any prohibited substance. The internal surveys of all Olympic team sports federations revealed that none of the team sports athletes tested positive for prohibited substances. The comparison of positive drug tests among different sports is currently not possible as the World Anti-Doping Agency (WADA) presents only adverse analytical findings in their published statistics rather than true positive results. The statistics include “therapeutic use exemptions” as well as elevated (>4) testosterone to epitestosterone (T/E) ratio which may be seen in normal athletes. Football accounts for the majority of doping controls performed worldwide. The current doping statistics demonstrate a very low incidence of positive tests and justifies the assumption that there is no evidence for systematic doping in football and most probably in any of the other Olympic team sports. Although no clear data are available from WADA about the distribution of in-competition and out-of-competition drug testing, it can be assumed that the majority are performed in competition. It has to be remembered that the professional football season in which the footballers are subject to random testing runs for 49 weeks a year in most football playing nations. There are several possible explanations for the low incidence of the positive findings of prohibited substances among football players:

- the stringent drug testing programme occurs during the entire football season in most countries
- football players worldwide understand that prohibited substances in sport will neither improve their physical performance nor their football specific skills and hence are reluctant to use agents that are not effective and subject to possible sanctions
- ongoing education campaigns by FIFA for doctors, administrators, officials and players have encouraged a drug-free culture in football

It is also possible that both in-competition and out-of-competition testing is insufficient to detect drug use. This is unlikely, given the large number of in-competition and out-of-competition drug tests occurring at all levels of professional sport over many years with relatively few positive results. Over the past six years FIFA, realising that the dimension of
misuse of prohibited substances is different from that in individual Olympic sports, has also developed close collaboration with the medical representatives of other Olympic team sports federations, as well as with the International Rugby Board. The medical representatives of these bodies expressed their collective opinion during the WADA meeting in March 2003 in Copenhagen, suggesting a possible revision of the World Anti-Doping Code given the different needs of international team sports federations and the lack of evidence of systematic doping in those sports. Furthermore, given the testing of over 20,000 doping controls in football annually worldwide, it became obvious that a close collaboration had to be developed with the accredited testing laboratories to understand the different examination methods and to keep abreast of new scientific developments. The close collaboration with the laboratories has resulted in these laboratories being considered equal partners in the global strategy against doping. It has also resulted in a number of research studies being performed on controversial issues such as nandrolone metabolism, analysis of the T/E ratio and the influence of age and ethnic differences on testosterone metabolism. It seems likely that the constantly increasing number of drug tests will not alter the incidence of positive findings. Unannounced testing at training grounds following the impressive example of UEFA with the Champions League teams could be introduced in all FIFA confederations to provide more information from possible misuse of prohibited substances between official matches. The absence of any positive tests in the UEFA testing to date makes it unlikely that this strategy will identify a significant number of drug cheats who are currently not being detected. Given these findings, the question that arises is whether there is a need for fundamental change in the strategy to fight doping in football? The FIFA Medical Committee is of the opinion that the educational process has to be intensified with the help of national associations and in particular, through team physicians. Team physicians play a central role in the educational programme as they have direct influence over player behaviour and have the knowledge to advise players, not only on the potential risks to health, but also of the effect that sanctions may have on a player's career if he or she is caught. The 32 team physicians of the 2006 finalists have once again confirmed their unconditional support of FIFA's strategy by signing their joint declaration prior to the 2006 FIFA World Cup Germany. The doping control officer at testing controls can also reinforce the educative aspect of the fight against doping [06009].

The doping control procedure
The full details of the FIFA doping control procedure are set out in the annually updated FIFA Doping Control Regulations (www.fifa.com/en/regulations/regulation/0,1584,9,00.html). With regard to the medicolegal aspects of doping control procedures, the process is as follows:

- once an A sample has tested positive, then the FIFA Doping Control Sub-Committee investigates the documentation of the case and prepares a report for the FIFA Chief Doping Control Officer. The FIFA Chief Doping Control Officer has to verify that the correct doping control procedures have been completed according to the doping control regulations. This process usually involves contacting the testing laboratory as well as the original doping control coordinator where the athlete was tested.
- if the analysis of the A sample is confirmed as positive by the FIFA Doping Control Sub-Committee's report, the FIFA General Secretary shall at once confidentially notify the chairman of the Disciplinary Committee, the Sports Medical Committee and the national association of the player concerned, which shall have the right to request a second analysis using the B sample within 24 hours of being notified.
- if a second analysis is requested, FIFA shall communicate this request immediately to the head of the laboratory where the B sample is being kept. An analysis of the B sample shall be carried out as soon as possible, by personnel who were not directly involved with the analysis of the A sample. The association concerned shall
have the right to have a representative present, in addition to the player concerned. The results of the analysis of the B sample shall be sent immediately to the FIFA Chief Doping Control Officer responsible, by fax or e-mail. If no request for a second test is made, the laboratory shall dispose of sample B after 30 days have elapsed.

In addition to the procedural roles above, the FIFA Chief Medical Officer and the Doping Control Sub-Committee also have to estimate the seriousness of the individual case from the medical point of view as to whether the violation was intentional (partially autonomous but not fully self-responsible), deliberate (fully autonomous) or negligent and examine whether any exceptional circumstances may apply. Finally, a written statement about the medical analysis of the case including an estimation of the medicolegal aspects has to be submitted to the FIFA Disciplinary Committee for consideration of sanctions. In cases where FIFA is asked by a national federation or a confederation to take over the sanction or decide about a sanction for the international level, the same procedure is carried out. The individual case management as outlined above is an integral part of FIFA's approach to doping control and based on Swiss sanction law. This means that there must be evidence that the player is personally guilty of the offence being sanctioned and the unjustness of his behaviour has to be obvious to him. Thus, every sanction inevitably contains a distinctive individual component [06002].

**FIFA Medical Assessment and Research Centre (F-MARC)**

The ongoing media debate surrounding the issue of doping in sport has raised public awareness of a problem that has been steadily developing over many years. This controversy reflects both the rapid development of various sports disciplines as well as the evolution of new doping methods and agents. The Fédération Internationale de Football Association (FIFA) introduced doping controls in football in 1970 to ensure that the results of national and international matches were a fair reflection of the ability of those taking part. Over the past 12 years, the FIFA Medical Assessment and Research Centre (F-MARC) has developed a worldwide network of specialists who are involved in the educational process within the regional football confederations and national associations as one facet of global anti-doping strategies. F-MARC has also been involved in the practical implementation of doping controls for FIFA competitions at all levels. FIFA has developed close collaboration not only with the confederations and member associations, but also with other team sports federations, particularly with the accredited drug testing laboratories. These relationships have helped to understand the extent of doping, which in turn forms the basis of a global, harmonised strategy in the fight against doping in football [06010].

**FIFA's approach to doping in football.**

FIFA's anti-doping strategy relies on education and prevention. A worldwide network of physicians guarantees doping control procedures that are straightforward and leave no place for cheating. FIFA actively acknowledges its responsibility to protect players from harm and ensure equal chances for all competitors by stringent doping control regulations, data collection of positive samples, support of research, and collaboration with other organisations. One article aimed to outline FIFA's approach to doping in football. Data on positive doping samples per substance and confederation/nation documented at the FIFA medical office from 1994 to 2005 are provided. According to the FIFA database, the incidence of positive cases over the past 11 years was 0.12 percent, with about 0.42 percent in 2004 (based on the assumption of 20,750 samples per year) and 0.37 percent in 2005. Especially important in this regard is the extremely low incidence of the true performance
enhancing drugs such as anabolic steroids and stimulants. However, there is a need for more consistent data collection and cross checks among international anti-doping agencies as well as for further studies on specific substances, methods, and procedures. With regard to general health impairments in players, FIFA suggests that principles of occupational medicine should be considered and treatment with banned substances for purely medical reasons should be permitted to enable players to carry out their profession. At the same time, a firm stand has to be taken against suppression of symptoms by medication with the aim of meeting the ever increasing demands on football players. It was concluded that the incidence of doping in football seems to be low, but much closer collaboration and further investigation is needed with regard to banned substances, detection methods, and data collection worldwide [06001].

FIFA introduced doping controls in 1970 to ensure that the results of national and international matches were a fair reflection of the ability of those taking part. The FIFA Sports Medical Committee is responsible for implementing doping controls at all FIFA competitions and also for coordinating with confederations and member associations. The overall management of doping controls is conducted by the FIFA administration (Medical Office and the FIFA Sports Medical Committee). Over the past 12 years, the FIFA Medical Assessment and Research Centre (F-MARC) has developed a worldwide network of specialists who are involved in the educational process within the confederations and national associations as well as in practical performance of doping controls for national, international, and FIFA competitions. The medical doctors/sports physicians, following their Hippocratic Oath as well as their professional and ethical values, play key roles in FIFA’s long term strategy in the fight against doping. Many of these doctors are also team physicians in their national associations. The fight against doping in football focuses on education and prevention with regular in-competition and out-of-competition controls. In past years, approximately 15,000 doping controls were performed annually on footballers, with over 20,000 performed in both 2004 and 2005. FIFA articulated its unyielding position in the fight against doping prior to the world cup competition in both 1998 and 2002 and reinforced its strategy in the FIFA Magazine in March 2004. Physicians demonstrated their strong support of the FIFA long term strategy in its fight against doping before the 2002 FIFA World Cup Japan/Korea. The team physicians of all 32 finalists signed a joint declaration in the fight against doping, supporting FIFA’s decision to introduce routine blood sampling to analyse for blood doping and erythropoietin (EPO). This was a firm message to the football community and demonstrated the excellent collaboration and cooperation between the FIFA Sports Medical Committee and the team physicians taking care of the players before and during the competition. The team physicians of all the finalists of the 2006 FIFA World Cup Germany again reinforced the fight against doping with a joint declaration signed on 5 March 2006 to keep this unique event free of doping [06001].

FIFA is a global organisation that unites over 250 million footballers in 207 countries. Around 40 million of these players are female. Currently, confederations, national associations, or both that fall under FIFA’s management, carry out their own doping controls at the competitions they stage. However, the urine or blood samples, or both must be analysed at laboratories accredited by FIFA/World Anti-Doping Agency (WADA). These laboratories send reports on any “chemically positive” A samples to the member associations, and FIFA headquarters for management and WADA for information. Once the FIFA medical office receives a positive A sample report, it requires follow up information from the national association/confederation in question, or both – that is, the results of the possible B sample decision made by the particular disciplinary committee. If the information is not provided, the FIFA disciplinary committee takes appropriate action. Since the 1994 FIFA World Cup in the USA, the FIFA Medical Office has undertaken stringent registration of analysed samples. A new doping control policy for FIFA competitions was introduced at the FIFA U-17 World
Championship in New Zealand in 1999. Since then, during tournaments, two players from each team are randomly selected to undergo doping tests after each match. Between 1994 and 2005, 3327 doping controls (men and women) were performed during three consecutive FIFA world cups (USA, France, Korea/Japan), two consecutive Olympic games (Sydney, Athens) as well as the last Women's World Cup (USA, 2003), the FIFA U-19 in Thailand, the FIFA U-17 World Cup in Peru, the FIFA Confederations Cup in Germany, the FIFA Club World Cup in Japan, the FIFA Beach Soccer World Cup in Brazil, the FIFA U-20 World Cup in the Netherlands, and FIFA World Championship in Futsal, Chinese Taipei, as well as during the World Cup 2006 preliminaries. Only four samples tested positive during this period: one for ephedrine and pseudoephedrine in 1994 one for cannabis and one for nandrolone during the FIFA World Youth Championship 2003 held in the United Arab Emirates, and one for ephedrine in Angola. This reflects an overall incidence of 0.12% positive cases over the past 11 years. The extremely low incidence of positive cases during FIFA competitions indirectly confirms the FIFA long term strategy in the fight against doping: that education and prevention play a key role in keeping high profile competitions free of doping.

It can only be assumed that team sports such as football are not as prone to misuse of performance enhancing substances as are individual sports. During the 2004 Olympic Games in Athens, there were 27 positive cases – all in individual athletes and none in any team sport participants. It might be hypothesised that the close collaboration of the team sport medical committees since the 2000 Olympic Games in Sydney, positively influenced the attitude of fairplay among team sports during the Olympic Games in Athens. Close collaboration between accredited laboratories, the reporting system, and the central control system is an important tool for statistical recording of the extent of doping in football in the future. Although several prominent footballers have tested positive for drugs in recent decades, the true extent of the problem is unknown. Even if we assume that doping is still not a major issue in team sports such as football, any estimation of the problem can be considered as merely an unscientific hypothesis or speculation. To meet the challenges brought about by this situation, FIFA has taken action to develop closer collaboration between the medical committees of the various confederations. In October 1999, the FIFA Sports Medical Committee and the Union of European Football Associations (UEFA) Medical Committee met to discuss the latest sports medicine issues with the aim of not only combating doping but also developing educational programmes designed to meet the fundamental objectives outlined above. Similar meetings have been conducted between the representatives of the FIFA Sports Medical Committee and the medical committees of the Confederation of North, Central American and Caribbean Association Football (CONCACAF) (North and Central America, 2000, 2001), Asian Football Confederation (AFC) (Asia, 2001, 2002, 2005), and Confederation Africaine de Football (CAF) (Africa, 2003, 2004). During 2005, meetings were conducted with the newly established Oceania Football Confederation (OFC) Sports Medical Committee and Confederación sudamericana de Fútbol (CONMEBOL) with the aim of harmonising doping control procedures, improving the understanding of the scientific background of doping, and enhancing the FIFA network of doping control officers (DCOs) who fulfil educational duties as a part of their responsibilities.

According to the statistics of the International Olympic Committee (IOC) (until 2003) and WADA accredited laboratories (as of 2004), approximately 20 750 doping controls are performed annually on football players. The majority of the controls are done in Europe and North and South America. The numbers of doping controls continue to increase in the other confederations. In this respect, FIFA developed its own database to keep records on the substances being reported as positive to allow online control of management of these samples within the different confederations and member associations. During 2004 and 2005, 88 (0.42 % based on the assumption of 20 750 samples per year) and 78 (0.37 %)
positive samples, respectively, were registered at FIFA. The increase is probably because of improved reporting systems used by the laboratories as a result of the implementation of WADA (March 2004). The majority of the positive cases were detected or reported by the European laboratories which receive most of their samples from the European national associations [06001].

The FIFA database will allow a continuous cross-check with the WADA database (ADAMS, Anti-Doping And Management System), once that is operational, not only to control the reporting system of the WADA accredited laboratories, but also to allow prospective studies on sanctions related to the different substances, the severity of the violation, or both. Just before the FIFA World Cup in France in 1998, a number of well known players tested positive for small amounts of nandrolone metabolites in their urine. Nandrolone (chemical name nortestosterone) is an anabolic steroid often encountered in bodybuilding doping cases. In general, this compound is taken in high doses and its degradation products (metabolites) remain detectable in urine for up to several months. Before the 1998 World Cup, FIFA commissioned an independent anti-doping laboratory (LAD) to carry out a collaborative study to obtain a true picture of the situation in football. With the agreement of national and international bodies, every player from every team in the top national leagues in Switzerland (A and B leagues) was tested after a game (356 players in total over two weekends) in collaboration with the Swiss anti-doping committee. The results were compared with those obtained by testing amateur footballers and students. Without revealing anything about the origin of these products, the study showed some players had nandrolone metabolites in their urine after the game. The traces of metabolites in the urine of those players were very small, and all were below the limits of a positive reading. On the basis of this study, FIFA was able to organise the anti-doping programme for the 1998 World Cup with a degree of assurance to provide reliable information to the competing teams to rule out any occurrence of false positive tests. With FIFA’s support, this study into nandrolone and its derivate substances continued. Extraordinary variability in excretion was shown, making the relation between dosage, time delay, and urine concentration critical. Involvement of a world governing body in such a research programme is essential if any worthwhile progress is to be made in this area. The players can also be given the assurance that, scientifically and ethically, they start a match on an equal “playing field” with their opponents as far as doping is concerned [06001].

Future challenges

In 2006, FIFA launched a new developmental programme, the Futuro III. The FIFA Medical Committee undertook to implement the mandate of Mr Joseph S Blatter, President of FIFA, and the FIFA Executive Committee, to educate more than 3000 physicians worldwide in football medicine over the next three years. Anti-doping education is an integral part of the instructional courses, which were launched in February 2006 in Oceania and then held again at the CONMEBOL confederation (South America) in April 2006. The active participation within the instructional courses will entitle physicians to become members of the worldwide network of FIFA medical officers, not only to deal with optimal management and prevention of injuries, but also to act as FIFA doping control officers throughout the 207 member associations of FIFA in collaboration with their national anti-doping organisations. In this respect, FIFA is of the opinion that the doping control programmes have to be carried out by the members of the international sports federations and are obligatory for physicians. There is no need to delegate this important work to commercial companies. The experience of FIFA clearly indicates that employing physicians to perform the doping controls is not only effective but can be done at low cost and most probably will reduce the risk of potential corruption as the physicians have to follow their professional ethical codes of conduct and have medicolegal constraints. Another challenge is the continuous search for identification of new
performance enhancing drugs being distributed on the market via the internet and in this respect medical science, in close collaboration with laboratory experts and the scientific committee of the World Anti-Doping Agency, might help to identify possible new drugs and sanction their misuse accordingly. Arguably the major challenge for the future lies in genetic doping and its detection. There is no doubt that we cannot stop the development of medical science as the development of altered genetic information seeks to benefit the many patients with incurable diseases. Yet it could be hypothesised that this scientific advancement might be misused for performance enhancement in sport. In this regard, the education and cooperation of team physicians forms a crucial link in the chain to prevent athletes adopting such strategies [06009].

The lowering of the threshold for the ratio of testosterone to epitestosterone (T/E) from 6 to 4 has led to intense discussion with the accredited laboratories and raised concerns on behalf of FIFA. According to the FIFA database 2005 none of the samples with elevated ratios between 4 and 6 showed evidence of exogenous intake in the gas chromatography isotope ratio mass spectrometry (GC-IRMS) tests. In face of the logistic impact and additional costs FIFA should strongly advocate detailed statistical analysis of the WADA data, examining the incidence of exogenous intake of testosterone in samples with T/E ratios between 4 and 6. Furthermore, legal difficulties arise in cases where the T/E ratio is between 4 and 6 but GC-IRMS does not verify exogenous intake [06002].

Recent years have shown a constant increase of positive tests for recreational drugs. While this finding reveals rather a social than a doping problem in the sense of the word, an important legal aspect has to be considered too: the consumption of marihuana presents a severe offence against the law in some countries especially in Africa and Asia, even if consumed abroad. Here, the publication of a positive result may lead to serious consequences for the respective player including a prison sentence. Anti-doping bodies should therefore carefully reconsider the unconditioned ban of recreational drugs, preferably based on a juridical expert's opinion [06002].

While the World Anti-Doping Code and the Doping Control Regulations of FIFA offer a comprehensive basis for the fight against doping, the permanent progress in the development of new substances as well as laboratory methods calls for regular review and update of adopted policies. Whereas harmonisation of the strategies of national and international anti-doping agencies is reinforced, the legislation and politics of different countries constitute a permanent obstacle. Any regulation concerning medicolegal aspects should therefore be based on scientific evidence and juridical expertise and has to be supported by close collaboration of national and international bodies [06002].

**WADA-accredited doping laboratories**

In 2013 there were 35 WADA-accredited laboratories. Their locations were [13003]:

<table>
<thead>
<tr>
<th>Continent (n)</th>
<th>County (city)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asia (6)</td>
<td>China (Beijing), India (New Delhi), Japan (Tokyo), Korea (Seoul), Malaysia (Penang), Thailand (Bangkok)</td>
</tr>
<tr>
<td>Africa (2)</td>
<td>Republic of South Africa (Bloemfontein), Tunisia (Tunis)</td>
</tr>
<tr>
<td>Australia (1)</td>
<td>Australia (Sydney)</td>
</tr>
<tr>
<td>Europe (20)</td>
<td>Austria (Seibersdorf), Belgium (Ghent), Czech Republic (Prague), Finland (Helsinki), France (Paris), Germany (Cologne and Kreischa), Great Britain (London), Greece (Athens), Italy (Rome), Norway (Oslo), Poland (Warsaw),...</td>
</tr>
</tbody>
</table>
Portugal (Lisbon), Romania (Bucharest), Russia (Moscow), Spain (Barcelona and Madrid), Sweden (Stockholm), Switzerland (Lausanne), Turkey (Ankara), Canada (Montreal), United States (Los Angeles and Salt Lake City)

North America (3)

South and Central America (3)

Brazil (Rio), Colombia (Bogota), Cuba (Havana)
EPIDEMIOLOGY OF DOPING

Given the profile of drug use in high-performance sport, it is left to wonder about the use of anabolic steroids in the playgrounds, gymnasia and arenas of our neighbourhoods. However, several years ago, it was noted that the use of anabolic steroids had crept into community sport [014]. A recent survey documented that 3.7 percent of grade 12 Canadian students reported having used steroids [08020].

The use of anabolic-androgenic steroids (AAS) by young athletes has been a primary concern of sports governing bodies because of the implications for unfair advantage in performance and the potential for adverse side effects. Research over several decades indicated a lifetime prevalence of AAS use for adolescent males of 4-6 percent and for females of 1½-3 percent, indicating a problem involving millions of athletes and a potential epidemic of AAS-related pathologies. However, recent studies have questioned the presumption that participation in organised sport is the primary risk factor for AAS use in adolescents as well as the extent estimates of the magnitude of the problem. Increasing evidence indicates that AAS use is associated with non-athletes and is linked to a broader syndrome of problem behaviours rather than efforts to achieve sporting success, and that sports participation may be protective against AAS use. Moreover, employing lifetime prevalence to gauge AAS use limits accurate evaluation of the personal and public health risk as the majority of respondents are not habitual users. Previous studies may have also inflated prevalence values through ambiguously worded survey questions and other design flaws, and few data are available on actual dosages. Prevention efforts need to be focused beyond organised sport and target the general adolescent population rather than athletes and should be founded on interventions with demonstrated efficacy for delinquent, antisocial and self-destructive behaviours rather than the ethical imperative of fair play [10017].

There is evidence to suggest that the prevalence of anabolic-androgenic steroids (AAS) is higher among young people than the general population. The purpose of one study was to examine the proportion of students who reported lifetime and past-year AAS use, explore other drug use among those who reported AAS use, and investigate demographic correlates of AAS use. Data was taken from a cross-sectional survey of a representative sample of Australian secondary students. A stratified two-stage probability sampling methodology was employed and schools were randomly sampled from each Australian State and Territory. A total of 376 schools participated in the survey. Lifetime AAS use was reported by 2.4% of 12-17-year-old students; use was more common among 12-15-year olds then 16-17-year olds. Regardless of age, being male, speaking a language other than English at home, not be at school on the previous school day, and rating own scholastic ability as below average were all associated with a greater likelihood of using AAS in their lifetime and in the past year. Those who reported AAS use also reported the use of a range of other substances, suggesting that AAS use may be part of a broader experimentation with substances. Interventions towards these groups regarding AAS may best be placed within a larger substance use intervention rather than being AAS-specific. In light of the low levels of AAS use among this group, more detailed research into AAS use among adolescent sporting groups may be warranted [10317].

Estimated number of unreported cases

Recent studies have suggested that the use of doping substances and particularly of anabolic androgenic steroids (AAS) is often practised by fitness centre visitors. These studies employed direct interview techniques and questionnaires to assess the estimated
number of unreported cases of doping. Because people hesitate to provide compromising information about themselves, these techniques are subject to response errors. In one study it was applied an alternative interview technique to assess more accurately unreported cases of doping in fitness centres. The investigation employed the randomized response technique (RRT) to reduce response errors. A cohort of 500 people from 49 fitness centres participated in this study. The RRT revealed a high prevalence of doping (13 %). In addition, and most importantly, the present RRT study revealed an alarmingly high prevalence of illicit drug use, specifically of cocaine use, that has been severely underestimated by previous studies. The RRT confirmed previously estimated rates of AAS use assessed by direct interview techniques and voluntary questionnaires, but uncovered a much higher usage rate of illicit drugs among fitness centre visitors. This outcome enabled us to construct a ‘probability’ rating for the use of doping substances in fitness centre visitors. Given its high prevalence and the predominant use of AAS, doping among fitness centre visitors is an issue of extreme relevance for the health care system. The study may help to characterize further doping substance users and to develop and apply prevention and intervention programmes specifically to individuals at high risk [06019].

**Doping in the community**

Prevalence of abuse of androgens in individuals of the general population has reached alarming dimensions. Use of androgens is no longer limited to competitive sports, but has spread to leisure and fitness sports, bodybuilding, and nonathletes motivated to increase muscular mass and physical attractiveness. Alarming studies from Germany demonstrated that members of the healthcare systems provide illegal androgens to 48 percent of abusers visiting fitness centers. The new trend to combine androgens with growth hormone, insulin, and insulinstatic milk protein-fortified drinks may potentiate health risks of androgen abuse. The use of androgens has changed from being a problem restricted to sports to one of public health concern. The potential health hazards of androgen abuse are underestimated in the medical community, which unfortunately contributes to illegal distribution of androgens. Both the adverse effects of current androgen abuse especially in young men as well as the chronic toxicity from past long-term abuse of now middle-aged men has to be considered as a growing public health problem. In the future, an increasing prevalence of androgen misuse in combination with other growth-promoting hormones and insulinstatic milk protein products has to be expected, which may have further promoting effects on the prevalence of chronic western diseases [09018].

The incidence of the abuse of illicit drugs in sport may be a useful indicator of the extent of the phenomenon among the youth population. Many drugs of abuse are included in the WADA (World Anti-Doping Agency) Prohibited List, and are therefore routinely tested for in antidoping controls. This study presents the data obtained in tests carried out in the period 2003- 2007 at the Antidoping Laboratory of Rome, on 44781 samples analysed. The methods used are those developed by the Laboratory for routine antidoping analyses. The percentage of positive test results ranges from 1.1 to 2.0 percent, with a high incidence of stimulants and drugs of abuse. The substance most frequently found is THC metabolite, which accounts for 0.2-0.4 percent of the total, followed by cocaine metabolites, accounting for 0.1 percent. Other stimulants found are ephedrines, carphedon, modafinil, and anorexic compounds such as phendimetrazine and nortenfluramine. No amphetamines or amphetamine-like designer drugs have been detected. These data may be indicative of the widespread incidence of cocaine and cannabis abuse among the young Italian population, bearing in mind that the phenomenon is underestimated in this study, due to the fact that drugs of abuse are investigated only in samples involved in competitive sport, and especially to the fact that people doing such activities take more care of their health and are subject to
greater control in their behaviour and habits with respect to the “normal” population [09019].

Pioneering studies regarding epidemiology of doping in the US were done in the early 1980s, when it was interviewed 3403 male high school seniors nationwide [08021]. The report in 1988 indicated that 6.6 percent of respondents had used steroids and more than two-thirds of the group started using steroids when they were 16 years old or younger. Twenty percent reported that health professionals were the primary source for obtaining steroids and 38 percent used injectable steroids. Pope et al studied 1,010 college men for use of steroids and also reported their findings in 1988 [08022]. The study found that only 2 percent of the respondents reported using steroids. The authors qualified their finding as potentially underestimating the true prevalence of steroid abuse. A review of published reports concluded that 3-12 percent of high school students in the 1990s used steroids, and of the group of abusers about half were adolescent females [08023, 08024]. Contrary to popular belief and supported by Pope's early findings, steroid abuse is not exclusively related to performance enhancement. DuRant et al reported in 1993 that steroid abuse in ninth graders was associated with use of cocaine, injected drugs, alcohol, marijuana, cigarettes and smokeless tobacco [08023]. They then reviewed the 1991 Centers for Disease Control and Prevention Youth Risk Behavior Survey of over 12,272 male and female public and private high school students, and confirmed the earlier finding that there is an association between steroid abuse and multiple drug abuse. In a later review of the 1997 Centers for Disease Control and Prevention Youth Risk Behavior Survey of 16,262 high school students, Miller et al reported no significant correlation in male or female steroid-abusing high school students with physical activity, nor were athletic participation or strength conditioning alone associated with lifetime steroid abuse [08024]. Steroid abuse may also include a wider population of non-athletes who have behavioral problems and may experiment with these now easily available performance-enhancing drugs. Their motivation may not be athletic enhancement, but rather cosmetic and body shaping purposes. To maintain youthful appearances, weekend athletes may experiment with hormones encouraged by "anti-aging" marketing, while adolescent females desirous of the long, lean female media images of "adult women" may use steroids and growth hormone to reduce fat and increase muscle tone [08025].

Drug abuse by adolescents has been investigated in various surveys that reported correlations between age, gender, and activity. However, none of these studies included chemical analyses to help substantiate the statements of participants. In one study, the urine specimens of 964 students (439 females, 525 males; mean age 22 years), who applied to study sports sciences at university, were assessed for anabolic steroids, stimulants, and selected drugs prohibited in sports. In total, 11 percent of the urine specimens provided contained drugs covered by doping controls. The most frequently detected compound was the major metabolite of tetrahydrocannabinol (9.8 %) followed by various stimulants related to amphetamine and cocaine (1.0 %). Indications of anabolic steroid use were found in 0.4 percent of urine samples but originated from contraceptives containing norethisterone. The present study provided unambiguous data on the status quo of drug (ab)use by adolescents hoping for a career related to elite sport or sports sciences. No use of anabolic steroids was detected. However, evidence for stimulants and tetrahydrocannabinol administration was obtained, although not reported by any participant, which highlights the issue of under-reporting in surveys based solely on questionnaires [08026].

The 1999 cross-sectional European School Survey Project on Alcohol and Other Drugs (ESPAD). Data collection by standardized methodology using anonymous self-administered questionnaires completed in the classroom from national probability samples of a total of 18,430 16-year-old high school students from six European countries (Bulgaria, Croatia, Cyprus, Greece, the Slovak Republic, and the U.K.) Besides anabolic steroid use and physical exercise, questionnaire items selected for this study included tobacco, alcohol, and
illicit drug use, indicators of other deviant behavior (self-harming thoughts and behavior, truancy, aggressive behavior), friends' use of steroids, and perceived availability. Backward elimination with likelihood ratio tests was used to select the variables to be retained in a multifactorial model. Interactions of other independent variables with country were checked. Logistic regression analysis of lifetime anabolic steroid users compared to nonusers showed that the odds of lifetime AS use are 1.4 times higher for students who exercise almost daily and 1.8 times higher for boys compared to girls. Significant associations of steroid use were also found with current frequent alcohol use, lifetime use of tranquilizers/sedatives and cannabis, and with the perceptions of friends' use of anabolic steroid and of easy availability of the substance. The authors concluded that findings indicate that daily exercising appears to increase the risk of anabolic steroid use in adolescents. However, a more general pattern of closely interlinked deviant types of behavior, such as other drug use and aggressive behavior, is prominent. Preventive interventions are needed targeted towards adolescents involved in intensive exercise and sport. These should take into account both the idiosyncrasy and setting of the sporting culture and the special characteristics of this group [08027].

Epidemiological confounding factors and false consensus effect (FCE)

The “False Consensus Effect” (FCE), by which people perceive their own actions as relatively common behaviour, might be exploited to gauge whether a person engages in controversial behaviour, such as performance enhancing drug (PED) use. In a study it was assumed that people's own behaviour, owing to the FCE, affects their estimation of the prevalence of that behaviour. It was further hypothesised that a person's estimate of PED population use is a reliable indicator of the doping behaviour of that person, in lieu of self-reports. Over- or underestimation was calculated from investigating known groups (i.e. users vs non-users), using a short questionnaire, and a known prevalence rate from official reports or sample evidence. It is proposed that sample evidence from self-reported behaviour should be verified using objective biochemical analyses. In order to find proofs of concept for the existence of false consensus, a pilot study was conducted. Data were collected among competitive UK student-athletes (n=124) using a web-based anonymous questionnaire. User (n=9) versus non-user (n=76) groups were established using self-reported information on doping use and intention to use PEDs in hypothetical situations. Observed differences in the mean estimation of doping made by the user group exceeded the estimation made by the non-user group (35 % vs 15 % for general doping and 34 % vs 26 % in hypothetical situations, respectively), thus providing preliminary evidence in support of the FCE concept in relation to doping. The presence of the “False Consensus Effect” in estimating doping prevalence or behaviour in others suggests that the FCE based approach may be an avenue for developing an indirect self-report mechanism for PED use behaviour. The method may be successfully adapted to the estimation of prevalence of behaviours where direct self-reports are assumed to be distorted by socially desirable responding. Thus this method can enhance available information on socially undesirable, health compromising behaviour (i.e. PED use) for policy makers and healthcare professionals. The importance of the method lies in its usefulness in epidemiological studies, not in individual assessments [00828].

The false consensus effect (FCE) is the tendency for people to assume that others share their attitudes and behaviours to a greater extent than they actually do. The FCE has been demonstrated for a range of health behaviours, including substance use. The study aimed to explore the relationship between elite athlete's engagement in recreational drug use and their consensus estimates (the FCE) and to determine whether those who engage in the behaviour overestimate the use of others around them. The FCE was investigated among 974 elite Australian athletes who were classified according to their drug use history.
Participants tended to report that there was a higher prevalence of drug use among athletes in general compared with athletes in their sport, and these estimates appeared to be influenced by participants' drug use history. While overestimation of drug use by participants was not common, this overestimation also appeared to be influenced by athletes' drug use history. The results suggest that athletes who have a history of illicit drug use overestimate the prevalence of drug use among athletes. These findings may be helpful in the formulation of normative education initiatives [12028].

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Despite the growing body of literature and putative links between the use of ergogenic nutritional supplements, doping and illicit drugs, it remains unclear whether, in athletes' minds, doping aligns with illicit behaviour or with functional use of chemical or natural preparations. To date, no attempt has been made to quantitatively explore athletes' mental representation of doping in relation to illegality and functionality. A convenience sample of student athletes from a large Australian university responded to an on-line survey. Competitive athletes (n=46) were grouped based on self-reported use as follows: 'none used' (30 %), supplement only (22 %), illicit only (26 %) and both supplements and illicit drug use (22 %). Whereas no athlete reported doping, data provided on projected supplement-, doping- and drug use by the four user groups allowed evaluation of doping-related cognition in the context of self-reported supplement- and illicit drug taking behaviour; and comparison between these substances. The False Consensus Effect was found for illicit substance use and was evident as a trend for ergogenic supplement use. It is unclear whether the results point to a relationship between doping and either or neither of the other substances. The results associated with respondents who used supplements suggested that doping estimates may be influenced by ergogenic supplement use. Individuals who used supplements tend to inflate the percentage of individuals who dope but to a much smaller degree than those who use other substances. In addition to these main results, illicit drug use and doping were overestimated. This indicates that, self-report notwithstanding; Australian university athletes may have unrealistic perceptions of illicit drug use and doping. While the pilot nature of this study, especially the small sample, curtails generalisability. The results are therefore interpreted under a generalisability caveat and are intended to inform the broader research program with regard to the observed trends. The presence of the FCE within rather than between substances provides an indication that nutritional supplement use and illicit drug use come from different behavioural domains. Individuals who admitted using one particular type of drug tend to inflate the percentage of individuals who uses the same drug to a much larger degree than those who use other substances. This suggests the FCE could provide an expedient way of identifying when interventions designed to influence one behaviour could influence another. The results indicate that interventions aimed at illicit drug use are unlikely to have much effect on supplement use, and vice versa. Estimates of those who used both
supplements and illicit drugs were more akin to illicit drug users, suggesting illicit drug use may be a dominant behavioural domain. Users of “both” illicit drugs and nutrition supplement gave similar social projections (67%) for illicit drug use to the projected figure of those who use illicit drugs only (74%), compared to a definitely lower estimate (55%) given by nutrition supplement-only users, suggesting that users of ‘both’ may have behaved like illicit drug users due to that domain requiring a different psychological mechanism. For example, users of both may do so as a function of substance use, whereas supplement only users may do so for ergogenic reasons. The dominance of illicit drug use may have implications for the domain specificity of doping behaviour. A reversed pattern was observed for nutrition supplement use projection, where nutrition supplement-only users gave higher estimation (46%) compared to those who use both (34%), thus providing further evidence that in mixed behavioural categories, and the self-anchored behavioural domain is context specific. This pilot study provided an indication that harnessing the FCE might be a fruitful avenue to further examine whether doping behaviour has more in common with illicit drug use or ergogenic supplement use. The pilot nature of the study suggests that doping may be an ergogenic phenomenon, however further testing with an improved research design and sample is needed to establish any such claim. The importance of having a precise picture of the mental representation of doping is underscored by the increasing need for effective anti-doping prevention and intervention. Further research is required to establish if some athletes project their own behavioural tendencies or actual behavior onto other athletes and assume that many others feel or do the same and indeed are using prohibited performance enhancing substances. As a consequence, their own doping tendency or behaviour appears normal and normative, so that they can follow it without compromising their own self-esteem and social acceptance. In this vein, FCE has importance beyond being a useful vector to understanding the position of doping in athletes' minds. These considerations, coupled with the mental representation of doping in athletes' minds, suggest possible intervention strategies to increase compliance with anti-doping initiatives [11013].

One of the key constraints in designing effective anti-doping programs is the lack of conceptual clarity of the psychological mechanisms that influence doping behaviour. For example, preventing doping use in sport on the basis of fair play versus cheating naturally lends to prevention and intervention programs that focus on the ethics of anti-doping and values, coupled with the consequences of being caught – not necessarily limited to sanctions but including dishonour, shame and guilt. Other programs may emphasise the potential hazards and detrimental health effects as consequences of doping use, which are omnipresent, independently from doping testing and sanctioning. In the recent years, a number of athletes have talked publicly about their reasons and motives for doping use, contrasting perceived obligations and duty to perform well with guilt and the shame of lying. Studies conducted among professional athletes, particularly cyclists, offer valuable insight into how athletes perceive doping; and how this perception varies in different contexts. In addition to the fact that many athletes consider doping as part of professional sport, most openly talk about experimentation with non-prohibited substances such as over-the-counter painkillers and non-steroidal anti-inflammatory drugs, caffeine and other non-prohibited stimulants. Nutritional supplement use, which has been considered as a gateway to doping by many is common among emerging and elite athletes and has raised concerns on its own account owing to potentially harmful interactions from combined use and high dosage. The question of whether doping behaviour has the character of illicit substance use, ergogenic substance use, neither or both has been recently raised in connection with anabolic steroids. The ongoing debate is around whether the use of prohibited ergogenic substances aligns with behaviours associated with illicit substance use (e.g. psychoactive controlled drugs) or with nutritional (ergogenic) supplement use. Resolving which behavioural domain doping belongs in athletes' mind provides valuable insight for primary prevention activity. As doping has been categorised as an illicit (illegal) activity, it follows illicit drug models. Thus, the
current anti-doping prevention follows typical demand control models seen for illicit drug use that focus on health education. It may be that the behaviour is functional with regard to its performance enhancing qualities. There is currently little in the way of ergogenic supplement primary prevention. Finally, doping may be an entirely new class of drug use behaviour, requiring a new set of primary prevention activities to be developed [11013].

Previous results investigating social projection in performance enhancing and illicit drug use suggest that projected prevalence of doping and drug use was higher among self-admitted users respectively but absent for nutritional supplement use, and that this social projection was domain specific. Domain specificity refers to an observed phenomenon that admitted doping use came with high estimations of doping use among other athletes with illicit drug use remaining unaffected; and conversely illicit drug users gave higher estimations of illicit drug use among others with estimated doping use remaining unaffected. Although differentiating between cause and effect between social projection and behaviour in data from cross sectional research is impossible, the relationship is clearly present in self-reported data. Interestingly, this phenomenon is only observable within the cognitively controlled information when athletes admitted the use of one or both of these drugs. The importance of the social project lies with the question of whether an elevated and potentially distorted social projection leads to a congruent behavioural choice or resulted from it. The fact that social projection aligns with self-reported behaviour but not necessarily with actual behaviour is intriguing, but more importantly it reveals something about athletes' cognitive processes relating to these substances. Thus, this may be used to gain insight into athletes' implicit mental representation of these, often concomitantly used, substances. Descriptive norms are individuals' perceptions of how common a particular behaviour is. These norms are likely to be affected by some degree of projection (i.e. x % of athletes use doping). In particular, the projection may suffer from a social bias coined the “False Consensus Effect” (FCE) which peoples' perception of their environment (including the behaviours of others) is distorted, thus resulting in a higher estimation. The FCE is a perceptual bias where people who engage in particular behaviours tend to overestimate the proportion of the population who also engage in that behaviour. People who abstain either underestimate or correctly estimate prevalence. For example, marijuana users tend to overestimate the proportion of the population who use marijuana, and non-users are more accurate or underestimate. A further characteristic is that the FCE is domain specific as it works within rather than across different categories or domains of behaviour. Therefore, if doping was an ergogenic phenomenon, users of nutritional supplements should overestimate doping and vice versa (the positive case). Conversely if doping belongs to another domain, then the estimates of doping would occur independently of nutritional supplement use (the negative case). This suggests the relatedness of doping with either illicit or ergogenic substance use behaviours can be determined by emergent patterns of the FCE across behaviours. It may be assumed mutually exclusive categories (i.e. a nutritional supplement user is not a doping user or illicit drug user, etc.). In reality, it is likely that athletes use substances from two or even all three of these substance categories, thus making mixed categories with testable differences in their estimations of drug, doping and nutrition supplement use. For example, athletes construct doping as illicit drug use and therefore athlete illicit drug users also overestimate doping. The legality of the substances may have a confounding influence [11013].

Elites

High use of medication and nutritional supplements has been reported in several sports. To document the use of prescribed medication and nutritional supplements in female and male junior, youth, and adult track and field athletes depending on their sports discipline a
A descriptive epidemiology study was performed. Analysis of 3,887 doping control forms undertaken during 12 International Association of Athletics Federations World Championships and 1 out-of-competition season in track and field was done. There were 6,523 nutritional supplements (1.7 per athlete) and 3,237 medications (0.8 per athlete) reported. Nonsteroidal anti-inflammatory drugs (NSAIDs; 0.27 per athlete, n=884), respiratory drugs (0.21 per athlete, n=682), and alternative analgesics (0.13, n=423) were used most frequently. Medication use increased with age (0.33 to 0.87 per athlete) and decreased with increasing duration of the event (from sprints to endurance events; 1.0 to 0.63 per athlete). African and Asian track and field athletes reported using significantly fewer supplements (0.85 vs 1.93 per athlete) and medications (0.41 vs 0.96 per athlete) than athletes from other continents. The final ranking in the championships was unrelated to the quantity of reported medications or supplements taken. Compared with middle-distance and long-distance runners, athletes in power and sprint disciplines reported using more NSAIDs, creatine, and amino acids, and fewer antimicrobial agents. The use of NSAIDs in track and field is less than that reported for team-sport events. However, nutritional supplements are used more than twice as often as they are in soccer and other multisport events; this inadvertently increases the risk of positive results of doping tests. It is essential that an evidence-based approach to the prescribing of medication and nutritional supplements is adopted to protect the athletes' health and prevent them from testing positive in doping controls [09020].

The aim of one study was to describe qualitatively and quantitatively dietary supplements and medication use in elite athletes. Athletes (n=912) reported medications and dietary supplements taken within 3 days before doping control. It was analyzed data collected from 2006 to 2008, indentified and classified substances. Total of 75 percent athletes reported use of at least one substance, 61 percent took dietary supplements (3.2 per user) and 41 percent took medications. Among users, 21 percent reported the use of six and more different products, and one took 17 different products at the same time. Majority of medication users took non-steroidal anti-inflammatory drugs (NSAID) (25 %), and 22 percent used more than one NSAID. It was found no gender differences in dietary supplements use. Individual sport athletes used more dietary supplements. The study showed widespread use of dietary supplements and drugs by elite athletes in Serbia. Consumption of dietary supplements with no evident performance or health benefits, demonstrated the need for specific educational programs focused on dietary supplements use. Amount, quantity and combination of the reported products raised concern about the risk of potential side effects [09021].

**Use of drugs during the Olympics**

It was gathered data and examined the use by elite Olympic athletes of food supplements and pharmaceutical preparations in total and per sport, country, and gender in the Athens 2004 Olympic Games. Data from two sources were collected: athletes' declaration of medications/supplements intake recorded on the Doping Control Official Record during sample collection for doping control, and athletes' application forms for granting of a therapeutic use exemption (TUE) and through the abbreviated TUE process (aTUE). 24 percent of the athletes tested for doping control declared no use of medications or food supplements. Food supplements (45 %) continue to be popular, with vitamins (43 %) and proteins/aminoacids (14 %) in power sports being most widely used. Nonsteroidal antiinflammatory agents and analgesics were also commonly used by athletes (11 % and 4 %, respectively). Laboratory analysis data reveal that of the aTUEs received for inhaled glucocorticosteroids, only budesonide was detectable in significant percentage (10 %). Only 7 percent of the 445 athletes approved to inhale beta2-agonists led to an adverse analytical finding [09022].
**Student athletes**

There is a general perception that use of performance-enhancing substances (PESs) does not fit the standard profile of substance use. One study sought to determine whether users of PESs report high-risk patterns of alcohol and other drug use and demonstrate risk behaviors associated with problematic substance use. Anonymous self-report questionnaires were administered to a sample of 234 male student athletes. PES users were defined as college athletes who reported past-year use of a broad array of PESs (including stimulants, hormone precursors, and nutritional supplements). Male athlete PES users (n=73) compared with nonusers (n=160) reported more problematic alcohol-use behaviors and more alcohol- and drug-use-related problems. The former compared with the latter was also more likely to report past-year use of tobacco products, marijuana, cocaine, psychedelics, and prescription drugs without a prescription. In addition, PES users demonstrated higher sensation seeking, and greater coping and enhancement motivations for drinking and marijuana use than non-PES users. Although banned PESs are not typically viewed as having a high addiction potential, male athletes who use these drugs may be more likely to participate in other problematic substance-use behaviors. Importantly, the male athletes in this study who reported PES use also participated in substance-use behaviors that can have profound negative effects on athletic performance [09023].

**Adolescents**

The use of anabolic-androgenic steroids (AAS) by young athletes has been a primary concern of sports governing bodies because of the implications for unfair advantage in performance and the potential for adverse side effects. Research over several decades indicated a lifetime prevalence of AAS use for adolescent males of 4-6 percent and for females of 1.5-3 percent, indicating a problem involving millions of athletes and a potential epidemic of AAS-related pathologies. However, recent studies have questioned the presumption that participation in organised sport is the primary risk factor for AAS use in adolescents as well as the extant estimates of the magnitude of the problem. Increasing evidence indicates that AAS use is associated with non-athletes and is linked to a broader syndrome of problem behaviours rather than efforts to achieve sporting success, and that sports participation may be protective against AAS use. Moreover, employing lifetime prevalence to gauge AAS use limits accurate evaluation of the personal and public health risk as the majority of respondents are not habitual users. Previous studies may have also inflated prevalence values through ambiguously worded survey questions and other design flaws, and few data are available on actual dosages. Prevention efforts need to be focused beyond organised sport and target the general adolescent population rather than athletes and should be founded on interventions with demonstrated efficacy for delinquent, antisocial and self-destructive behaviours rather than the ethical imperative of fair play. It has been 50 years since the first report of AAS use by a high school athlete, and since then considerable research has characterised AAS use in young athletes. It appears that illicit use of AAS may be less problematic in young people in organised sport than in the general adolescent population and that prevention efforts would be more effective utilising a public health model rather than one focused on sports-specific concerns such as “fair play”. Yesalis and Bahrke reviewed anabolic steroid use by adolescents up to 1999 and reported that lifetime prevalence in 16 regional studies from 1989-1993 in the USA ranged from 1.6 to 4.4 percent (with higher values for males; in three studies, the prevalence for males was reported as 6.3-6.5 %); in two sets of USA national studies from 1991 to 1997, prevalence ranged from 1.9 to
3.7 percent (with higher values for males), with the values in a third national survey from 1991 to 1994 indicating prevalence from 0.3 to 0.7 percent. The authors’ summary of nine studies from 1990 to 1999 of anabolic steroid use among high school-aged students in a variety of other countries (Canada (three); Sweden (two); South Africa (two); Australia (one); UK (one)) indicated lifetime prevalence ranging from 0.6 to 3.7 percent (with higher values for males). In a review including eight studies (seven from the USA; one from Canada) it was reported prevalence ranging from 1.0 to 5 percent for boys (although two studies reported values of 11 % and 15 %, respectively) and 0.8-2.8 percent for girls (with the same study that reported 15.3 % in boys indicating a prevalence of 6.7% for girls). Of 16 more recent works, six were conducted in the USA (four national samples; two regional studies); nine were in Europe (one trans-European study involving six countries; two national studies (Poland, Norway); six regional (Sweden (three); Norway, Germany, France (one each)); and one regional study from Brazil. As with all of the previously published work on this topic, the majority of new studies differed significantly in their methodology, including the time frame of use (lifetime; previous 30 days; previous 6 months; previous 12 months), sample (middle-school students; high school students; athletes; community-dwelling youth) and source of data (most used researcher-developed questionnaires, although few provided the actual wording of the AAS use questions; internet solicitation). The lack of a standardised approach makes it difficult to compare across studies or acquire an accurate picture of the phenomenon. In Sweden, the Center for Alcohol and Narcotic Information (CAN) questionnaire was used for regional studies in 1995, 1998 and 2000, and indicated prevalence for males of 2.1 percent, 2.9 percent and 1.2 percent, respectively, and for females of 0.2 percent and 0.0 percent (1995 and 1998, respectively). Findings from other studies varied widely. For example, it was reported only 0.8 percent for a national follow-up study of 8508 Norwegian middle and high school students, whereas in another study it was found 3.6 percent for males and 0.6 percent for female high school students in a smaller regional study in Norway [09024].

One article explores the issue of performance-enhancing drug use in adolescent athletes. The article describes current substances that are being used by adolescent athletes, explains their positive and negative effects, examines factors contributing to their increased use in adolescent athletes, and discusses approaches to educating adolescents about alternate means of enhancing their athletic performance. It is hoped that this information will be useful toward encouraging young athletes to pursue, safe, healthy, and natural means of performance enhancement, such as practice and strength training, to improve sports performance in a safe, effective manner [06021].

Ergogenic drugs are substances that are used to enhance athletic performance. These drugs include illicit substances as well as compounds that are marketed as nutritional supplements. Many such drugs have been used widely by professional and elite athletes for several decades. However, in recent years, research indicates that younger athletes are increasingly experimenting with these drugs to improve both appearance and athletic abilities. Ergogenic drugs that are commonly used by youths today include anabolic-androgenic steroids, steroid precursors (androstenedione and dehydroepiandrosterone), growth hormone, creatine, and ephedra alkaloids. Reviewing the literature to date, it is clear that children are exposed to these substances at younger ages than in years past, with use starting as early as middle school. Anabolic steroids and creatine do offer potential gains in body mass and strength but risk adverse effects to multiple organ systems. Steroid precursors, growth hormone, and ephedra alkaloids have not been proven to enhance any athletic measures, whereas they do impart many risks to their users. To combat this drug abuse, there have been recent changes in the legal status of several substances, changes in the rules of youth athletics including drug testing of high school students, and educational initiatives designed for the young athlete. One article summarized the current literature regarding these ergogenic substances.
and details their use, effects, risks, and legal standing [06003].

**Versus those not participating in sports**

One study examined the relationship between high school sports participation and the use of anabolic steroids (AS) and legal performance-enhancing dietary supplements in young adulthood. Additionally, the relationship between the use of AS and legal dietary supplements was explored. Data on approximately 15,000 adolescents from the National Longitudinal Study of Adolescent Health were used. School sports participation was assessed when adolescents were in grades 7-12. AS use and legal performance-enhancing dietary supplement use were assessed six years later. Males were more likely than females to use AS and legal supplements. A sport by gender interaction emerged for the use of AS, indicating that the gender differences in AS use were greater for those who participated in sports during high school. High school sports participation was associated with increased likelihood that adolescents would use legal supplements in young adulthood. Finally, there was a positive relationship between the use of legal dietary supplements and AS use. The study highlights the important role that the social environment during adolescence has on future health behaviors. Results suggest that the sporting context experienced during early adolescence may have lasting effects on the use of performance-enhancing substances. The use of legal performance-enhancing dietary supplements appears to be more prevalent than the use of AS, and there seems to be a positive relationship between the use of AS and legal performance-enhancing dietary supplements [06022].

**Disableds in sports**

Activities concerning the fight against doping with regard to the Paralympic Games have been initiated in 1984, when first doping controls were conducted. The foundation of the International Paralympic Committee exactly 20 years ago (1989) considerably supported systematic sports drug-testing programs specifically designed to meet the particular challenges related to disabled sports, which yielded a variety of adverse analytical findings (e.g., with anabolic steroids, diuretics, corticosteroids, and stimulants) especially at Paralympic Summer Games. In Germany, doping controls for handicapped athletes were established in 1992 and have been conducted since by the National Paralympic Committee Germany and the National Anti-Doping Agency. Also here, various analogies in terms of antidoping rule violations were found in comparison to doping controls of nondisabled athletes. In the one article, available numbers of samples analyzed at Paralympic Summer and Winter Games as well as within the doping control program for disabled sports in Germany were summarized, and particularities concerning sample collection and the doping method termed boosting were presented [09025].

To examine the use of food supplements and pharmaceutical preparations by elite Paralympic athletes in the Athens 2004 Paralympic Games data obtained from athletes' declaration of intake of drugs/supplements recorded on the Doping Control Official Record during sample collection for doping control, and athletes' application forms for granting of a therapeutic use exemption. Sixty-four percent of the athletes tested for doping control declared use of medications or food supplements, and 81 percent of these athletes declared intake of fewer than four preparations. Non-invasive routes of administration dominated. Food supplements (42 %) were popular, and drugs used to treat several pathological conditions noted. Non-steroidal anti-inflammatory agents and analgesics were commonly used (10 % and 6 %, respectively). The prevalence of inhaled beta2-agonist use (5 %) was higher than expected and exceeded that at the Athens Olympic Games. The first
examination of elite Paralympic athletes shows a more rational approach to the use of medication and food supplements, but a similar consumption pattern to that of athletes at the Athens Olympic Games. Because of the dearth of such studies, consumption trends among Paralympic athletes remain unclear. Fewer Paralympic athletes declared the use of medications and food supplements and, in general, a more rational intake pattern was recorded than for Olympic athletes. Drugs used to treat several pathological conditions were recorded, with a higher prevalence of drugs for insulin-dependent diabetes mellitus than in recent Olympic Games. The prevalence of inhaled beta2-agonist use at the Athens Paralympic Games was higher than expected and exceeded that at the Athens Olympic Games. The need to counsel athletes with disabilities on their nutritional needs is confirmed, and close monitoring by healthcare professionals is recommended [09026].

Issues in paraolympics

Autonomic dysreflexia (AD) is unique to individuals with spinal injuries (SCI) at T6 or above and can be voluntarily induced. Although AD improves wheelchair racing performance in some athletes, it also elicits exaggerated blood pressure, which could be dangerous. The International Paralympic Committee considers AD doping and banned its use. Purpose. The purpose of one study was to evaluate AD knowledge, incidence and attitudes (KIA) of Paralympians with SCI. An existing questionnaire was modified to include questions of AD KIA, validated by three experts and piloted with a small sample. It was administered on-line, mailed to members of a scientific network and distributed during the Beijing Paralympic Games. Of 99 participants, 55 percent had previously heard of AD while 39 percent were unaware; 17 percent, all males, had used AD to enhance performance. Participants reported that AD was (1) useful for middle (79 %) and long distance (71 %), marathon (64 %) and wheelchair rugby (64 %); (2) somewhat dangerous (49 %), dangerous (21 %) or very dangerous (26 %) to health. Results were not influenced by age, injury level or injury duration. The findings indicate the need for educational programmes directed towards enhancing the AD knowledge of rehabilitation professionals, coaches and trainers working with spinal injuries individuals [10319].

Paralympic medicine describes the health-care issues of those 4500 or so athletes who gather every 4 years to compete in 20 sports at the Summer Paralympic Games and in five sports at the Winter Paralympic Games. Paralympic athletes compete within six impairment groups: amputation or limb deficiencies, cerebral palsy, spinal cord-related disability, visual impairment, intellectual impairment, or a range of physically impairing disorders that do not fall into the other classification categories, known as les autres. The variety of impairments, many of which are severe, fluctuating, or progressive disorders (and are sometimes rare), makes maintenance of health in thousands of Paralympians while they undertake elite competition an unusual demand on health-care resources. The increased physical fitness of athletes with disabilities has important implications for cardiovascular risk reduction in a population for whom the prevalence of risk factors can be high [12036].

One study reports in detail on the antidoping program of the Paralympic Movement to improve knowledge and optimize intervention programs, including educational and awareness initiatives. Data retrieved from annual statistics reports and historical records are complemented with personal observations. An overall incidence proportion of <1 percent of antidoping rule violations in the Paralympic Movement is reported, mainly resulting from urine testing during in-competition periods. This led to a total of 60 antidoping rule violations (of which 37 in the sport of International Paralympic Committee powerlifting) since 2000. A critical analysis of these data allows for an assessment of risk factors by sport. An efficient transfer of knowledge indicates the need to strengthen educational awareness, preferably imbedded in a multidisciplinary approach toward athletes’ health. The particular case of
autonomic dysreflexia is addressed as a separate theme [12037].

Gym users

The use of performance- and image-enhancing drugs/substances (PIED) outside elite sports appears to be increasing, although the current knowledge of the nature of PIED use among recreational athletes is scarce. The present study analyzed enquiries that were submitted to the Danish Anti Doping Agency (ADD) over an 18-month period, to gain knowledge of PIED use among individuals who exercise recreationally in Denmark. One thousand three hundred ninety eight queries were examined with respect to the age and gender of the enquirer, affiliation to sport or exercise and substance in question. The key findings were that the ADD information service is generally used by males in their mid-20s who exercise in gyms and are not engaged in competitive sports. Approximately 15 percent of the enquirers were users of anabolic androgenic steroids (AAS) or other substances banned within elite sports by the World Anti Doping Agency, and an additional 15 percent considered using such substances. The present results suggest that there is a pronounced interest in the use of AAS and other PIEDs among Danish gym members [09027].

State-based sports institute

The purpose of one investigation was to examine the nutritional supplement intake of athletes from a state-based sports institute. Athletes (n=72) from seven sports (kayaking, field hockey, rowing, waterpolo, swimming, athletics and netball) completed a questionnaire detailing their daily usage and rationale therefore. The large majority (63/72; 88 %) of surveyed athletes reported using nutritional supplements, with no difference between female and male athletes. Kayakers (6.0) consumed a higher number of nutritional supplements than swimmers (4.0), field hockey (1.5), rowing (2.4), waterpolo (2.3), athletics (2.5) and netball (1.7) athletes. The athletes believed that nutritional supplements are related to performance enhancements (65 %), positive doping results (63 %), and that heavy training increases supplement requirements (65 %). The cohort was equivocal as to their health risks (56 %) or their need with a balanced diet (53 %). The most popular supplements were minerals (46 %), vitamins (43 %), other (32 %), iron (31 %), caffeine (22 %), protein (17 %), protein-carbohydrate mix (14 %), creatine (13 %) and glucosamine (4 %). The majority of supplementing athletes (n=63) did not know their supplements active ingredient (62 %), side effects (57 %), or mechanism of action (54 %) and admitted to wanting additional information (57 %). Only half of the athletes knew the recommended supplement dosages (52 %). The performance enhancing perception may explain the large proportion of athletes that reported using nutritional supplements, despite over half of the athletes believing that supplements are not required with a balanced diet and can cause positive doping violations [10021].

Pharmaco-epidemiology of anabolic steroids

The first report of AAS abuse dates from 1954 when members from the Soviet Union’s world champion’s weight lifting team were found to abuse these substances inadvertently. As late as 1975, AAS abuse was classified as doping. According to the world anti-doping agency report in 2008, AAS were the most commonly identified prohibited drugs among all, comprising 59 percent of all reported findings. Unfortunately, AAS abuse occurs frequently not only amongst professional athletes, but also in the general population, particularly high school students. Also, hypogonadism is considered to affect 2-4 million men in the United
States. In a recent Endocrine Society clinical practice guideline, AAS replacement therapy was indicated to treat men suffering from hypogonadism correlated with low testosterone blood levels. Next to relevant clinical indications of AAS in physiological doses (28-56 mg/week), illegal abuse of high doses of AAS as a muscle strengthening agent in eugonadal men is reported in high rates, which results in blood levels of androgenic compounds 10 to 100 times above the physiologic and therapeutic range [12125].

Comparison with a prison population

Use of anabolic androgenic steroids (AAS) has been associated with adverse psychiatric effect, violent behavior, and criminality. The aim of one study was to further investigate the motives for and consequences of AAS use, with focus on violent and antisocial behavior. Fifty-nine prisoners were interviewed on their use of AAS, and their history was mapped with Addiction Severity Index interviews. Of these prisoners, 56 percent admitted previous use of AAS, of whom 24 percent declared to have committed violent crimes in connection with use of AAS. However, the only significant difference between users and nonusers with regard to criminal history when measured with the Addiction Severity Index was that the AAS users more often stated that they had been prosecuted for crimes labeled as “other crimes,” which did not include violent crimes. The reported side effects of AAS corresponded well to those previously reported. These results indicate that use of AAS is common among Swedish prisoners and that the motives and consequences of such use are similar to what has been observed in other AAS-using populations. Furthermore, the study supports earlier notions that misuse of AAS might cause violent behavior, but only in certain individuals and mainly in combination with other substances [10025].

Drug Information Database

To analyse enquiries made in the Drug Information Database (DID) to develop a better understanding of athletes’ interests and concerns regarding the prohibited status of available substances a retrospective analyses of anonymous enquiries recorded in the DID in 2006 and 2007 of athletes and supporting personnel was performed. The DID recorded 223 717 enquiries with 200 of the >6000 UK licensed pharmaceutical products receiving over 100 enquiries each. The majority (79 %) of these enquiries were in the pharmaceutical product category, followed by recreational drugs (10 %). A variety of common medications were subject to enquiry, with anti-inflammatory agents, decongestants and bronchodilators being most common; a trend in keeping with reported medication use by athletes. Of all enquiries, 42 percent were not found owing to misspelled words or enquiries about unregulated substances. The proportion of enquiries about substances not listed in the database is relatively high and has increased over the 24 month observation period. The DID is a well-used information resource with some 10 000 enquiries being made each month. Of the about 60 percent of successful enquiries, the major focus was on pharmaceutical products. With some 73 percent of enquiries being made by the athletes themselves, further investigations are warranted to explore enquiry patterns in relation to specific sports. Of the unsuccessful enquiries, a large number were related to nutritional supplements, which warrants further investigation. The DID database appears to be a valid mirror of athletes’ chemically assisted practices and may be successfully used to inform health professionals as well as antidoping prevention programmes [09028].

The widespread use of chemically assisted performance enhancements in sport is a growing concern, with the emphasis on the need for harm reduction policies and intervention,
underpinned by empirical evidence from clinical trials. Considerable advances have been, and continue to be, made in the development and application of the analytical sciences that underlie the detection of prohibited performance-enhancing substances (PES) both in and out of competition. Practices of chemically assisted performance enhancements reach beyond using prohibited drugs and methods. Recent studies show that a noteworthy proportion of athletes take nutritional supplements daily and the majority of users of supplements in sports fail to take appropriate supplements to achieve their desired health-maintenance or performance-related outcomes. This mismatch between rationale underlying supplement use and the outcomes of the chosen supplements could form part of an educational programme to eradicate misplaced supplement use in sports. However, these literature reports may also be useful in designing new trials and testing programmes to evaluate the side effects of supplements. In addition to supplements, athletes competing at international sport events reported an alarmingly high use of a number and combination of nutritional supplements, over-the-counter (OTC) and prescription-only medications, a pattern that is mirrored in sub-elite athlete populations. Among Belgian athletes, the reported use of OTC medication has increased from 20 percent to 25 percent in 3 years (2002-2005), with the proportion of users above 35 percent in certain sports (such as corticosteroids in cycling). Whilst antidoping prevention programmes target high-performing athletes, the use of performance-enhancing substances has spread beyond elite sports. The complexities of ranges of prescription and over-the-counter medicines, social drugs and supplements, along with the broad range of products which contain them, necessitate an ever-watchful eye from athletes and coaching teams so that inadvertent doping does not occur. The risk to athletes is twofold: in addition to the scenario of inadvertently failing a doping test, several prescription and OTC medicines have known side effects that may hinder an athlete’s performance. This concern is exemplified by the well-known gastric irritation caused by some non-steroidal anti-inflammatory drugs (NSAIDs). Owing to the enormous number and variety of supplements, prescription and OTC medicines available, along with a lack of clear information regarding doping, further research is required for information on which studies should be undertaken in terms of drug types in order to inform WADA testing and antidoping prevention programmes. One approach to this conundrum is to utilise the drug information databases to study patterns of athlete enquiries, assuming that enquiries are true reflections of interest and behaviour or behavioural intention. While enquiries do not necessarily equate to uses, it is reasonable to assume that athletes make enquiries about the prohibited status of the drugs or substances they are taking or considering for medical or performance-enhancing reasons [09028].

Specifically, the most frequent enquiries were on variants of salbutamol (1882), caffeine (1702) and ingredients of pain/fever relievers (ibuprofen and paracetamol, 1412 and 1349, respectively) and cold medications (ephedrine, 1151). Both ephedrine and salbutamol are bronchodilators and their in-competition use is prohibited. However, salbutamol may be used under the therapeutic use exemption (TUE) scheme. The high frequency of enquiries about NSAIDs and bronchodilators is in keeping with the literature precedents. One possible explanation for the relatively high prevalence of decongestants and asthma medications among the enquiries is the exercise-induced asthma that has been documented among winter endurance athletes. It is equally possible that these substances have performance-enhancing effects but are allowed if prescribed with evidence of medical need under the TUE scheme. Therefore, further investigation is warranted into sport-specific enquiries about potentially performance-enhancing substances (i.e. salbutamol, corticosteroids, etc). The most frequent “unlisted” enquiry was creatine (24 %), followed by various commercial products for muscle building (HMB, Maximuscle, Methoxyisoflavone) and some form of sugar (dextrose, glucosamine) at 9 percent and 8 percent, respectively. In this survey among UK athletes, respondents believed that unintentional doping offences are mainly caused by inadequate labelling, changes in composition of supplements and lack of information, and felt
that increased awareness of the UK Sport website and regularly updated list of acceptable supplements would help to prevent inadvertent doping. However, nutritional supplements, herbal remedies and other non-herb non-mineral substances are not covered in the DID [09028].

Different countries

The most common steroids involved with doping are testosterone and nandrolone based on WADA data and retrospective autopsy data. In 2006, 34 WADA-accredited laboratories analyzed almost 200,000 urine samples with positive findings in approximately 2 percent. Of the positive samples, AASs accounted for 45 percent of the adverse findings with the most common AASs being testosterone, nandrolone, stanozolol, and methandienone [13003].

Sweden

A recent doping report performed by the Swedish National Institute of Public Health estimated that at least 10,000 people in Sweden, among 9 million people, have used AAS in 2008. However, higher estimations of 50,000–100,000 users have also been made. Different questionnaires among gym customers showed a lifetime prevalence between 2.7 and 4.8 percent. Among the males 3.8-6.0 percent had tested AAS/doping agents at some time and 0-4 percent of the females. In an investigation the prevalence of abuse among young adolescents, 16 and 17 years old, in one Swedish county, by an anonymous multiple-choice questionnaire, indicating a misuse of 2.9 percent among the male population. The Swedish Customs and the National Criminal Police in 2010 reported an increasing illegal import of AAS into Sweden. In particularly, the import of pure substances, used in local production of ampoules and tablets for the black market, has increased markedly over the last years [12025].

Forensic study in Sweden

Anabolic Androgenic Steroids (AAS) are controlled substances in Sweden. The law was passed in 1992 and concerns synthetic anabolic steroids, testosterone and its derivatives, growth hormones and chemical substances that increase the production or release of testosterone and its derivatives or of growth hormones. Even the use of AAS is prohibited in Sweden since 1999, which is quite unique compared to other countries in Scandinavia and also worldwide. Traditionally AAS have been used by elite athletes to enhance performance, but in recent years it has become an increasing problem outside elite sport among athletes, bodybuilders and criminals. Use of AAS is associated with psychiatric side effects such as aggression, depression and violent behavior. Supraphysiological doses and long term use can cause serious physical harm such as cardiovascular toxicity and even premature death. It was investigated and evaluated the drug analytical findings in forensic cases from suspected perpetrators in cases from the police where a screening for AAS was requested to get information about the prevalence of AAS use and the occurrence of poly-drug abuse. The study was based on samples submitted from the police authorities to the Department of Forensic Toxicology in Sweden during the period 1999–2009. Urines were analyzed by methods based on GC–MS and LC–MS–MS. It was also analyzed the prevalence of AAS use at the prison and probation services. A total number of 12,141 urine samples (6362 police cases and 5779 inmates) were analyzed and 34 percent of the cases from the police and 12 percent of the inmates were tested positive for AAS. Nandrolone was the most frequently detected AAS, found in 1249 (62 %) of the positive cases followed by testosterone found in 719 (36 %) cases. Methandienone, stanozolol, boldenone, trenbolone and drostanolone are also commonly misused. Thirteen different AAS substances were detected
In 2009, an addition of eight new substances since 1999 when only five different types of AAS were detected. Including oxymesterone, which only has been identified once in 2003, gives a total number of fourteen different AAS identified during the study period. Nandrolone, testosterone, methandienone and stanozolol have overall been the most frequently used AAS and are currently still in lead. 1120 (60 %) of the positive cases were found to have used more than one type of AAS. The number of AAS found simultaneously has increased from 1 (48%) or 2 (52 %) in 1999 up to eight different AAS in year 2009. High concentration levels, more than 30,000 ng/mL urine, were measured for some of the metabolites and even unchanged steroids were detected at several thousand ng/mL. The most commonly illegal drugs found were cannabis and amphetamines (including methamphetamine and MDMA), both of which were detected in 440 (37 %) cases each. Benzodiazepines were detected in 339 (28 %) cases, cocaine in 218 (18 %) cases, opiates (morphine and codeine) in 77 (6 %) cases with 6- acetylmorphine present in 9 of these cases indicating intake of heroin, ephedrine in 68 (6 %) and GHB in 61 (6 %) cases. It was also investigated the drug abuse pattern of the AAS positives (n=921) compared to the 148,585 cases of petty drug offences, analyzed 1999–2009, where AAS were not measured. The drug abuse pattern for the confirmed AAS users corresponds to the pattern for other drug addicts with some minor differences. Amphetamine and cannabis were most frequently used by both groups, but to a wider extent noted for the non-AAS group. Also opiates were most commonly used within the non-AAS group. Cocaine and benzodiazepines were on the other hand used more broadly by the AAS-users. Thus, the illegal use of AAS in Sweden has increased over the last eleven years. Of the individuals suspected of doping offence, more than 30 percent were tested positive for AAS. At the correctional institutions, 11 percent of the inmates tested, were using AAS. The AAS users in Sweden are primarily young men (99 %) with a median age of 25 years. The high concentrations of AAS and their corresponding metabolites measured in urine, reveals that supraphysiological doses of AAS are administered. The incidence of poly-drug abuse of 60 percent unequivocally demonstrates that AAS are commonly used together with other drugs of abuse, which is in accordance with the drug abuse pattern of the average drug addict. AAS have to be considered as an equally serious social problem as narcotics in Sweden [12026].

**In Swedish prisons**

Anabolic Androgenic Steroids (AAS) are considered drugs of abuse and are controlled substances in Sweden since 1999. Traditionally AAS have been used by elite athletes to enhance performance, but in recent years it has become an increasing problem outside elite sport among athletes, bodybuilders and criminals. Use of AAS is associated with psychiatric side effects such as aggression, depression and violent behavior. Supraphysiological doses and long term use can cause serious physical harm such as cardiovascular toxicity and even premature death. It was investigated and evaluated the drug analytical findings in forensic cases from suspected perpetrators in cases from the police where a screening for AAS was requested to get information about the prevalence of AAS use and the occurrence of poly-drug abuse. The study was based on samples submitted from the police authorities to the Department of Forensic Toxicology in Sweden during the period 1999-2009. Urines were analyzed by methods based on GC-MS and LC-MS-MS. We also analyzed the prevalence of AAS use at the prison and probation services. A total number of 12,141 urine samples (6362 police cases and 5779 inmates) were analyzed and 33.5% of the cases from the police and 12 percent of the inmates were tested positive for AAS. The users of AAS were mainly in 99 percent men with a mean age of 26 years whereas the women were 30 years old. The most frequently used AAS was nandrolone followed by testosterone and methandienone. Other illicit and licit drugs were detected in 60 percent of the cases from the police, strongly indicating a frequent poly-drug abuse among users of AAS [12118].

**Denmark**
From 2003 to 2009, the number of cases testing positive in Denmark for use of doping increased from 11 to 181. The cases were observed exclusively among individuals exercising in fitness centers. Consequently Anti Doping Danmark initiated a population based survey about individual knowledge, attitudes and use of muscle enhancing drugs. The survey focused in particular on young males aged 15 to 25 years old exercising in fitness centers. 5,010 individuals aged 15-60 years were selected at random and asked to respond to a postal or web-based questionnaire. 1,703 individuals (34 %) responded to the questionnaire. The most important results from the survey included:

- 1.5% reports that they currently use or have been using muscle enhancing drugs. The proportion amounts to 44,000 individuals aged 15-60 years in the general population. The prevalence of use among individuals that exercises in a fitness center was 3.3 %, which amounts to approximately 16,500 individuals
- 6% have considered using muscle enhancing drugs. This proportion amounts to 150,000 individuals
- more than half of those that have considered using muscle enhancing drugs knows of other people in their social network, that uses the drugs
- a substantial proportion of the population opposes the use of muscle enhancing drugs
- attitudes to muscle enhancing drugs among individuals exercising in fitness centers are no different from others
- 19 % of the population knows where to acquire muscle enhancing drugs. Among those that have considered using the drugs, 49 % report that they know where to get the drugs
- the majority of those using anabolic steroids do not discuss their abuse with their own doctor.

In addition, results showed a more relaxed attitude to muscle enhancing drugs and a higher level of knowledge about the effect of the drugs among the 6 percent of the respondents that at some point have considered using the drugs. Users of muscle enhancing drugs are found more frequently in fitness centers than other sport facilities. Muscle enhancing drugs are more frequently a topic of discussion among respondents exercising in fitness centers, and several report about supervision in use of muscle enhancing drugs being available in the fitness center. At the same time, significant more individuals in the fitness environment report knowing about someone in their social network that use muscle enhancing drugs, compared to other respondents. A positive results is the rarity of offers of muscle enhancing drugs among individuals exercising in fitness centers, which occurs equally seldom as among other respondents exercising elsewhere. Also positive is the opposing attitude to muscle enhancing drugs among respondents exercising in fitness centers, as among others. The rarity of offers and the opposing attitudes among respondents exercising in fitness centers points to a good social environment. In sum, the positive results observed should be included in the preventive activities [10429].

Individuals exercising to for a more muscular and athletic body or to gain more self confidence, report that use of muscle enhancing drugs is acceptable if use of drugs was not associated with health hazards. Individuals that exercise to increase their muscle volume are more frequently observed in fitness centers and other areas where excessive weight training is a supplement to the main sport. The positive attitude to muscle enhancing drugs among individuals exercising for a more muscular and athletic body is contrasting the attitude among individuals for whom such motives for exercising are of no importance. For this reason,
information and education about the health hazards associated with use of muscle enhancing drugs should be continued and increased in strength – both within and outside commercial fitness centers. As argued above, information about short- and long term health hazards associated with use of muscle enhancing drugs should be included as an integrated part of the preventive initiatives. In contrast, information on legal consequences should be included to a lower degree – if at all. Narratives that illustrate the health and social risks associated with use of muscle enhancing drugs could form the core of the information and education parts of the preventive work. The prevalence of attitudes to and use of muscle enhancing drugs is documented in the present survey. Results indicate a possible higher prevalence of use in some environments – including fitness centers. Since the latest survey on use of muscle enhancing drugs in Denmark published in 1999, other surveys have been conducted. Any results and conclusions that are based on self-reported behavior that is perceived a criminal act and social unacceptable will necessarily face major methodological challenges. The results of the now presented survey are no exception. Notwithstanding these caveats, the overall conclusion of the surveys conducted within the past ten years is that the abuse of muscle enhancing drugs at best remains stable or worse is increasing. Nothing points to a decrease in use of the drugs [10429].

In general practice
The goal of one study was to investigate the use and side effects of anabolic androgenic steroids (AASs), growth hormone (GH), erythropoietin (EPO) and other enhancing drugs by patients in general practice. In a questionnaire, 702 general practitioners (GPs) in Denmark were asked to estimate the number of their patients who within the preceding year had admitted to using or were highly suspected of having used AASs, GH, EPO or other enhancing drugs. In addition, they were asked to describe the possible side effects of their use. Of 571 eligible GPs, 119 had within the preceding year treated patients who had admitted to or were highly suspected of having used AASs, GH, EPO or other enhancing drugs. 182 users were reported by the GPs during that period; 180 (99 %) were males, and 156 (86 %) were between 20 and 40 years of age. 125 of the patients (69 %) had admitted the use to the GP; AASs had been used by 123 patients (98 %). EPO was not reported in any case, but GH had been used by 9 patients (7 %). 127 (70 %) of the patients had experienced side effects, and 87 (49%) had contacted the GP due to these side effects. The use and side effects of AAS are commonly reported in general practice, whereas the use and side effects of GH and EPO seem to be uncommon. Their use is mainly by men under 40 years of age. The use of AAS is often connected with dermatological and musculoskeletal side effects. It is recommended also to consider possible use of AASs when consulted by a patient with unexplained symptoms of cardiovascular disease, psychological or sexual dysfunction, gynaecomastia or liver dysfunction [06026].

Norway

Adolescents
To prospectively study the stability of anabolic androgenic steroid (AAS) use and predictors of AAS use, and to investigate whether AAS use alters the risk of later emotional and behavioral problems a survey of a national sample of Norwegian high school students (age 15-19) in 1994 was followed up in 1999 (n=2924). Measures of frequent alcohol intoxication (50+ times per 12 months), cannabis use (12 months), hard drug use (12 months), being offered cannabis, eating problems, conduct problems, sexual debut before age 15, BMI, involvement in power sports, perceived physical appearance, and satisfaction with body parts were obtained. Life-time prevalence of AAS use were 1.9 and 0.8 percent in the follow-up period. Multivariate logistic regression revealed that future AAS use was predicted by young age, male gender, previous AAS use, involvement in power sports, and frequent
alcohol intoxication. AAS use did not predict future emotional or behavioral problems other than reducing the risk of future frequent alcohol intoxication. It was concluded that frequent alcohol intoxication and involvement in power sports appear to predict future AAS use. At the population level there was little stability in individual AAS use from adolescence to early adulthood. No detrimental effects of AAS use could be detected in this study, but low statistical power limits this conclusion.

A total of 1351 high school students (52 % males, 48 % females) with mean age 18 years from randomized school classes in Hordaland County, Norway, participated in an Internet survey conducted in 2004 about the lifetime use of anabolic steroids and personal acquaintance with at least one user of anabolic steroids. In addition to questions about anabolic steroids the participants completed the Hospital Anxiety and Depression Scale and the Alcohol Use Disorders Identification Test. They also answered questions about demography, smoking, and narcotic use. The lifetime prevalence for use of anabolic steroids was 3.6 percent for males and 0.6 percent for females. In all, 28 percent of the respondents reported having at least one acquaintance that used or had used anabolic steroids. Use of anabolic steroids and having acquaintances using such drugs were strongly related to use of other drugs such as alcohol, nicotine, and narcotics. Implications for prevention are discussed and the study's limitations are noted.

Finland

The aim of one study was to describe the lifetime occurrence and associated factors of anabolic-androgenic steroids (AAS) among young Finnish males. Of the 10 829 male conscripts (median age 19), 10 396 (96 %) answered a questionnaire during the first days of their conscription in the years 2001-2007. The main outcome was lifetime AAS use. It was also studied associations between 13 socioeconomic, health, and health behavioral background variables and AAS use by logistic regression. Eighty-nine (0.9 %) respondents reported having used AAS. In addition, 26 (0.3 %) respondents reported that they would use AAS if they could obtain them. In multivariate analysis, which included all significant variables and age, the strongest associated factors were weight training at fitness centers more than three times a week (odds ratio 11.8; 95 % confidence interval 7.1 to 19.6), low educational status (odds ratio 3.7; 95 % confidence interval 2.0 to 7.0), and weekly drunkenness as drinking style (odds ratio 2.4; 95 % confidence interval 1.4 to 4.5). Sports other than weight training were not associated with AAS in the sample. The use of AAS is relatively uncommon among Finnish males. It is strongly associated with weight training at fitness centers but also with lower educational status and a drunkenness-oriented lifestyle. Prevention should be targeted at those males participating in weight training.

UK

In an English report the House of Commons Science and Technology Committee says that more needs to be done on every level to combat the use of illegal substances by athletes. The cross party group of MPs says that athletes caught cheating by using chemicals or biological agents should be banned from sport for four years and ordered to repay any financial gains they have made since their last clean test. Athletes should also have to state where they obtained the banned substances before they are allowed to return to competitive sport. The committee concluded that official figures on the incidence of illegal doping may not accurately reflect the problem, and it called for more research into the true scale of the problem. Figures from the World Anti-Doping Agency show that 2.1 percent of tests for banned substances resulted in "adverse analytical findings" in 2005; in the UK 1.3 percent of 7968 tests proved positive in 2005-6. To make it easier to detect performances improved by
illegal substances all UK athletes should be made to compete on the international circuit during the 12 months before the Olympics, says the report. And a new agency that is independent of UK Sport and other national sporting bodies should be set up to test athletes for drug use. The agency should also monitor and evaluate potential new illegal substances and methods as they are developed. The report also recommends a pilot project to examine the feasibility of a physiological or doping passport to be carried by all athletes. This would record the results of doping tests and natural concentrations of hormones such as erythropoietin during their careers, which would make it easier to detect any substance abuse, the committee says. The committee also expressed concern at the ease in which banned and potentially dangerous substances can be obtained for use by athletes. It recommended that the government review regulation in this area [07035].

Figures from the Department of Health in the United Kingdom showed that 0.2 percent of young people had tried anabolic steroids in 2001-4 and 0.5 percent in 2006. A questionnaire study of 3403 12th grade students (final year of secondary school) in the United States found that 6.6 percent admitted to taking anabolic steroids. Worryingly, two thirds of those who started using anabolic steroids when they were aged 16 or younger. A study of homosexual men who regularly attended gyms in London in 2000 found that 15 percent of the 792 men surveyed had used anabolic steroids in the preceding year, with 12 percent of them having injected the drugs. Two thirds of the respondents used more than one agent (so called "stacking"). The high prevalence of use of injectable anabolic steroids correlates with a local experience, where 43 percent of new registrations for needle exchanges were users of anabolic steroids. This figure might be an underestimate as many users of anabolic steroids will collect needles and syringes for friends and other users as well [12025].

**Greece**

Doping use is an ongoing problem in contemporary sports. Despite efforts to detect and control doping, research on its etiology is limited, especially among elite-level athletes. One study used an integrated social cognition model to examine the predictors of doping intentions. Structured anonymous questionnaires were completed by 1075 Greek adult elite-level athletes (medium age = 25 years, 36 % females) from both team and individual sports. Multiple regression and mediation analyses showed that attitudes, normative beliefs, situational temptation, and behavioral control significantly predicted doping intentions. A normative process was identified whereby situational temptation mediated the effects of normative beliefs on intentions. The findings provide the basis for future social cognition research in doping use, and set the framework for the development of evidence-based preventive interventions [10436].

One article concerns the analysis of the adverse analytical findings (AAFs) and the appropriate alterations made during the period 2005-2011, so that the Doping Control Laboratory of Athens (DCLA) obeys the updated World Anti-Doping Agency (WADA) List of Prohibited Substances. The percentage AAFs of the DCLA was compared with those of WADA-Accredited Laboratories. In 2008, the term Atypical Finding was introduced by the WADA representing a reported but inconclusive result. A characteristic example is when a testosterone-to-epitestosterone ratio is >4 followed by a negative gas chromatography/combustion/isotope ratio mass spectrometry result. In a total of about 30,000 athlete samples, 136 athletes were found with an increased testosterone/epitestosterone ratio and 43 with tetrahydrocannabinol metabolite (THCCOOH) of 427 reported AAFs. Twenty-one athletes in total were found positive with methylhexaneamine, the 11 found after a batch of 1000 samples was reprocessed. Besides, there were AAFs below their Minimum Required Performance Level (MRPL). The increasing need for higher detectability imposed new
apparatus, e.g., liquid chromatography/quadrupole/time-of-flight mass spectrometry, whereas that for lowering the capital costs and reporting times led to the unification of the screening method which includes stimulants, diuretics, anabolics and other substances [0125].

**Italy**

The objective of one study was to assess the prevalence of illicit drugs use among young adults, in particular elite athletes. The study considers the data obtained from anti-doping analyses performed on nearly 100,000 urine samples from 2000 to 2009 by the World Anti-Doping Agency accredited Italian Anti-Doping Laboratory. The percentage of adverse analytical findings varies on a yearly basis, but it is in the range 1.0-1.8 percent (not considering atypical findings, such as an altered endogenous steroid profile). Among positive results, there is a high prevalence of stimulants and drugs of abuse. The drug of abuse found most frequently is the tetrahydrocannabinol (cannabis) metabolite, accounting for 0.2-0.4 percent of the total samples analysed (18 % of the positive results). The second most frequently encountered drug is cocaine, as detected from cocaine metabolites, accounting for 0.1 percent of the total samples analysed (7 % of positive results). Other stimulants found included amphetamines, ephedrines, carphedon, modafinil, and anorexic compounds. No amphetamine-like designer drugs were detected. These data are indicative of the widespread prevalence of cocaine and cannabis use among the young adult population. However, due to the particular population studied, it must be considered an underestimation of the phenomenon among elite athletes with respect to the general population [11016].

**France**

To describe the prevalence of doping and its progression in a cohort of preadolescent athletes during a 4-year follow-up a prospective cohort study was performed. It was a self-questionnaire survey where all of the pupils entering the first year of secondary school (sixth grade) in the Vosges Département (east France) and followed for 4 years was asked about drug use (prohibited substances, tobacco, alcohol, cannabis), intention to use, reported health hazards, perceived drug effectiveness, self-esteem, trait anxiety. At the beginning of the study, 1.2 percent (95 % confidence interval 0.8 to 1.6) stated that they had taken doping agents at least once in the preceding 6 months, and this had risen to 3.0 percent four years later, which was a statistically significant difference. Of those who had used doping agents, 4 percent reported that they had experienced a health problem related to doping, and 44 percent reported that they had won at least one sports event as a result of using the drug.

Use of doping agents is linked to the number of hours of practice per week, intention to use, use of other drugs, self-esteem and trait anxiety. The results show that doping does exist in preadolescent athletes who train every day [07034].

The French report of the National Academy of Medicine named "Sport and Health" underlines the medical, social and educational dimensions of sporting activities. Various kinds of sporting practices are described: they concern the approximately 7,000 high level athletes, around 8,000 professional (licensed) sportsmen, and sporting club members (approximately 15 millions people). A large number of amateurs do not practice in any structure and therefore are neither managed in their activities nor medically followed. Some characteristics of sporting practice at various stages of life have been documented. Around 50 percent of the teenagers from 12 to 17 years have a sporting practice out-of-school besides the weekly three hours applied at school or college; however, the withdrawal of sporting practice by a high number of teenagers results in a sedentary lifestyle with overweight and obesity, major risks factors for health. Elderly people take a profit from a regular and medically controlled physical activity. Functional capacities are thus improved,
cardiovascular risks factors among other, which results in better quality of life of the aged and delays their dependence. The benefit upon public health of sporting practice has been pointed out in the primary prevention of cardiovascular and respiratory diseases, osteoporosis, obesity, diabetes, breast and colon cancer, and mood disturbances. It is currently well acknowledged that sporting practice is an important component of public health in both primary and secondary prevention of many diseases. Deleterious effects of which the most serious is the sudden death related to a cardiovascular anomaly, which generally occurs during an important physical effort. An important sport drift is the practice of doping to improve performances through the use of hormones, anabolics, EPO, transfusions, ... When a person exceeds his/her capacities of adaptation, because of a badly adapted or a too intense drive, this overtraining results in a reduction in physical capabilities, stress, behavioral issues and sleep-wake disorders. All of those issues often lead sportsmen to doping with the aim to improve their capabilities, rapidly installing an overtiredness state resulting in a fall of performances. A major aim from the view point of public health is to reinforce the fight against doping since it concerns a large number of people, sportsmen and amateurs, with teenagers among them. Lastly, the report underlines that sport medicine is practically not taught in the initial training of medical doctors [09029].

The purpose of one investigation was to determine the substances used, and the attitudes towards doping of high school athletes. A four-page, self-completed questionnaire was designed to determine the drugs used (licit, illicit and doping substances) along with beliefs about doping and the psychosociological factors associated with their consumption. The questionnaire was distributed to all the high school students enrolled in a school sports association in the Lorraine region in Eastern France. The completed forms were received from 1459 athletes: 4 percent stated that they had used doping agents at least once in their life (their main source of supply being peers and health professionals). Thirty-four percent of the sample smoked some tobacco, 66 percent used alcohol, 19 percent cannabis, 4 percent ecstasy, 10 percent tranquilizers, 9 percent hypnotics, 4 percent creatine and 41 percent used vitamins against fatigue. Beliefs about doping did not differ among doping agent users and non-users, except for the associated health risks which were minimized by users. Users of doping agents stated that the quality of the relations that they maintain with their parents is sharply degraded, and they reported that they are susceptible to influence and difficult to live with. More often than non-doping agent users, these adolescents are neither happy, nor healthy, while paradoxically, they seem less anxious and they are more self-confident. The findings suggest that doping prevention among young athletes cannot be limited uniquely to the list of banned drugs [10441].

Germany

Goal-directed measures to prevent doping and drug abuse in sports requires empirical data. In this connection, a cross-sectional analysis was carried out in 2004. The purpose of the study, on the one hand, was to register reliable data of the current situation in Thuringia, and, on the other hand it was to give information on possible interventional steps with scientific support. Within three months, 2319 adolescents from 16 Thuringian schools (5 regular schools, 4 secondary schools, 3 sport schools and 4 vocational schools) were surveyed. Three hundred and forty-six (15 %) students out of 2287 students (26 students without a statement) indicated use of prohibited substances from the WADA list in the previous year: 16 (0.7 %) anabolic-androgenic steroids (AAS), 10 (0.4 %) growth hormones, 56 (2.4 %) stimulants, 305 (13.2 %) cannabis, 2 (0.1 %) diuretics, 52 (2.2 %) cocaine/heroin and 6 (0.3 %) erythropoietin. Moreover, nonathletes (n=490) reported a substance use that was approximately 5 percent higher than that of recreational athletes (n=1254) and nearly three times higher than that of competitive athletes (n=497). All three groups (nonathletes, recreational athletes and competitive athletes) performed poorly on a knowledge test
regarding doping in general with an average below 60 percent in each case. Another main aspect of the study was to determine factors influencing substance use in sports. Besides the doping specific knowledge, age contributed as well as anti-doping attitude, to the resulting variance. Gender, however, played no role. The findings of the study point towards the need for improvement of specific knowledge of doping among students and that their attitude towards doping must be altered. The goal in this case is to test the effectiveness of appropriate scientific intervention [07033].

Doping controls are key factors for fair and clean sports. The developments of German activities in the national antidoping fight were evaluated over a period of 18 years (1989-2006) with regard to in-competition and out-of-competition testing. The quantity of respective controls conducted by federations and antidoping organizations, the ratio of in- and out-of-competition controls, the number of athletes per squad (and thus per-capita tests) as well as adverse analytical findings were summarized in a review. The available data demonstrated a constantly increasing effort, particularly regarding the amount of out-of-competition controls, but also discrepancies in per-capita analyses between different federations. In light of recent doping scandals and confessions in 2007, a critical review of national antidoping actions is considered necessary [08031].

In the context of house searches in Germany, numerous drugs were confiscated and subjected to chemical analysis, including anabolic agents such as various anabolic-androgenic steroids (stanozolol, testosterone derivatives, trenbolone esters, etc.) and clenbuterol, as well as agents with anti-estrogenic activity (tamoxifen, clomiphene), drugs stimulating virility (sildenafil, tadalafil), and unlabeled plastic bags. Liquid chromatography-tandem mass spectrometry, gas chromatography-mass spectrometry with nitrogen-phosphorus specific detection, gel electrophoresis, and immunological tests were employed to test for the effective content of 70 products. In 18 cases (26 %), the declared ingredients differed from the actual content, in particular concerning anabolic-androgenic steroids. Nandrolone and trenbolone esters, for instance, were frequently substituted or complemented by various testosterone derivatives, and several testosterone depot formulations originally composed of four different esters were found to contain fewer or wrong components. Except for those drugs supposedly originating from so-called underground labs, fake packings were hardly or not distinguishable from original boxes by visual inspection [08032].

**Adolescents**

Goal-directed measures to prevent doping and drug abuse in sports requires empirical data. In this connection, a cross-sectional analysis was carried out in 2004. The purpose of the study, on the one hand, was to register reliable data of the current situation in Thuringia, and, on the other hand it was to give information on possible interventional steps with scientific support. Within three months, 2319 adolescents from 16 Thuringian schools (5 regular schools, 4 secondary schools, 3 sport schools and 4 vocational schools) were surveyed. Three hundred and forty-six (15 %) students out of 2287 students (26 students without a statement) indicated use of prohibited substances from the WADA list in the previous year: 16 (0.7 %) anabolic-androgenic steroids (AAS), 10 (0.4 %) growth hormones, 56 (2.4 %) stimulants, 305 (13.2 %) cannabis, 2 (0.1 %) diuretics, 52 (2.2 %) cocaine/heroin and 6 (0.3 %) erythropoietin. Moreover, nonathletes (n=490) reported a substance use that was approximately 5 percent higher than that of recreational athletes (n=1254) and nearly three times higher than that of competitive athletes (n=497). All three groups (nonathletes, recreational athletes and competitive athletes) performed poorly on a knowledge test regarding doping in general with an average below 60 percent in each case. Another main aspect of the study was to determine factors influencing substance use in sports. Besides the doping specific knowledge, age contributed as well as anti-doping attitude, to the resulting
variance. Gender, however, played no role. The findings of the study point towards the need for improvement of specific knowledge of doping among students and that their attitude towards doping must be altered. The goal in this case is to test the effectiveness of appropriate scientific intervention [06023].

**Serbia**

Socio-economic changes that occurred in the wake of dismemberment of former Yugoslavia resulted in the appearance of social pathology, one of which was the increase in the use of psychoactive substances. A study was carried out among 1011 elementary school children (seventh and eighth grades) and secondary school children (all four grades) in the area of Belgrade from 2003 to 2004. Out of the total number 457 (45 %) were elementary school pupils and 554 (55 %) secondary school pupils. There were 524 (52 %) boys and 487 (48 %) girls, aged from 12 to 18 years (the average age being 15 years). The method used was the European School Survey Project on Alcohol and Other Drugs Questionaire. Totally 14 percent examinees tried psychoactive substances. The most frequent drug used at the first contact was marijuana (10.8 %) at the age of 15 tried by 13 percent examinees, inhalants (4.4 %), amphetamines (4.1 %), sedatives (3.7 %), alcohol combined with marijuana (3.9 %), then cocaine (2.8 %), heroin (2.3 %), alcohol combined with sedatives (2.2 %), and ecstasy (1.6 %), followed by anabolic steroids, heroin, diethylamid lisergic acid (LSD) and magic mushrooms. It was determined that going out in the evening, smoking and binge form drinking were directly connected with the use of psychoactive substances [08030].

**Croatia**

Substance use and misuse (SUM) and the relation to physical activity/exercise/athletic participation (sport factors) and scholastic achievement are rarely studied in Croatia. The aim of this study was to investigate the SUM habits in Croatian adolescents (17-18 years of age, 254 males, and 218 females), and to study potential gender-specific interrelationships between scholastic and sport factors in relation to SUM. The testing was done using an extensive, anonymous, self-administered questionnaire that consisted of scholastic variables, sport factors, and SUM data. Descriptive statistics, counts, and proportions were calculated. Gender differences were established using the Kruskal-Wallis test. Gender-specific correlations within and between studied variables were established using the Spearman's correlation. The incidence of smoking habits and alcohol consumption among Croatian adolescents was alarming, and a serious intervention program should be developed to address this issue. Educational achievement was negatively related to SUM, with no gender-specific relationships. The data indicated some "protective" effects of the sport factors against SUM in boys, but a significant positive correlation between alcohol drinking and sport participation in girls was also noted [11019].

**Poland**

To estimate the prevalence of anabolic-androgenic steroids (AAS) abuse among adolescent and young adults in Poland 3,687 men (48 %) and women (52 %), median age 23 (interquartile range 19-30 years) participated in a survey via a "pop-up window" which appeared on two popular Polish internet portals during one month. Questions concerning their body image, exercise behaviour, education level and use of anabolic-androgenic steroids were asked. The prevalence of anabolic-androgenic steroids use was 6.2 percent among males and 2.9 percent among females. Male AAS users, compared to non-users, were more often concerned about their physical appearance, were less educated and often engaged in some sport activity. Among female AAS users, no significant differences
concerning self-body image satisfaction or participation in sports were found. However, compared to non-users, female AAS users were less educated. It was concluded that the abuse of AAS is a reality in Poland and may become a serious health concern among adolescents and young adults [06027].

**Europe, combined countries**

Data on doping among young non-professional athletes are scarce. In order to estimate the prevalence and predictors of doping use, a standardized, anonymous questionnaire was self-administered by 2650 tertiary education students from five European Union countries (Finland, France, Germany, Greece, Italy) and Israel. The reported usage rate of a doping agent (at least once) was 2.6%, with no significant variation in the frequency of doping reporting among the participating countries. Doping was, however, less common among students of biomedical schools (OR: 0.49) and was higher among males (OR: 2.16). Students, who use to drink coffee or recall frequent occasions of involvement in drunkenness episodes, were more likely (twice and three times, respectively) to report doping, and students using nutritional supplements or having participated in a major athletic event were more likely (four times and twice, respectively) to report doping in comparison with students who do not. Of note is the high odds ratio for reporting individual doping when having a friend who uses doping (OR: 8.61). Given the large size of the physically active young individuals in the population and the small number of professional athletes, doping in the general population may be, in absolute terms, as sizeable problem as it is among the professional athletes. There was evidence that high-risk behaviour and supplement use increased the risk of doping [06028].

**Jordan**

One study was conducted to measure the extent of androgenic steroids abuse among two targeted groups in Jordan, college students and athletes, and the risk factors associated with this abuse. Five hundred and three Jordanian collegiate students and 154 bodybuilding athletes completed a three section questionnaire that investigated demographic information, prevalence of anabolic-androgenic steroids and attitude towards steroids abuse. Of the investigated collegiate students, 4.2 percent were current users, while the percentage rose to 26 percent among the athletes; the mean age of users in the two groups was 20 and 28 years, respectively. Almost one-third of the students started abusing anabolic steroids before the age of 15 years while more than half of the athletes started between the ages of 15 and 18 years. Knowing where and how to get the drugs has not been a problem for either the students or the athletes as their friends and coaches were the major sources. The main reasons for using anabolic steroids have been found to help improving athletic performance and physical appearances [08029].

**Iraq**

The majority of men's sports need high levels of strength and power. The effects of any given type of performance-enhancing substance are mostly directly related to its ergogenic effects (enhanced strength, higher energy production, and better recovery), anabolic potential (increased protein synthesis, especially in muscles), and/or stimulating properties (increased attention and loss of fear), which give a competitive advantage to athletes. A descriptive correlational study was conducted to identify bodybuilders' and athletes' perception toward substance use and to identify the relationship between substance use and those athletes' sociodemographic characteristics of age, level of education, social status, and monthly income. A purposive "nonprobability" sample of 172 bodybuilding athletes were recruited.
from gym users of Baghdad city. The study found that two fifths of those who used anabolic-androgenic steroids (AAS) were 19 years old or younger, less than one half were overweight (body mass index 25-30), two fifths of participants enjoyed exercise/training to an extreme level, two fifths of study participants highly perceived the improvement of athletic performance, two fifths of the study participants highly perceived the importance of improving athletic performance, less than half of the study participants used AAS, one quarter of the study participants who used AAS had been influenced by their coaches to use such substances, and more than one third of the study participants who used AAS were using such substances in the form of oral tablets and intramuscular injection together [12027].

Korea

Athletes report frequent use of various dietary supplements (DSs). The objectives of one study were to obtain information about Korean Olympians' DS use during the training period for the Beijing 2008 Summer Olympic Games and immediately before their Olympic events, to obtain DS-intake reasons and DS providers, and to obtain information on athletes' doping education, knowledge, and educators. Korean Olympians completed 2 questionnaires 1 week before the opening and within 1 week after the closing of the Beijing 2008 Summer Olympic Games. Results showed that 79 percent of male and 82 percent of female Olympians take more than 1 dietary supplements during the training period and that vitamins and Oriental supplements are the 2 top-ranked DSs. Reasons for dietary supplements use were to improve recovery ability (66 %) and muscle performance (22 %), and sources of obtaining dietary supplements were parents (36 %) and coaches (35 %). Furthermore, 79 percent of Korean Olympians reported receiving regular education on antidoping regulations from Olympic-sponsored education classes (64 %) and coaches (15 %) [11021].

USA

Although various surveys have tracked the prevalence of anabolic-androgenic steroid (AAS) use in American teenagers and young adults, no recent surveys have assessed the lifetime prevalence of AAS use in Americans overall. It was therefore analyzed serial youth-survey data to derive estimates of the lifetime prevalence of AAS use in the current American general population. It was first determined the distribution of age of onset of AAS use, based on pooled data from nine studies. Using this distribution, we then developed equations to project the eventual lifetime prevalence of AAS use among young survey respondents, once they aged and completed the period of risk for initiating AAS. It was similarly calculated the denominator of lifetimes of risk for AAS use in the total American population. It was next applied these equations to four independent national youth datasets to derive current American general-population estimates for lifetime AAS use. Finally, using data from 10 pooled studies, it was estimated the lifetime prevalence of AAS dependence among AAS users. Age-of-onset studies consistently showed that AAS use begins later than most drugs, with only 22 percent of users (95 % confidence interval 19 to 25 %) starting before age 20. Applying the age-of-onset findings to national youth datasets, it was estimated that among Americans currently age 13-50 years, 2.9-4.0 million have used AAS. Within this group, roughly 1 million may have experienced AAS dependence. Although subject to various limitations, the estimation techniques suggest a surprisingly high prevalence of AAS use and dependence among Americans [13030].

Because 57 percent of all high school students play on formal sports teams, the use of both illicit and legal ergogenic drugs to enhance performance in amateur athletics is of significant concern today. Furthermore, up to one third of high school students who use anabolic steroids are in the population of nonathletes who use steroids to improve their appearance.
Drug and supplement use is not uncommon today. It is estimated today that 1 to 3 million US athletes are taking steroids, and 2500 tons of creatine were consumed in 1999. Many substances are being used by today’s youths, commonly without recognizing any risks of such drugs. Even as this problem increases, there are concerns that pediatric residents are receiving minimal education in the field of sports medicine during both medical school and residency. Although the focus of pediatric sports medicine is typically proper training and managing common injuries, an emerging issue is learning about the drugs that are chosen by young athletes to improve athletic performance [06003].

The US National Institute on Drug Abuse has called for increased research into the use of physical activity in substance abuse prevention, specifically research into physical activity type and context. One paper examined the relationships between secondary school student substance use and exercise in general and school athletic team participation, and examines such relationships over time. Nationally representative cross-sectional samples of 8th-, 10th-, and 12th-grade students were surveyed each year from 1991 to 2009. Substance use measures included past 2-week binge drinking and past 30-day alcohol, cigarette, smokeless tobacco, marijuana, and steroid use. Analyses were conducted during 2009-2010. Across grades, higher levels of exercise were associated with lower levels of alcohol, cigarette, and marijuana use. Higher levels of athletic team participation were associated with higher levels of smokeless tobacco use and lower levels of cigarette and marijuana use across grades and to higher levels of high school alcohol and steroid use. Exercise helped suppress the undesired relationship between team participation and alcohol use; exercise and athletic team participation worked synergistically in lowering cigarette and marijuana use. Observed relationships were generally stable across time. There appear to be substantive differences between exercise and team sport participation in relation to adolescent substance use. These findings from cross-sectional data suggest that interventions to improve levels of general physical activity should be evaluated to determine if they help delay or reduce substance use among youth in general as well as among student athletes [11020].

Data on the actual prevalence of AAS use are limited by recall and reporting biases associated with illicit drug use. Many studies of AAS use focus on adolescents. In a nationwide study of 3403 12th-grade male students in 46 private and public US high schools during the 1980s, 6.6 percent of the respondents admitted to ever using AASs based on their response to questionnaires. The individual participation rate was low (50%). Almost 40 percent of the respondents admitted to the administration of 5 or more cycles of AASs. Further studies performed at local and statewide levels have confirmed similar findings indicating that 3-12 percent of high school male adolescents use AASs at some time in their lives. These studies indicate that about one-third of the high school students use AASs for appearance rather than athletic performance. In 1999, the National Institute on Drug Abuse Monitoring the Future study indicated that approximately 2.7 percent of US 8th and 10th graders and 2.9 percent of 12th graders admitted to the use of AASs at least once. For 10th graders, the prevalence of AAS use increased to 2.7 percent in 1999 from 2.0 percent in 1998. For all 3 grades, the 1999 levels represented a significant increase from 1991, when 1.9 percent of 8th graders, 1.8 percent of 10th graders, and 2.1 percent of 12th graders admitted AAS use at least once. The data on adolescent use of AAS in 2004 and 1999 were similar with 2.5 percent of 12th graders reporting the use of AASs at least once. Risk factors for the use of AAS among adolescents include male gender, participation in strength-related sports, and use of other illicit drugs. In college and professional athletics, the prevalence of AAS abuse is less defined because of the ramification of AAS use. However, the prevalence of AAS use among these groups is probably substantially higher than in high school students, particularly in football players and male track-and-field-athletes. Between 1972 and 2000, there were 29 athletes disciplined for positive AAS drug tests. However, the abuse of AASs is probably more widespread among these athletes because of the difficulty detecting
all AAS use and the small number of doping charges compared with the number of positive drug tests. Current trends suggest that most AAS abusers are non-athletes using these drugs for cosmetic purposes [13003].

**Origin of AAS in the United States**

AASs are DEA schedule III drugs of the Controlled Substances Act. The primary sources for illicit AASs in the United States are manufacturing facilities in Mexico. Traffickers from the United States enter Mexico and purchase AASs at pharmacies in Baja, California, where AASs are available by prescription. They subsequently smuggle the AASs back in to the United States. Other sources of illicit AAS in the United States include eRussia, Poland, Hungary, Spain, Italy, Greece, Canada, and the Netherlands as well as some clandestine labs in the United States. The diversion of legitimate pharmaceutical preparations provides a much smaller portion (i.e. 10-15%) of illicit AASs compared with other illicit sources. Information on the administration and procurement of AASs is widely available on the Internet [13003].

**Australia**

There is evidence to suggest that the prevalence of anabolic-androgenic steroids (AAS) is higher among young people than the general population. The purpose of one study was to examine the proportion of students who reported lifetime and past-year AAS use, explore other drug use among those who reported AAS use, and investigate demographic correlates of AAS use. Data was taken from a cross-sectional survey of a representative sample of Australian secondary students. A stratified two-stage probability sampling methodology was employed and schools were randomly sampled from each Australian State and Territory. A total of 376 schools participated in the survey. Lifetime AAS use was reported by 2 percent of 12-17-year-old students; use was more common among 12-15-year olds then 16-17-year olds. Regardless of age, being male, speaking a language other than English at home, not be at school on the previous school day, and rating own scholastic ability as below average were all associated with a greater likelihood of using AAS in their lifetime and in the past year. Those who reported AAS use also reported the use of a range of other substances, suggesting that AAS use may be part of a broader experimentation with substances. Interventions towards these groups regarding AAS may best be placed within a larger substance use intervention rather than being AAS-specific. In light of the low levels of AAS use among this group, more detailed research into AAS use among adolescent sporting groups may be warranted [11017].

One study aimed to investigate the prevalence of illicit drug use among elite Australian athletes with a focus upon cannabis, ecstasy, meth/amphetamine, cocaine, GHB and ketamine; explore perceptions concerning the extent of drug use among this group; ascertain opinions regarding specific drugs of concern; and investigate predictors of recent drug use. Data were taken from surveys with 974 elite athletes. One-third of the sample had been offered or had the opportunity to use illicit drugs in the past year; despite this, the self-reported prevalence of all six drugs under investigation was lower than that reported by the general population. Sixteen percent of athletes believed that there was a drug of concern in their sport, with ecstasy, cocaine and alcohol being nominated. Knowing other athletes who use illicit drugs, being offered or having the opportunity to use drugs and identifying as a “full-time athlete” were significant predictors of recent drug use. The study found that one-third of the athlete sample had been offered or had the opportunity to use illicit drugs in the past year; despite this, there was low self-reported drug use. Despite media discussion regarding alcohol use in sport, alcohol was nominated as a drug of concern only by a small proportion of athletes, and further research investigating this issue may be warranted [11018].
One study aimed to determine through a questionnaire applied to interviewers, the current or past use of anabolic androgenic steroids (AAS), as well as other hormones, and other medicines, food supplement and illicit drugs among strength training apprentices in the city of Porto Alegre, RS. It was interviewed 288 subjects draw from a sample of 13 gyms. The prevalence of current and past use of AAS was about 11 percent (32/288), other hormones 5 percent (16/288) and other medicine 4 percent (12/288). The most used AAS were nandrolone and stanozolol; the other hormones were gonadotropin, triiodothyronine (T3). The most frequent side-effects were behavioral such as humor oscillation, irritability and hostility, and endocrine disturbances such as acne and increased or decreased libido. When analyzed together with other hormones in a variable named "hormonal agents" (AH), AAS presented a statistical difference among genders considering that the most frequent use of hormones occurred among men and those who consume food supplements. The comparison of these findings to other national and international results is difficult due to the epidemiological design. Even if it is considered, the observed prevalence suggests that preventive attitudes as well as special care in the orientation and education of this population must be taken [07036].

Different sports

Prevalence of use depending on type of sports

Prior research shows that college athletes have higher rates of substance use, especially alcohol, than do college students who are not involved in athletics. To augment the literature, the author sought to determine which sports/teams are at the greatest risk for substance use. The author used data from the 1999 Harvard School of Public Health College Alcohol Study, a national survey of college and university students in the United States. Findings indicated that male hockey and female soccer athletes were the most likely to report substance use and that male basketball and cross-country/track athletes reported lower levels of substance use. There is variation in substance use on the basis of sport/team affiliation, and future researchers should examine why certain groups of athletes have higher rates of substance use [07037].

Correlation to selected socio-demographic, health-related, and sports-related predictors

Conducted researches recognize various risk factors, as well as protective factors against doping behaviour in different sports i.e. sports disciplines or activities. The main goal of one research was to identify the correlation between selected socio-demographic, health-related, and sports-related predictors with doping factors in three different types of sports, which are (1) highly energetic demanding sports (weightlifting), (2) highly technical demanding sports (racquet sports), and (3) highly tactical demanding sports (sailing). The research consisted of three separate studies, each one of them researching one of the sports. The sample of subjects included altogether 293 athletes, senior level competitors (older than 18 years of age). In total, the sample comprised three homogenous sub-samples, as follows: athletes in highly energetic demanding sports (weightlifters and power lifters; n=27), athletes in highly technical demanding sports (table tennis, tennis and badminton players; n=188), and athletes in highly tactical demanding sports (sailing; n=78). The first study involved weightlifters where it should be pointed out the existence of high doping behaviour In this study, religiousness was interpreted as the most significant protective factor against doping behaviour, while
sports factors are not found to be significantly related to doping. The study involving racquet
sport athletes suggests a high risk of doping behaviour among those athletes who observe
doping behaviour in their sport. It was noticed low levels of athletes' trust in their coaches'
and physicians' opinions on doping issues. This is an issue which should be researched in
the future, because the underlying cause has not been studied as yet. Briefly, it seems that
either the athletes are not convinced of their coaches'/physicians' expertise regarding doping
issues, and/or they do not believe in their good intentions. It is particularly important, as the
previous research has shown that with the increased trust in coaches and physicians, the
chance that an athlete will use doping decreases. As expected, it is characteristic for sailing
that it has a low likelihood of potential doping behaviour, although the consumption of dietary
supplements is high. Substance abuse in sports spreads beyond those that enhance athletic
performance. All of these issues should be studied in more detail in the future and, if
appropriately validated, incorporated into anti-doping intervention programs [13031].

Professional ballet dancers

One study investigated substance use and misuse among 16 female and 9 male Croatian
ballet professionals in 2008 using an original questionnaire. It was analyzed social, personal,
activity- and training-related, and educational factors, and criteria such as: binge alcohol
drinking, cigarette smoking, appetite suppressant consumption, analgesic use, and actual
and potential "doping" habits. Frequency tables and rank-order correlation were calculated.
More than one third of the male dancers reported binge drinking, while 20 percent of the
females smoked more than a box of cigarettes per day. Almost 25 percent of these dancers
would use "doping" if it will ensure successful ballet performance, regardless of negative
health consequences. In males, the risk of potential "doping" behavior increased with age. In
females, education level was negatively related to cigarette smoking, but positively correlated
to potential "doping" habits and behavior. In both genders, religiousness was the factor
negatively related to potential "doping" behavior and belief that "doping" exists in
professional ballet. Results suggest that there is evident need for more specific medical
and/or psychological services in professional ballet [10318].

Track and field

To document the use of prescribed medication and nutritional supplements in female and
male junior, youth, and adult track and field athletes depending on their sports discipline
analysis of 3,887 doping control forms undertaken during 12 International Association of
Athletics Federations World Championships and 1 out-of-competitions season in track and
field was performed. There were 6,523 nutritional supplements (1.7 per athlete) and 3,237
medications (0.8 per athlete) reported. Nonsteroidal anti-inflammatory drugs (NSAIDs; 0.27
per athlete, n=884), respiratory drugs (0.21 per athlete, n=682), and alternative analgesics
(0.13, n=423) were used most frequently. Medication use increased with age (0.33 to 0.87
per athlete) and decreased with increasing duration of the event (from sprints to endurance
events; 1.0 to 0.63 per athlete). African and Asian track and field athletes reported using
significantly fewer supplements (0.85 vs 1.93 per athlete) and medications (0.41 vs 0.96 per
athlete) than athletes from other continents. The final ranking in the championships was
unrelated to the quantity of reported medications or supplements taken. Compared with
middle-distance and long-distance runners, athletes in power and sprint disciplines reported
using more NSAIDs, creatine, and amino acids, and fewer antimicrobial agents. It was
concluded that the use of NSAIDs in track and field is less than that reported for team-sport
events. However, nutritional supplements are used more than twice as often as they are in
soccer and other multi-sport events; this inadvertently increases the risk of positive results of
doping tests [10018].
Football

Big sports events like the 2008 European Football Championship are a challenge for anti-doping activities, particularly when the sports event is hosted by two different countries and there are two laboratories accredited by the World Anti-Doping Agency. This challenges the logistics of sample collection as well as the chemical analyses, which must be carried out timeously. In the paper it was discussed the handling of whereabouts information for each athlete and the therapeutic use exemption system, experiences in sample collection and transportation of blood and urine samples, and the results of the chemical analysis in two different accredited laboratories. An overview of the analytical results of blood profiling and growth hormone testing in comparison with the distribution of the normal population was also presented [09033].

Tennis

The sport of tennis has had its own experiences regarding nutritional supplements and doping. Bohdan Ulihrach and Greg Rusedski are rare examples of athletes who tested positive in a doping case, but were exonerated because they might have used contaminated supplements. The complexity in these cases lies in the fact that the source of their positive tests might have been supplied by the testing authority itself: the Association of Tennis Professionals (ATP). The tribunals, special enquiries, and task forces that studied these cases all named the nutritional supplements provided by the ATP organisation as the most likely source of the nandrolone metabolite. However, many minerals and vitamins that were available on the ATP tour have actually been tested, and the true source has never been confirmed. Because there were more (anonymous) cases within the ATP that were all linked, as demonstrated by an analytical anomaly noticeable in the mass spectrogram of the urine analysis, all of these tennis players have been cleared. Such exoneration by an anti-doping tribunal is extremely rare [07025].

Table tennis

Substance use and misuse was studied in athletes competing at the Slovenian Nationals 2008-2009 (responding rate was 100 percent; 50 males and 29 females; aged 18 years or older). The anonymous questionnaire for studying the use and corresponding educational, socio-demographic, and sport factors was used. More than 90 percent of all the athletes included in our study do not rely on coaches’ and/or physicians’ opinion regarding nutritional supplements and doping. Chi-square test revealed higher prevalence of binge drinking, cigarette smoking, and potential doping behavior in males. In both genders, with the advancement of the sport status, the probability for potential doping behavior increases [10022].

Combined racket sports

Furthermore, it is apparent from annual lab statistics that the doping-testing programmes concentrate the analyses on Olympic rather than in Non-Olympic sports, and on sports which are already associated with doping (e.g. physically demanding sports). For example, in 2009, 26,593 urine and blood samples from track and field athletes yielded 398 total findings of suspicious substances. At the same time, 467 tests of curling athletes resulted in only 14 total findings. The higher absolute number of adverse or atypical analytical findings in “highly physically demanding” track and field athletics than in “less-demanding” curling seems unsurprising (398 vs 14). But, the surprising pattern is evident for aquatics (13,995 total
samples: 156 total findings, or 0.65 \% of samples) in comparison to shooting (24/2,630; 0.91 \% of samples) or archery (14/976; 1.44 \% of samples). Doping in Olympic racket sports was found to range from 0.17 to 0.94 percent in the following order: badminton: 2/1,175, tennis: 17/3,945 and table tennis: 10/1,066. Racket sport games are characterised by a handheld racket that is used to propel a missile between two (or four) players with the purpose of placing the missile in such a position that one player is unable to return it successfully. These sports are also characterised by an area of play that has a specified size, within which the missile must be contained, and by the presence of a net that the missile must pass above on each play. The unique sizes and shapes of the area of play, the height of the net and the type of missile and racket used give character to each variant of the game. Racket sports are unique due to the fact that players can modify the physiological demands of the game by controlling the rest intervals between rallies, games and sets. Average oxygen consumption for single-match duration badminton is reported to be 39.6 ± 5.7 ml/kg/min (73 \% VO_{2\text{max}}); oxygen consumption for table tennis is 26 ± 4 ml/kg/min (47 \% of VO_{2\text{max}}), and consumption for tennis is 29 ± 6 ml/kg/min (51 \% of VO_{2\text{max}}). Of course, average match duration must also be considered. In the 2006 badminton World Championship in Madrid, the average match duration was 33:35 minutes. At the Olympic Games in Beijing, the average table tennis match lasted for 27:31 minutes. The average duration of tennis matches depends on the type of court but typically ranges from 120 to 180 minutes. For example, at Wimbledon in 2005, the average duration of tennis matches was 137 minutes, whereas the average match in the Australian Open that same year lasted for 154 minutes. A major determinant of the outcome of a game is an individual's physical fitness, which can be influenced by SUM. Although SUM is regularly investigated in sports as a whole, SUM is rarely studied in racket sports. Apart from studies dealing with sports and physical activity in youth and related SUM issues in which racket sports (tennis mostly) were not studied systematically, there are only a few papers addressing SUM in Olympic racket sports. Briefly, Kondric it was reported on SUM habits in Slovenian table tennis players. Also, Maquirriain it was analysed offences to the Doping Code committed by tennis players between 2003 and 2009. When studying SUM problems in sports, previous investigations noted that SUM is gender-, sociodemographic-, culture-, and sport-specific and, therefore should be studied accordingly. Apart from the fact that SUM is rarely investigated among racket sports, we determined that these sports would be particularly suitable for our study based on several factors. First, table tennis, tennis and badminton are Olympic Sports that fall directly under WADA jurisdiction and anti-doping legislation. Second, all three sports share similar competitive characteristics as they are all individual sports with no physical contact between opponents. However, these sports are also diverse in terms of physiological demands [11557].

It was studied a total of 188 participants divided into three groups: table tennis players (n=78), badminton players (n=83), and tennis players (n=27). All players were 18 years of age or older and had participated in at least one of the two most recent competitions at the highest national level for their sport (e.g. Slovenian Nationals). The number of tennis players is almost half the size of the other two groups because mature tennis players (+18) are typically professionals and rarely compete in the Slovenian Nationals. SUM and its corresponding educational, sociodemographic, and sport-specific factors were investigated using a previously developed and validated questionnaire for studying SUM. Substance use and misuse data consisted of questions on binge drinking (7-point scale from "I do not drink alcohol" to "I binge a few times a week"), cigarette smoking (7-point scale from "not smoking" to "2+packs daily"), consumption of drugs and opiates (use of different drugs and opiates were inquired after but subjects reported only marihuana and hashish use). Doping factors were evaluated with questions concerning the athlete's opinions on doping practice in their sport (4-point scale from "I do not think doping is used" to "Doping is often"), potential doping habits (4-point scale from "I do not intend to use doping" to "I'll use it if assured it will help me"), and trust in their coach regarding doping and trust in their physician regarding doping
Nutritional supplements were reported separately and included the consumption of isotonics, proteins, carbohydrates, and recovery supplements. Additionally, we asked athletes to indicate who advised them to use NS with the coach, physician, friend, and self-decided as choices. Badminton players reported the highest level of binge drinking. Statistically significant differences were found in cigarette use, but this was mostly because of the high proportion of smoking cessation among badminton and tennis players. No significant differences were found for doping factors, although 1 in 10 badminton players said they would use doping if they were assured that it will improve their performance without any negative health consequences. Data revealed that most female athletes do not trust their own coaches regarding doping issues (mistrust in coaches ranges from 61% in badminton to 83% in tennis), whereas their trust in their physicians' opinions on the same issue was somewhat higher. Approximately 50% of females declared no use of nutritional supplementation. Female racket sport athletes reported using vitamins, minerals and isotonics almost exclusively. The reported use of other substances was very low among tennis players who were mostly advised by coaches or medical professional to consume nutritional supplements, whereas athletes in table tennis and badminton were not. In male athletes, there was no statistically significant difference between players of different racket sports in their perception of doping behaviours. One-third of the studied athletes thought that doping is used in their sport. Sixty to 90% of the male athletes reported that they do not trust coaches' or medics' opinions regarding doping issues and problems. A minority of athletes (10% in badminton, 15% in table tennis and 24% in tennis) indicated that they would use doping if assured that it would help them achieve competitive results without any negative health consequences. However, 5 to 10% of the studied male athletes declared that they might potentially dope regardless of the possible health hazard. Nutritional supplement use was mostly frequently reported by tennis players, followed by badminton and table tennis. More than half of the tennis and badminton players were formally advised by a coach or medical professional to use nutritional. Players in this sample reported varied levels of substance use with binge drinking and cannabinoids use reaching a concerning level with 40 and 30% binge drinking and 16 and 21% using cannabinoids, form males and females respectively. This level of use is comparable with the national statistics as detailed below. Tobacco use, in contrast, appears to be a male phenomenon. It was revealed an overall trend among racket sport athletes showing that the most significant overlap between self-reported use of these substances exist between binge drinking and opioid use, reaching 11 percent (males) and 7% percent (females). More precisely athletes who reported either binge drinking or opioid use were more likely to also use the other. Although these activities take place outside of the controlled sporting arena, the extent to which athletes reported these activities is concerning. Almost half of the athletes in the sample reported NS use. Interestingly, there was very little overlap between current NS use and willingness to use prohibited substances. The majority of those who indicated that they would be willing to use doping did not report current supplement use. Even more disturbing is the fact that more than 80% of the tennis and table tennis players and 60% of the badminton players report not trusting physicians' opinions on doping issues. Additionally, sports physicians are mostly focused on orthopaedic and locomotor injuries in sports and are rarely systematically educated regarding nutritional supplementation and doping. Consequently, athletes do not consider them to be reliable, leading to low levels of trust regarding the information they provide on doping issues. These problems must not be overlooked because those who trust physicians' and coaches' opinions on doping are less prone to doping behaviour in the future.

**Biking**

The human physiological system is stressed to its limits during endurance sports competition events. It was described a whole body computational model for energy conversion during...
bicycle racing. About 23 percent of the metabolic energy is used for muscle work, the rest is converted to heat. It was calculated heat transfer by conduction and blood flow inside the body, and heat transfer from the skin by radiation, convection and sweat evaporation, resulting in temperature changes in 25 body compartments. It was simulated a mountain time trial to Alpe d'Huez during the Tour de France. To approach the time realized by Lance Armstrong in 2004, very high oxygen uptake must be sustained by the simulated cyclist. Temperature was predicted to reach 39°C in the brain, and 39.7°C in leg muscle. In addition to the macroscopic simulation, it was analysed the buffering of bursts of high adenosine triphosphate hydrolysis by creatine kinase during cyclical muscle activity at the biochemical pathway level. To investigate the low oxygen to carbohydrate ratio for the brain, which takes up lactate during exercise, it was calculated the flux distribution in cerebral energy metabolism. Computational modelling of the human body, describing heat exchange and energy metabolism, makes simulation of endurance sports events feasible [11561].

Imagine a medicine that is expected to have very limited effects based upon knowledge of its pharmacology and (patho)physiology and that is studied in the wrong population, with low-quality studies that use a surrogate end-point that relates to the clinical end-point in a partial manner at most. Such a medicine would surely not be recommended. The use of recombinant human erythropoietin (rHuEPO) to enhance performance in cycling is very common. A qualitative systematic review of the available literature was performed to examine the evidence for the ergogenic properties of this drug, which is normally used to treat anaemia in chronic renal failure patients. The results of this literature search show that there is no scientific basis from which to conclude that rHuEPO has performance-enhancing properties in elite cyclists. The reported studies have many shortcomings regarding translation of the results to professional cycling endurance performance. Additionally, the possibly harmful side-effects have not been adequately researched for this population but appear to be worrying, at least. The use of rHuEPO in cycling is rife but scientifically unsupported by evidence, and its use in sports is medical malpractice. What its use would have been, if the involved team physicians had been trained in clinical pharmacology and had investigated this properly, remains a matter of speculation. A single well-controlled trial in athletes in real-life circumstances would give a better indication of the real advantages and risk factors of rHuEPO use, but it would be an oversimplification to suggest that this would eradicate its use [13063].

The 1990-2010 period in professional cycling is labeled by some as the epo epidemic. Surprisingly, performance enhancement by epo and blood doping is not that clear-cut for endurance athletes, leading to the question whether doping indeed strongly influenced cyclists' performances from the 1990s onwards. It was examined the records (1947-2008) of the Tour de France, Giro d'Italia, and Vuelta a España (n=181) and assessed the time it took riders to win the race. The findings revealed normally distributed performances and linear and quartic relationships in victors' performances over time that correspond with Brewer's (2002) sociohistorical analysis of professional cycling suggesting that effects of the epo epidemic on professional cyclists' achievements may be overestimated [11562].

Professional cycling has suffered from a number of doping scandals. The sport's governing bodies have responded by implementing an aggressive new antidoping program known as the biological passport. Cycling's biological passport marks a departure from traditional antidoping efforts, which have focused on directly detecting prohibited substances in a cyclist's system. Instead, the biological passport tracks biological variables in a cyclist's blood and urine over time, monitoring for fluctuations that are thought to indirectly reveal the effects of doping. Although this method of indirect detection is promising, it also raises serious legal and scientific concerns. Since its introduction, the cycling community has debated the reliability of indirect biological-passport evidence and the clarity, consistency, and
transparency of its use in proving doping violations. Such uncertainty undermines the legitimacy of finding cyclists guilty of doping based on this indirect evidence alone. Antidoping authorities should address these important concerns before continuing to pursue doping sanctions against cyclists solely on the basis of their biological passports [11563].

To understand why pharmacological enhancements should never be allowed in cycling, you need to understand that all spectator sports thrive by selling simple stories to their fans. The cycling story is that, with great talent and after years of training, the best riders ride faster than the others at the very limits of natural human endurance. In the Tour de France, this story has been told and retold for 100 years – over stages, tours and careers. It describes the overall winner, the best hill climber and even the failed solo breakaway. How could cycling’s story survive if pharmacological enhancements were allowed? Even if the time comes when botulinum toxin injections are available from vending machines, doping should never be allowed in cycling [07038].

Using a psychosociological approach, the purpose of one study was to identify and understand the use of doping substances by young elite cyclists. Semi-structured interviews were conducted with young cyclists who were hoping to find a professional team and cyclists who had recently become professional. All of the young cyclists interviewed took nutritional supplements and believed that they improved their performance, which has been shown by other scholars to be a risk factor for doping. These cyclists believed that doping at the professional level in cycling was acceptable but did not approve of it at the amateur level. They were attracted to doping; they were open to using doping substances themselves if it was the key to continuing their cycling career, but only after they became professional. Team staff, doctors, parents and friends helped to create a “clean” environment that prevented the young cyclists from doping before becoming professional. The more experienced cyclists, who doped or used to dope, transmitted the culture of doping to the young cyclists, teaching them doping methods and which substances to use. This study could help to improve prevention and help to detect doping, as it is clear that doping behaviors begin at the amateur level [10019].

After recent scandals in cycling involving doping, it has been asserted that top-level cycling is impossible without pharmacological support. An important prerequisite for successfully completing the Tour de France is maintaining energy balance. To compensate for the daily caloric expenditure of 23-25 MJ, conventional food must be supplemented with liquid food. Quick administration of liquid carbohydrates is essential for optimal recovery of glycogen stores in the liver and skeletal muscle. Androgenic anabolic steroids are a frequently used form of doping. In endurance sports, these drugs have not been shown to affect endurance performance, and there is little evidence to suggest that they enhance recovery. Although epoetin use can increase maximal oxygen uptake, its effects on maximal power output are less pronounced than what is generally assumed. A relationship between haemoglobin concentration and sport performance has not been proved. It has been found that growth hormone rather has a negative than a positive influence on the sport performance. The doping problem is due in part to superstition, hearsay and insufficient knowledge among the athlete's support personnel, which frequently leads to medical malpractice in sport. Education and quality control for sport professionals, including sports physicians, may help to control the doping problem [06029].

In 2006, a couple of professional cycling teams initiated their own testing programs. The objective of one study was to describe fluctuations in commonly measured blood parameters among top-level riders. From 2006 to 2007, a total of 374 blood samples and 287 urine samples were obtained from 28 elite, male cyclists. Blood was analyzed for hematocrit (Hct), hemoglobin concentration ([Hb]) and % reticulocytes. Seventy-six percent of all samples
were collected out-of-competition (OOC). From December 2006 to September 2007, the average Hct and [Hb] decreased by 4.3 percent point and 1.3 g/dL, respectively. After the end of the competitive season, the values increased back to baseline levels. During the Tour de France, the [Hb] decreased by 11.5 percent, with individual decreases ranging from 7.0 to 20.6 percent. Hct and [Hb] values were lower in-competition (40.9 % and 14.1 g/dL) compared to out-of-competition (43.2 % and 15.0 g/dL) and pre-competition (43.5 % and 14.9 g/dL). The results suggest that when interpreting blood sample results in an anti-doping context, the sample timing (OOC, pre- or in-competition) and time of year should be kept in mind [08033].

The protection of the health of athletes is one of the three criteria taken into account when registering a substance in the World Anti-Doping Agency prohibited list. Nevertheless, in elite-level cycling, banned substance use is widespread. The present research adopted a psychological approach to examine how or whether perceived health risks influence elite-level cyclists' decisions to use banned substances. Sixteen semi-structured interviews were conducted with cyclists hoping to join a professional team (n=6), neo-professional cyclists (n=2), and former professional cyclists (n=8). Although an evolution was observed in the organization of doping and perceptions of doping over the last decade, the perceived health hazards did not influence, most of the time, decisions to use banned substances among the sample of cyclists. There was a systematization of exogenous substance use in the cycling environment and a trivialization of the side effects of the banned substances. Finally, younger cyclists were not concerned about the long-term health consequences of banned substances; they were more focused on the short-term performance-enhancing benefits. There is a need to implement more effective preventive programs to change athletes' attitudes toward doping and its health risks [11015].

In 1998, the Festina scandal at the Tour de France provided the first proof of widespread doping in professional cycling. This doping scandal marked the end of team-organized doping in professional cycling and ushered in a new period marked by the increasing implementation of anti-doping measures. One article evaluates the impact of the anti-doping rules and tests instituted since the Festina scandal. It was adopted a psychosocial approach to analyze the organization of doping and the development of doping attitudes and practices in high-level cycling. Sixteen cyclists were interviewed, of which eight were young, current cyclists and eight were former cyclists who became professionals before the Festina scandal. The results show that although the fight against doping in the last decade has reduced doping use in high-level cycling, anti-doping measures have also had unexpected effects. The fight against doping in cycling is not over [13064].

**Indirect evidence of effects**

Since doping improves athletic performance, anti-doping policies should have the opposite effect. One analysis examined whether changes in the speed of major cycling races reflect recent anti-doping efforts. Average speeds of 5th place finishers of the Tour de France, Giro d'Italia, and Vuelta a España cycling races were obtained for the period 1990-2009. Between 1990 and 2004, the average speed had been significantly increasing by 0.16 km/h per year. In a downturn, since 2004, the average speed has significantly decreased by 0.22 km/h per year. The slowing down of professional cycling races is compatible with the hypothesis that recent anti-doping efforts in professional cycling have curbed the use of performance-enhancing substances [10020].

**Tour de France 2006**

The saga of the 2006 Tour de France is disappointing. Cycling fans have always thought that the greatest cycling competition in the world, next in terms of audience and media involvement to the Olympics and the football World Cup, was an endurance race in which
cycling performance and racing ability would have been the main requisites for wearing the yellow jersey in Paris. However, recent developments have profoundly altered this scenario, transforming the Tour into an elimination race. Just 24 h ahead of the prologue start in Strasbourg, nine riders from five teams were ruled out of the race, suspected of doping by an international probe based on blood transfusions involving more than 200 athletes from different sporting disciplines. This huge doping scandal, conventionally known as the “affair Fuentes”, began in an ordinary apartment in Madrid, where the Spanish police discovered the covert structure of an international performance-enhancement business; they seized more than 200 blood bags, along with doping records and several other doping substances. The investigators were able to match code names of athletes with their highly detailed doping records. In the prosecution, the American rider Floyd Landis, who wore the 2006 yellow jersey in Paris, tested positive for abnormal concentrations of testosterone after winning stage 17 and is now set to lose his title. If this were not enough, the runner-up Oscar Pereiro of Spain, who would probably have been awarded the yellow jersey, is under investigation for testing positive for the banned substance salbutamol! Metabolites of this drug, often prescribed for asthma, were discovered by the French antidoping body, AFLD, in Pereiro's urine sample after stages 14 and 16. Although the authority of the AFLD is limited to French soil only, Pereiro may not be allowed to take part in the 2007 Tour and could be stripped of second place in the 2006 race until he has been definitely cleared of this allegation. These disappointing events warrant further consideration. Firstly, the unexpectedly high number of cyclists still involved in doping cases clearly indicates that the strict control strategies adopted by sports federations and antidoping organisations will probably not modify this trend. It must be assumed that the athlete's desire to win, along with the vision of glory and money, will always overcome the risk of being found guilty. Different and multifaceted strategies are needed, which should be based on preventing rather than identifying or criminalising doping. Once the young athlete becomes familiar with unfair practices, it is difficult to modify this attitude. It is clear that controls, suspensions and civil and penal sanctions are not reliable deterrents [07030].

Floyd Landis

Another question is: are antidoping controls absolutely reliable? This is probably the foremost question that should be answered. In the specific case of the 2006 Tour de France, former winner Floyd Landis has denied ever taking performance-enhancing drugs. His key defence is the contention that the National Laboratory for Doping Detection in Paris made technical and analytical errors. In testosterone cases, the first level of testing is to measure the ratio of testosterone to epitestosterone (T/E ratio). A ratio higher than 4:1 triggers a further test based on the measurement of trace isotopes of carbon in the urine sample. The documents Landis has posted show that the laboratory measured the T/E ratio in his urine sample at least three times, producing three different results: 4.9:1, 5.1:1 and 11.4:1. A variety of additional reasons were offered for the failed test: one was that he drank whisky and beer on the night before the incriminating stage. Additional explanations were dehydration, anti-inflammatory injections for hip pain, and natural metabolism. The third crucial issue is the hullabaloo when an athlete tests positive. Doping cases are not supposed to be made public until they are resolved, but most become public through the media once a positive “A” test is confirmed. There is a tendency for there to be access to confidential information on previous tests of athletes before a definitive result, either positive or negative, is released by the competent sport authority and antidoping organisation. Enormous emphasis was given to the exclusion of the nine riders before the official start of the competition in Strasbourg. On some occasions, these athletes have been judged by the media in advance of the sport authorities and declared guilty on the basis of contradictory evidence, causing enormous human and financial problems and ultimately jeopardising their careers. None of these athletes has yet been charged. On the contrary, some have been completely cleared and are free to race. Landis criticised officials from the UCI and WADA for announcing the results of
his test without analysing the second sample, as normally takes place in the anti-doping procedure. He also claimed that the test was not conducted anonymously, saying he had evidence to prove that laboratory staff had access to the names of the cyclists whose samples were being tested. In the Pereiro case, it appears that the UCI had allowed him to use salbutamol under the therapeutic use exemptions scheme. However, AFLD claim that Pereiro's team did not complete the relevant medical forms. Regardless of the definitive conclusion of the cases, all these riders have been designated as doping athletes by the media, with potential detrimental economic and moral consequences. This is unacceptable, considering that a definitive test result may be unavailable for several months after the opening of the case [07030].

**Germany**

Doctors and politicians in Germany are demanding stricter laws for sports medicine after three doctors were discovered to have given performance enhancing drugs to professional cyclists. Two of the three doctors, from Freiburg University Hospital, were suspended in 2007 by the university when they admitted doping professional cyclists. In separate statements, Lothar Heinrich and Andreas Schmid said that they gave the blood cell stimulating hormone erythropoietin to the cycling team of the German telephone company Deutsche Telekom, now T-Mobile. The confessions were made after several cyclists had recently publicly admitted to taking drugs for performance and accused the doctors of involvement. The incident had spread to amateur ranks a few days later when another doctor from the Freiburg sports medicine department who had worked several times for German Olympic teams (not only cyclists) admitted giving performance boosting testosterone to riders as far back as 1980. The third doctor, Georg Huber, was suspended by both German cycling authorities and the University of Freiburg. Two former cyclists had triggered his resignation, naming him in a newspaper story and claiming that doping in amateur German cycling was widespread long before team Telekom. The German Sports Medicine Association decided that all its members who look after athletes, professional and lay, must sign a statement distancing themselves from any doping. In Germany 11,000 doctors are qualified in sports medicine [07039].

**Bodybuilding**

Bodybuilding is a sport in which competitors are judged on muscular appearance. One case study tracked a drug-free male bodybuilder (age 26-27 years) for the 6 months before and after a competition. The aim of one study was to provide the most comprehensive physiological profile of bodybuilding competition preparation and recovery ever compiled. Cardiovascular parameters, body composition, strength, aerobic capacity, critical power, mood state, resting energy expenditure, and hormonal and other blood parameters were evaluated. Heart rate decreased from 53 to 27 beats/min during preparation and increased to 46 beats/min within 1 month after competition. Brachial blood pressure dropped from 132/69 to 104/56 mmHg during preparation and returned to 116/64 mmHg at 6 months after competition. Percent body fat declined from 15 to 5 percent during preparation and returned to 15 months during recovery. Strength decreased during preparation and did not fully recover during 6 months of recovery. Testosterone declined from 9.22 to 2.27 ng/mL during preparation and returned back to the baseline level, 9.91 ng/mL, after competition. Total mood disturbance increased from 6 to 43 units during preparation and recovered to 4 units 6 mo after competition. The case study provides a thorough documentation of the physiological changes that occurred during natural bodybuilding competition and recovery [13062].

**Dancesport**
DanceSport is the competitive form of ballroom dancing, and even though it has more participants worldwide than ballet and modern dance, there is less peer-reviewed research. A review was conducted to identify all relevant literature to help researchers and clinicians gain an enhanced understanding of dancesport. Eight databases were searched, with 34 articles found in topics including participation motives, psychology, exercise physiology, fitness training, injuries and injury prevention, biomechanics, menstrual dysfunction, and substance use. The results indicate that researchers have been inconsistently recording and reporting anthropometric and dancesport data; for example, 31 studies separated participants by gender, 21 included the competition classification of dancers, 19 reported which style of dancesport participants competed in, and 13 described the participants as a dance couple. Common injuries affected the neck, shoulder, spine, knee, lower leg, and foot. Dancesport is in the very heavy to extremely heavy category in energy expenditure (mean heart rate: male 175.2 ± 10.7, female 178.6 ± 8.6 bpm) and utilizes both aerobic and anaerobic energy systems. Alpha-beta and heart rate variability intervention techniques are reported to successfully enhance performance in dancers. Dancesport participants also appear less likely to smoke cigarettes, but have little knowledge about anti-doping rules. During events, professionals danced farther (30 m) and faster (0.3 m/sec) than junior dancers. Female competitors were more likely to be eumenorrheic. Dancesport is a physically and mentally demanding competitive sport, but there is a need to standardize measurements in future studies to allow comparison [13065].

Recreational sports

Substance abuse has become increasingly widespread among athletes at sub competitive and recreational level, due in part to the lack of controls in form of doping tests. Hypertension and the many other side effects related to the illicit use of prescription drugs pose a substantial but often underestimated threat to public health. The symptoms are recognized late and are then mostly repressed or misjudged. Since the abuse is concealed to the doctor when help is finally sought, it results in extensive and expensive tests that can seldom lead to an effective treatment. Two case reports were presented to elaborate on this issue [10023].

Prevalence of use by fitness centre members

Studies on the use of performance enhancing drugs (PED) in fitness centres rely predominately on conventional survey methods using direct questioning. However, research indicates that direct questioning of sensitive information is characterized by under-reporting. The aim of one study was to contrast direct questioning of different types of PED use by Dutch fitness centre members with results obtained with the Randomized Response Technique (RRT). Questionnaires were conducted among members of fitness centres. PED were classified into the following categories: anabolic steroids, prohormones, substances to counteract side-effects, growth hormone and/or insulin, stimulants (to reduce weight), and miscellaneous substances. A total of 718 athletes from 92 fitness centres completed the questionnaire. The conventional method resulted in prevalences varying between 0 and 0.4 percent for the different types of PED with an overall prevalence of 0.4 percent. RRT resulted in prevalences varying between 0.8 and 4.8 percent for the different types of PED with an overall prevalence of 8.2 percent. The overall prevalence of the two survey methods differed significantly. The current study showed that the conventional survey method using direct questioning led to an underestimation of the prevalence. Based on the RRT results, the percentage of users of PED among members of fitness centres is approximately 8.2 percent. Stimulants to lose weight had the highest prevalence, even higher than anabolic steroids. The key task for future preventive health work is to not only focus on anabolic steroid use,
but also include interventions focusing on the use of stimulants to lose weight [13032].
THEORETICAL ASPECTS ON DOPING-TESTING

Overview

Testing results show that cheating athletes alter their drug abuse behaviors to avoid detection. For example, when a urinary test was developed for recombinant EPO, athletes changed doses and routes of administration to shorten the detection window. This is confirmed by information obtained from athlete blogs and government investigations. Thus, continued research into innovative test methods and strategies is necessary to deter drug abuse. Perhaps more importantly, the scientific contributions from antidoping research go beyond the field itself to affect other scientific disciplines, such as analytical chemistry, endocrinology, genetics, laboratory medicine, pharmacology, physiology, and sports medicine. More collaborative research with experts in these fields should enhance the rate of discovery of innovative approaches to solve doping problems. It is critical to recognize that new tests and methods must be fit for purpose. The tests and methods developed must make the transition into routine testing use. Thus, antidoping research is yet another field where translational research is a key component to applying new technology to solve problems [12006].

Doping in competitive sport is a peculiar phenomenon. The need for performance enhancement is emerging from the desire to maximise or even expand human capacities, and by doping to gain competitive edge in a situation where athletes’ performances are judged on two levels simultaneously: athletes compete against the opponents in situations where typically only one can win and are also automatically entered into a quest for breaking records which opens up the competitive arena including all from the past. From the array of substances with performance enhancing properties, a wide range represents fully acceptable means, whilst a defined set is prohibited by some authorities. In general terms, and for the purpose of this paper, the term “doping” refers to the latter category. From the system’s point of view, the current detection-based anti-doping policy does not automatically eradicate the use of prohibited substances, but rather presents a barrier with a quantifiable risk of being caught. It is easy to see that such a system leads to two primary strategies employed by the athletes [13016]:

- compliance, driven by respect for the rules, desire to compete clean or fear of being caught
- circumvention, i.e. outwitting the system by using not-yet-known or undetectable substances, masking or simply betting on chances of not being selected for testing

In order to design effective anti-doping measures, gaining insight into the driving forces behind doping behaviour is vital. Whilst the doping decision is very complex involving moral, economical and health considerations, theoretically this complexity can be distilled into a simple decision situation where pros and cons are weighed against each other in the context of unknown but assumed choices of the opponents. Based on the assumption that eradicating doping from sport requires a significant change in this decisional balance; by formulating and formally solving multi-player doping games, we aim to make a contribution to developing a better understanding of doping decisions [13016].

Theories on laboratory testing

A well-planned testing strategy for anti-doping organizations is a key element in order to
obtain representative samples and a correct biomatrix for efficient doping control, but the contribution of anti-doping laboratories is also fundamental to the overall success. Analytical methods may be needed to reveal potential masking attempts and fraud which could take place outside the sample collection session (e.g. haemodilution) to complement carefully supervised sample collection procedures. As pre-analytical processes, laboratories verify the authorized origin, evaluate the transport time and conditions, and also verify the identity and integrity of the biological samples at the time of receipt. In the actual analytical processes, fit-for-purpose methods and instrumentation are used for the detection of prohibited substances which are specified by WADA. The majority of the prohibited substances are exogenous, i.e. they are not naturally present in the body. In these cases, qualitative identification of the substance and/or its metabolite is sufficient for establishing an anti-doping rule violation. In the prohibited list, however, there are substances such as testosterone, erythropoietin (EPO), growth hormone (GH), and insulin-like growth factor 1 (IGF-1), which are available as pharmaceutical products for clinical purposes, but which are also produced endogenously. In these cases the analytical methods and result interpretation should be capable of discriminating between exogenous source and clinical or pathological conditions. Implementation of gas chromatography-combustion-isotope ration mass spectrometry (GC-C-IRMS) has enabled the analysis of endogenous anabolic steroids and their misuse, whereas methods based on electrophoretic techniques (IEF-PAGE and SDS-PAGE) and immunoassays have provided tools to detect large biomolecules, the concentration of which is still often below the sensitivity limits of mass spectrometric approaches. Research projects within these special topics require active and devoted scientists, and also provide significant improvements for routine analysis in the form of enhanced detection times and by facilitating the result evaluation [13078].

In general terms, technical improvements, for example enhanced chromatographic and mass spectrometric resolution, higher sensitivity, scan-to-scan polarity switching, and more powerful data analysis allow for faster, more sensitive, and more reliable screening strategies. According to the prevailing International Standard for Laboratories (ISL), the doping control samples can be re-analyzed within a time period of up to eight years following the reporting of the original result. This is particularly interesting when new instrument technologies are introduced in the analysis routine for the purpose of monitoring new drug-derivatives or new chemical entities emerging onto market, and also when new long-term excreted metabolites are reported for already known prohibited substances. Although the flexibility of the ISL increases the risk of an adverse analytical finding, it also requires adequate sample storage condition and knowledge on the long-term effects on various analytes. The list of prohibited substances is updated annually; active research and up-to-date analytical processes are mandatory in order to reveal the high-risk emerging compounds and to target the appropriate sample matrix. The laboratories’ assignment is to search for and to adapt the applicable parts of modern methodologies to the anti-doping context. In the success of these research projects and with efficient implementation of new techniques, international and interdisciplinary co-operation plays a major role. Similar to any branch of applied sciences, in doping control the science is not for science’s sake only. With respect to routine analysis and service to the customers, these improvements should be also practical, prompt, flexible, and cost-effective [13078].

Homo economicus: pay-offs and sanctions

Lay explanation for doping rests on the assumption that for those who engage in doping practices “winning is everything”, hence they use prohibited methods to ensure this outcome. This approach assumes that the choice is purely rational-economic and responds to externally imposed incentives and deterrents. As long as the perceived advantages from using doping constitute a far better scenario than any scenario with no doping, factoring in
the risk of being detected and its consequences, the only logical action is to dope. Contrary to the detection-sanction based deterrence methods, economical models recognise the importance of the prize structure, considering both benefits and costs. Prize structures can also be manipulated so the monitoring cost is kept low. These models suggest that eradicating doping would require changes in the external factors, such as increased dis-utility (including the chance of being detected and its consequences), decreased utility (reduced pay-off) or some combination of the two. In reality, it is unlikely that such change will be effectively implemented. Based on a review of economical models of doping, posit that rank order contests such as sport competitions with highly skewed prize structures inevitably lead to undesirable practices (i.e. doping) players may employ in order to enhance their chances to finish in positions with high pay-off. A follow-up study with empirical data from thirteen different athletic events reinforced the assumption that increase in competition increases doping, and consequently lead to equitable sanctions. The nature of doping makes policing difficult and leads to an imperfect but costly monitoring system, where externally imposed sanctions may inadvertently motivate doping use by indicating that doping is widespread, hence the need for harsh sanctions [13016].

**Aim of anti-doping**

The aims of the World Anti-Doping Programme and the Code are to care for the athlete’s fundamental right to participate in doping-free sport and thus promote health, fairness and equality for athletes worldwide, and to guarantee harmonized, coordinated and effective anti-doping programmes at the international and national level relating to the detection, deterrence and prevention of doping [13017].

**The Goldman dilemma**

Discussions of doping often report Goldman’s sensational results that half of the elite athletes asked would take a drug that guaranteed sporting success which would also result in their death in 5 years’ time. There has never been any effort to assess the properties of the “Goldman dilemma” or replicate the results in the post World Anti-Doping Agency context. This research evaluated the dilemma with contemporary elite athletes. Participants at an elite-level track and field meet in North America were segregated into an interview or online response. After basic demographics, participants were presented with three variant “Goldman dilemmas” counter-balanced for presentation order. Only 2 out of 212 samples (119 men, 93 women, mean age 20.89) reported that they would take the Faustian bargain offered by the original Goldman dilemma. However, if there were no consequences to the (illegal) drug use, then 25/212 indicated that they would take the substance (no death condition). Legality also changes the acceptance rate to 13/212 even with death as a consequence. Regression modelling showed that no other variable was significant (gender, competitive level, type of sport) and there was no statistical difference between the interview and online collection method. It was concluded that Goldman’s results did not match this sample. A subset of athletes is willing to dope and another subset is willing to sacrifice their life to achieve success, although to a much lesser degree than that observed by Goldman. A larger scale online survey is now viable to answer important questions such as variation across sports [13018].

**Forensic intelligence in anti-doping**
Today's approach to anti-doping is mostly centered on the judicial process, despite pursuing a further goal in the detection, reduction, solving and/or prevention of doping. Similarly to decision-making in the area of law enforcement feeding on Forensic Intelligence, anti-doping might significantly benefit from a more extensive gathering of knowledge. Forensic Intelligence might bring a broader logical dimension to the interpretation of data on doping activities for a more future-oriented and comprehensive approach instead of the traditional case-based and reactive process. Information coming from a variety of sources related to doping, whether directly or potentially, would feed an organized memory to provide real time intelligence on the size, seriousness and evolution of the phenomenon. Due to the complexity of doping, integrating analytical chemical results and longitudinal monitoring of biomarkers with physiological, epidemiological, sociological or circumstantial information might provide a logical framework enabling fit for purpose decision-making. Therefore, Anti-Doping Intelligence might prove efficient at providing a more proactive response to any potential or emerging doping phenomenon or to address existing problems with innovative actions or/and policies. This approach might prove useful to detect, neutralize, disrupt and/or prevent organized doping or the trafficking of doping agents, as well as helping to refine the targeting of athletes or teams. In addition, such an intelligence-led methodology would serve to address doping offenses in the absence of adverse analytical chemical evidence [13079].

Colored illicite tablets

The necessity of specific, confirmatory tests in the identification of seized illicit products was highlighted by the analysis of eighteen heart shaped, blue tablets confiscated by Police at a street control in the North East of Italy. The tablets responded as amphetamines to a preliminary color test (Marquis); a subsequent, confirmatory assay by gas chromatography-mass spectrometry revealed the presence of two anabolic androgen steroids (AAS), methandienone and methyltestosterone, in concentration of 1.7 and 1.5 mg respectively per tablet; no trace of amphetamine-like or nitrogen containing compounds was found. The observed orange coloration was due to the reaction of concentrated sulphuric acid, contained in the Marquis reagent, with the delta(4) C-3 keto group of steroids. The two AAS, banned under the world antidoping code, are not considered as psychoactive drugs of abuse in most countries, although their trafficking may entangle severe public health concerns [13083].

Theories on doping in sports

Human behavior occurs within a system, and as such, so do behaviors in performance-related domains (e.g. athletics, academics). Doping is a performance enhancement behavior that can be problematic because of the negative physical and psychological effects associated with the use of some substances and the common argument that doping is unfair. However, doping continues and may be increasing. Because a firm theoretical or empirical understanding of doping does not exist, one article proposed a conceptual, comprehensive, and innovative systemic model of doping behavior. The model is built from relevant empiricism supporting the idea that contemporary doping behavior is a function of systemic transactions between historical doping practices, the present environment, current antidoping interventions, one's genetic makeup, developmental milestones, social factors, and epigenetics [11424].

The fight against doping is a challenging task. Owing to the complexity of the doping phenomenon, simultaneous consideration of physiological, medical, pharmacological, psychological, ethical and systemic factors is required in order to be successful in this endeavor. The need for effective deterrence policy is underscored by the fact that the
The problem of performance enhancements has spread beyond the elite athlete population. It is well documented that groups other than competitive athletes are at risk of using doping agents, especially steroids. Furthermore, medical enhancement of non-sport performance (i.e., quality of life, appearance) appears to be widely acceptable among non-athlete population. For effective deterrence methods, individual, systemic and situational factors that make an athlete or athlete group more susceptible to doping than others should be fully investigated. Traditional behavioral models assume that the behavior in question is the ultimate end. However, growing evidence suggests that in doping situations, the doping behavior is not the end but a means to an end, which is gaining competitive advantage. Therefore, models of doping should include and anti-doping policies should consider attitudes or orientations toward the specific target end, in addition to the attitude toward the tool itself. Data were collected by questionnaires containing a battery of psychological tests among competitive US male college athletes (n=199). Of the 199 athletes, 15 (8%) reported having personal experience with doping and an additional 9 (5%) claimed to have used substances classified as doping for medical reasons. The same figures for current use of performance enhancing substances were lower: 5 (3%) and 1 (1%), respectively. Outcome measures included sport orientation (win and goal orientation and competitiveness), doping attitude, beliefs and self-reported past or current use of doping. A structural equation model was developed based on the strength of relationships between these outcome measures. Whilst the doping model showed satisfactory fit, the results suggested that athletes’ win and goal orientation and competitiveness do not play a statistically significant role in doping behavior, but win orientation has an effect on doping attitude. The structural equation modeling (SEM) analysis provided empirical evidence that sport orientation and doping behavior is not directly related. It was concluded that the considerable proportion of doping behavior unexplained by the model suggests that other factors play an influential role in athletes' decisions regarding prohibited methods. Future research, followed by policy development, should incorporate these factors to capture the complexity of the doping phenomenon and to identify points for effective anti-doping interventions. Sport governing bodies and anti-doping organisations need to recognize that using performance enhancements may be more of a rational, outcome optimizing behavior than deviance and consider offering acceptable alternative performance-enhancing methods to doping.

Despite the increased anti-doping effort, the relative number of adverse analytical findings has not decreased considerably in the past four years (written 2007). The appropriateness of education as a deterrent is questionable as it has been shown that doping specific knowledge is higher among doping users than among their non-user counterparts. While prevention, complemented with detection, will be likely to be the main approach to the doping problem, the ultimate goal for sport governing bodies should be creating policies for a truly effective deterrence. Setting detection aside, there is still a fundamental distinction between prevention and deterrence. It is suggested that prevention (and detection) create an environment where the chances of detection and punishment for using doping are uncomfortably high, hence keep athletes away from employing such means, regardless of their motives. On the other hand, value-based deterrence in its true, perhaps Utopian sense, is associated with the creation of an environment where athletes never feel motivated to use illegal means for performance enhancement.

### Doping Detection

Historically, the anti-doping movement has been based on detection and prevention, with the initial emphasis on detection. Organisational structures and standard operating procedures have been in place to ensure compliance with the anti-doping regulations. Detection relies on testing, which has been increasingly problematic in high performance sport. It has been argued persuasively that making testing effective as a deterrence method, either the volume
of tests conducted or the sanctions imposed have to be increased significantly, potentially to the level that is practically not feasible. The new technologies in both the development of undetectable methods and the detection of the new methods have led to rapidly escalating costs, bearing in mind that tests are currently not even available for all banned substances and methods. If the trend continues, costs of effective testing will soon became a prohibiting factor. Athletes, as they progress in their sports career, are gradually drawn into the vicious circle of the constant desire to enhance performance. In this process, some athletes may become more susceptible to doping than others, depending on the combination of their personality and the situation. Therefore, both the individual and systemic factors contributing to doping behavior should be fully investigated in order to underpin effective, targeted anti-doping intervention. In support of the argument against detection from a psychological perspective, it was provided empirical evidence for the failure of detection based deterrence showing that in a hypothetical situation, athletes first consider their moral beliefs, followed by the fear of negative health consequences and legal sanctions associated with the use performance-enhancing drugs. The effect of the threat of legal sanctions practically diminished when moral beliefs and health concerns were added to the behavioral model, directing policy makers to alternative deterrence methods. Additionally, many speculate that with gene doping on the horizon of competitive sport, detection based regulation will soon be seriously undermined [07004].

Doping prevention

The WADA and national sport governing bodies have added preventive measures to their detection programs. Examples for anti-doping prevention include: WADA’s Athlete Outreach Program (launched in 2001) targeting top performing athletes at major sporting events, the Anti-Doping Development Program (started in 2004), which aims to help countries and organizations to set up quality doping control, and the Educational Programme, which is a major tool of the WADA in an attempt to create a doping free culture by providing education to all stakeholders about the dangers of doping and its consequences. Congruently, the 100 percent me programme of UK Sport aims to promote positive attitudes and values of those who successfully competed drug-free and to provide accurate and relevant information on anti-doping. The 100 percent me is an educational program with three distinct but related strands. Outreach programme provides a framework for delivering accurate information and giving advice on anti-doping issues to athletes, athlete support personnel, and parents across the UK via sports events, workshops, training sessions and conferences. The accreditation programme allows interested individuals to gain knowledge in anti-doping and became a “100 % me” tutor. The 100 percent me is also a brand promoting the image of the “clean athlete” based on values of personal responsibility, choices, fairness and honesty. This image is linked to the Ambassador programme where successful drug-free athletes committed to anti-doping use the 100 percent me platform to promote drug-free sport among their fellow athlete. The Education Model Guidelines (EMG) are in place to help National Governing Bodies (NGBs) develop their own programmes using the 100 percent me framework. The UK model is one of the existing anti-doping national programmes. In the US, the U.S. Anti-Doping Agency (USADA) is responsible for similar testing and education programmes, and in place to eliminate conflict of interest of NGBs testing and sanctioning their own athletes. The Australian Sports Anti-Doping Authority (ASADA) has also launched a comprehensive the ASADA Education Service Charter in 2007. The Charter places an emphasis on developing athletes’ and support personnel’s understanding of the physical and psychological risks of doping to ensure that athletes and support personnel are aware of their rights and responsibilities [07004].

Explaining the doping behavior
Whether it is a realistic goal or not, effective deterrence is hindered as long as doping behavior is poorly understood. Before any serious consideration is given to deterrence methods, factors that make an athlete or athlete group more inclined to doping than others must be fully investigated. The WADA has only just started to channel funds to social science doping research to develop better understanding and consequently, more effective deterrence programs. Aiming to add to the body of knowledge on one possible cause of doping behavior (i.e. individual dispositions and attitudes) is congruent with the current priorities of the WADA Social Science Research Programme [07004].

Both the eminent literature and the official global sport organisational stance suggest that athletes’ attitudes are responsible for the deviant behavior of doping. Being overly competitive or exceedingly win-orientated is often used as a lay explanation for doping. Although gender, cultural and competitive level differences among athletes have been scrutinized since the late '80s the relationship between these factors and doping behavior has not been empirically tested, except in one project. In one study the classic “theory of planned behavior” (TPB) model provided a theoretical framework for a study among Italian adolescents, where attitude was found to be the strongest predictor for behavioral intention. The TPB model held across different levels of sport involvement and gender. Alternative theoretical models of doping have been developed attempting to explain the complex nature of doping. The models are based on existing general models from either health science or criminology but their application to the doping situation has not been empirically validated. The first among the few used the “Health Belief Model” to develop a theoretical drug control model. Although it was not explicitly stated, the model also incorporates some kind of economic rationality when it considers the balance between deterrence and incentives and availability and affordability of performance enhancing substances. According to the model, athletes' doping behavior is the ultimate function of this cost/benefit ratio, personality and morality, legitimacy of sanctioning organisation, social context (reference group) and attitude toward doping. The “Drugs in Sport Deterrence Model” also considered costs and benefits but used these concepts in a broader sense. Their model is based on “Deterrence Theory” used in criminology and costs and benefits include material and social consequences, as well as individual effects, such as health concerns, guilt or even satisfaction from sport achievement. Situational factors (i.e. prevalence perception, professional status, type of drug, experience with testing, etc.) were also thought to have an effect on the final decision regarding doping use. The common element of all three models is that subjective norms play a seemingly important role in doping behavior. As it is evidenced in a recent, WADA Social Science research funded extensive literature review, published research into athletes' motivation and reasons for doping use reveal an important factor that has been prominent in game theory models but overlooked in the existing doping behavior models: that doping behavior is not the ultimate end but rather a means to an end. It can be argued whether the ultimate end is winning or achieving a specific sport related goal (i.e. breaking a record); and it may vary from athlete to athlete. Nevertheless, if doping is a tool to achieve an end-goal, then models of doping should include attitudes or orientations toward the specific target end,
In consideration of the structural model of doping, the existing literature, more specifically the Theory of Reasoned Action (TRA), the Theory of Planned Behaviour (TPB), previous structural equation models of attitude and behavior and previous doping models were consulted. The Theory of Reasoned Action established a linear sequence of cognition (beliefs), affects (attitude), conation (behavioural intention) and behaviour. Later, the model has been criticized for the underlying and unrealistic assumption of absolute behavioural control, hence perceived behavioural control was added and the model expanded into the TPB. Models have been empirically tested and refined by showing interaction between the predictors and by questioning the generality of the model. An earlier model suggests an important notion, namely multiple factors influencing the behaviour. The notion of multiple factors is, of course, not new. In 1977, it was already mentioned multiple-act criterion, where attitudes toward a target (i.e. doping in general) is linked with observed heterogeneous behaviors (i.e. supporting the anti-doping movement but using doping at the same time). Influencing factors can be learned experiences (past behavior), perceived control, personality, cost/benefit ratio and most importantly: goals. Also, it was focused on past behavior (in general) whereas new doping models consider personality, availability, free choice of actions and situational factors as well as perceived control over behavior and free choice. Curiously, a situation where multiple attitudes are influencing a single behavior has not been considered in doping attitude-behavior modeling. If doping behavior is considered as a means to an end, attitude toward the end point should be taken into account. Support for this assumption can be found in the literature for at least two decades.

Others have suggested that doping may be used to achieve one or more of many goals, including reaching unattainable goals, breaking off the plateau, or even to signal group membership; or mark transition from being recreational to professional athlete. Contrary to the argument that the crucial element of doping is the intent to gain unfair advantage at the expense of other competitors, athletes do not necessarily see using doping as unfair or advantageous. Doping may be employed as a useful tool to improve performance to the level that is, or perceived to be necessary to have a reasonable chance for winning. When athletes assume that their competitors follow the same logic, the motivation for doping use is often reduced to the desire to level the playing field and ensure equal chances. These rational decisions regarding doping behavior could easily be against the general attitude toward doping, which is suppressed by other, stronger driving forces such as the desire to win, goal orientation or competitiveness.

One of the most important features of the present study was the finding that sport orientation is not strongly related to doping behavior, or to doping attitude. The only exception was win orientation, which showed a significant relationship with doping attitude. Thus the importance of winning may have influenced what athletes think about doping, but it does not necessarily manifest in their behavior. From the path coefficients, it was clear that athletes' desire to win, to achieve their personal goals or their competitive nature is not necessarily related to their decision regarding use of prohibited performance enhancements. None of the measures, except expressed belief, had a significant path to behavior. Apparently, athletes using prohibited means of performance enhancements do not have to be overly competitive or win-orientated. They do not have to endorse such pharmaceutical agents, or agree with the use of such substances in order to actually use them. Many athletes claimed that they would prefer not to use drugs and would not do it if they were certain that the competition was drug-free. The paranoia about other competitors using performance enhancement is a reappearing theme in these papers. In addition, it has been noted that athletes often feel an external pressure to win, most often in the form of warning about exceptionally good opponents. Hence, using doping agents may be more of a rational, outcome optimizing
behavior than deviance. If this is the case, sport governing bodies may do well if in addition to placing a ban on certain performance enhancing substances and methods, they provide athletes with acceptable alternatives. The small negative (but not significant) relationship between goal orientation and doping behavior was a logical connection because among the three sport orientation measures, goal orientation reflects an orientation to personal standards, regardless of the situation. The other two measures, desire to win and competitiveness reflect a tendency to enter and strive for success in a sport situation. Using banned performance enhancements in most athletes' view was expected to be against their standards as sportsmen. However, at the same time doping is often viewed as a means to an end; a 'tool' that is bad but necessary to ensure success in competition. Therefore, a positive relationship was expected. Of the two measures studied, competitiveness had a small but insignificant, positive path to doping behavior, whilst winning practically showed no relationship at all. On the other hand, the only statistically significant relationship with sport orientation measures and other factors was between win orientation and doping attitude. Sport orientation and attitude appear to be similar constructs and distinctly different from behavior. Athletes may think that doping is needed or not needed for winning but when it comes to actual behavior, it might be influenced by other factors more than attitude or orientation. This is not to say that personality, attitude, values should be discarded in order to make room for other factors. As probably no two individuals would react identically to the same combination of environmental factors, it is fair to assume that contextual contingencies are mediated through the combination of individual factors. Adherence to norms is a particularly difficult question. Decisions regarding doping use are influenced by at least two possibly competing norms:

- the general social norms, such as fair play, condemnation of cheating
- the special norms held by the athletes' immediate subcultures

One possible explanation for the strong path between belief and behavior is justification. Those athletes who use doping or wish to use such performance enhancements would prefer to do so without social stigmatization. Such a view is in keeping with previous research where athletes expressed their view of doping as a necessary means to a desired end and whilst they acknowledge rule breaking behavior, they do not consider themselves cheaters or more cheating than any other [07004].

The results of this research have both theoretical and practical implications. At the theoretical level, the findings of the paper are a step toward a comprehensive doping model. It highlights the need for the inclusion of other influencing factors and makes suggestions for future model testing. At the practical level, understanding the driving forces behind doping and how athletes wish to deal with these factors must be at the center of informed deterrence policies. Athletes are, by nature, highly motivated and achievement oriented individuals and have grown to appreciate methods for performance enhancement (training, nutrition, physiotherapy, equipment, etc.). The distinction between acceptable and prohibited methods must be made clear and convincing. To be effective, authorities must be able to

- justify the doping ban in general
- use evidence-based selection of substances and methods included into the prohibited list
- use the same criteria for all substances and methods
- communicate such decisions to all stakeholders

Suggesting anti-doping education and perhaps changes in attitudes to doping is a rather futile approach if the other influencing factors are kept constant. A value-based deterrence requires changes at all levels and in all stakeholders. Large scale research aiming to
understanding the driving forces behind doping behavior and gaining knowledge of effective deterrent factors is much needed and should be extended beyond the athlete population to include coaches, managers and officials. Sport governing bodies and anti-doping organisations are in the unique position to endorse and foster such research. International and national anti-doping organisations should make targeted funding opportunities for doping-related research aiming at increasing knowledge regarding both the doping behavior and alternative acceptable means of performance enhancement. Constant improvement of performance is, after all, the core characteristic of competitive sport [07004].

Multiple drug use

Because the use of anabolic steroids remains illicit, detailed knowledge of their side effects is unknown. Reported side effects emanate from case reports, retrospective studies, and comparisons drawn from prospective studies of steroid therapy in patients with a variety of wasting syndromes. Further confusing the specific side effects of a given anabolic steroid is the common practice that many athletes take multiple drugs simultaneously and in multiple administration routes. Additionally, physiologic differences between patients with wasting syndromes and athletes limit our ability to extrapolate the results of controlled studies to anabolic steroid use in sports. One other compounding factor in determining which side effects arise from anabolic steroid use is that users also ingest other illicit drugs including cocaine, marijuana, alcohol, and tobacco. Doctors and training staff must be aware of the potential harms in order to both recognize and more importantly educate patients about these possibly life-threatening adverse effects [06031].

Athletes often alternate or combine multiple forms of AAS in an effort to maximize benefits while minimizing side effects. Users often take an average of five different drugs to achieve supraphysiologic anabolic levels exceeding 40 to 100 times the normal physiologic hormonal effects. This use of a combination of products is commonly referred to as stacking, and it is this stacking that makes it difficult to pinpoint the direct effect of different agents. The effects of AAS tend to follow a linear doseresponse curve for both anabolic and androgenic actions, although at high doses there appears to be a plateau to their physiologic effects. The supraphysiologic doses used by athletes far exceed the saturation point of the androgenic receptors. Therefore, there must be additional mechanisms to explain why supraphysiologic doses of steroids seem to enhance strength. There are three different physiologic mechanisms through which AAS may exert their effects [06031]:

- First, AAS improve the body's utilization of ingested protein, which favorably alters nitrogen balance. They mainly stimulate protein synthesis by turning on gene transcription after binding to androgenic receptors at the cellular level. Androgens exert their effects on a number of varied tissues including bone, adipose tissue, skeletal muscle, brain, prostate, liver, and kidney, as well as reproductive tissues. Therefore, a more complex understanding at the cellular level is needed to explain the variety of effects, knowing that their actions are mediated by only one androgen receptor. These receptors appear to be identical in muscle and various other organs, but their absolute number and affinity for various types of AAS potentially explain the variety of different effects in multiple organ systems and from various AAS products. Some effects of testosterone are mediated through conversion to other bioactive compounds including dihydrotestosterone and estradiol.
- A second effect of steroids is to displace glucocorticoids from binding to their receptors, thus exerting an anticatabolic effect. Because glucocorticoids usually depress protein synthesis, this antagonistic effect explains muscle mass gain and also the utility of these drugs in wasting syndromes.
- A third postulated effect of steroids is that they confer a psychologic benefit to athletes wishing to gain strength. AAS users describe a euphoria or high following ingestion that allows them to work harder during workouts and recover more rapidly, which allows them both to intensify their training and become more aggressive in competition.

Co-operation in drug testing

There are many areas of common interest between anti-doping laboratories and those working in the clinical, legal and forensic fields. In addition to methodological similarities, there are aspects of the findings in sport drug testing that overlap with other fields in such a way that sport drug testing and clinical, legal or forensic work may benefit from mutual interaction. Three recent examples are presented from the author’s experience. Case report 1 concerns the clinical relevance of hCG findings in sport drug testing as potential indicators of the presence of a (testicular) tumour in athletes. Case report 2 refers to difficulties that accredited laboratories can encounter due to differences between national legal systems and the administrative regulation systems of sport authorities. The example involves a network of blood collection for further autologous transfusion. Case report 3 relates to additional forensic-type investigations needed to interpret a situation where intoxication of a whole delegation was responsible for apparent doping cases. Clinical, legal and forensic fields must recognize the added value that some results and developments coming from anti-doping laboratories may have. At the same time anti-doping analysts should be aware of new issues, methodologies and problems appearing in related fields [10438].

Methodology for investigation of doping in the society

To date, there are estimates for the percentage of unknown cases of doping and illicit drug use in fitness sports, but not for elite sports. This can be attributed to the problem of implementing questionnaires and surveys to get reliable epidemiological estimates of deviant or illicit behaviour. All athletes questioned were subject to doping controls as members or junior members of the national teams. In order to estimate the prevalence of doping and illicit drug abuse, the athletes were either issued an anonymous standardized questionnaire (SQ; n=1394) or were interviewed using randomized response technique (RRT; n=480). It was used a two-sided z-test to compare the standardized questionnaire and RRT results with the respective official German NADA data on the prevalence of doping. Official doping tests only reveal 0.81 percent (n=25,437; 95 % confidence interval 0.70 to 0.92 %) of positive test results, while according to RRT 6.8 percent (n=480; 95 % confidence interval 2.7 to 10.9 %) of our athletes confessed to having practiced doping. Standardized questionnaire and randomized response technique both revealed a prevalence of about 7 percent for illicit drug use, but SQ failed to indicate a realistic prevalence of doping (0.20 %; 95 % confidence interval 0.02-0.74 %). It was thus demonstrate for the first time that data from official doping tests underestimate the true prevalence of doping in elite sports by more than a factor of eight. The results indicate that implementing randomized response technique before and after anti-doping measures could be a promising method for evaluating the effectiveness of anti-doping programs [09017].

Understanding athletes’ attitudes and behavioural intentions towards performance enhancement is critical to informing anti-doping intervention strategies. Capturing the complexity of these attitudes beyond verbal declarations requires indirect methods. One pilot study was aimed at developing and validating a method to assess implicit doping attitudes.
using an Implicit Associations Test (IAT) approach. The conventional IAT evaluation task (categorising "good" and "bad" words) was combined with a novel "doping" versus "nutrition supplements" category pair to create a performance-enhancement related IAT protocol (PE-IAT). The difference between average response times to "good-doping" and "bad-doping" combinations represents an estimate of implicit attitude towards doping in relation to nutritional supplements. 111 sports and exercise science undergraduates completed the PE-IAT, the Performance Enhancement Attitude Scale (PEAS) and answered questions regarding their beliefs about doping. Longer response times were observed in the mixed category discrimination trials where categories "good" and "doping" shared the same response key (compared to "bad-doping" combination on the same key) indicating a less favourable evaluation of doping substances. The PE-IAT measure did not correlate significantly with the declared doping attitudes, indicating a predictable partial dissociation. Action-oriented self-report expressed stronger associations with PE-IAT; participants who declared they would consider using doping showed significantly less implicit negativity towards banned substances. Similarly, those who reported more lenient explicit attitudes towards doping or expressly supported legalizing it, showed less implicit negativity towards doping in the sample, although neither observed differences reached statistical significance. Known-group validation strategy yielded mixed results: while competitive sport participants scored significantly lower than non-competitive ones on the PEAS, the two groups did not differ on PE-IAT. It was concluded that the results suggest a potential of the PE-IAT method to capture undeclared attitudes to doping and predict behaviour, which can support targeted anti-doping intervention and related research [08019].

To date, there are estimates for the percentage of unknown cases of doping and illicit drug use in fitness sports, but not for elite sports. This can be attributed to the problem of implementing questionnaires and surveys to get reliable epidemiological estimates of deviant or illicit behaviour. All athletes questioned were subject to doping controls as members or junior members of the national teams. In order to estimate the prevalence of doping and illicit drug abuse, the athletes were either issued an anonymous standardized questionnaire (SQ; n=1394) or were interviewed using randomized response technique (RRT; n=480). It was used a two-sided z-test to compare the standardized questionnaire and RRT results with the respective official German NADA data on the prevalence of doping. Official doping tests only reveal 0.81 percent (n=25,437; 95 % confidence interval 0.70 to 0.92 %) of positive test results, while according to RRT 6.8 percent (n=480; 95 % confidence interval 2.7 to 10.9 %) of our athletes confessed to having practiced doping. Standardized questionnaire and randomized response technique both revealed a prevalence of about 7 percent for illicit drug use, but SQ failed to indicate a realistic prevalence of doping (0.20 %; 95 % confidence interval 0.02-0.74 %). It was thus demonstrate for the first time that data from official doping tests underestimate the true prevalence of doping in elite sports by more than a factor of eight. The results indicate that implementing randomized response technique before and after anti-doping measures could be a promising method for evaluating the effectiveness of anti-doping programs [09401].

Current antidoping policy, essentially a costly repressive zero-tolerance approach in elite sport, will continue to be hampered by the limits of technology. False negatives and false positives are inherent possibilities with testing technology and current protocols do not adequately address the problem of biological and pre-analytical variability, which both may lead to unreliable test results. This uncertainty is acceptable in the field of therapeutic medicine but problematic in sport because athletes can never be considered truly clean, whereas false accusations should be avoided at all cost. Pragmatically, the introduction of additional analyses to the already huge armamentarium of antidoping tests is questionable, both in ethical and economical terms, especially when the diagnostic efficiency of the new tests is not proven [08045].
Enforcement of the antidoping-code is performed by doping controls. For this purpose, blood and urine samples of athletes are collected and analysed. In 2006 approximately 200,000 samples were analysed worldwide, with 1.96 percent being tested positive [08046].

Drug-testing practices are based on separation of a sample's constituents by chromatography and detection of target compounds by mass spectrometry, one of the workhorses in anti-doping research. Identification is reported as positive when the test and reference sample signals agree within a particular tolerance window. However, the size of this window is not constructed with an acceptable risk of false positives in mind. Rather, fixed decision criteria hold, regardless of the quality of the laboratory or the signal properties of the target compound. However, a laboratory that produces relatively precise results should deploy stricter criteria. Likewise, target compounds should be differentiated so that information in their signals can be respected [08047].

Always deploying the same rigid criteria leads to a probability of false positives that depends on the particular laboratory and target compound in an undefined way. This situation is frustrating because the statistical solution - flexible criteria that account for various complications - was already published and thoroughly tested five years before these rigid criteria were introduced. So, is it ignorance of the literature or failure to understand the analytical problem at hand that underlies the ongoing usage of these arbitrary decision rules? Laboratories, as well as their clients and (re-)accrediting organizations, should start reflecting on their accountability with regard to this avoidable malpractice [08047].

Already, in the run-up to the Olympic Games, vast amounts of time, money and media coverage have been spent on sports doping. Several doping experts have contended that tests aren't sensitive enough and let dozens of cheaters slip through the cracks, but only some athletes are facing sanctions. For example upon testing positive for clenbuterol, US swimmer Jessica Hardy was held back from the 2008 Olympic team and faces a two-year ban from the sport. China has already banned several athletes, some of them for life, on doping charges. Indeed, many world-class athletes will find their life's accomplishments and ambitions, their integrity and their reputations hinging on urine or blood tests [08048].

One factor at play in many cases that involve statistical reasoning, is what's known as the prosecutor's fallacy. At its simplest level, it concludes guilt on the basis of an observation that would be extremely rare if the person were innocent. Consider a blood test that perfectly matches a suspect to the perpetrator of a crime. Say, for example, the matching profile occurs in just 1 out of every 1,000 people. A naive prosecutor might try to convince a jury that the odds of guilt are 999:1, that is, the probability of guilt is 0.999. The correct way to determine odds comes from Bayes rule and is equal to 999 times P/(1-P) where P is the "prior probability" of guilt. Prior probability can be difficult to assess, but could range from very small to very large based on corroborating evidence implicating the suspect. The prosecutor's claim that the odds are 999:1 implies a prior probability of guilt equal to 0.5 (in which case P and 1-P cancel). Such a high value of P is possible, but it would require substantial evidence. Suppose there is no evidence against the suspect other than the blood test: he was implicated only because he was from the city where the crime occurred. If the city's population is one million then P is 1/1,000,000 and the odds of his guilt are 1001:1 against, which corresponds to a probability of guilt of less than 0.001 [08048].

Therefore it must be asked if, when an athlete tests positive, he or she without doubts is guilty of doping? The international Court of Arbitration for Sport upheld doping charges against cyclist Floyd Landis, stripping him of his title as winner of the 2006 Tour de France and suspending him from competition for two years. The court agreed with the majority
opinion of a divided three-member American Arbitration Association (AAA) panel and essentially placed a stamp of approval on a laboratory test indicating that Landis had taken synthetic testosterone. Despite that there might be inherent flaws in the testing practices of doping laboratories. Close scrutiny of quantitative evidence used in Landis’s case show for example it to be non-informative. This says nothing about Landis's guilt or innocence. It rather reveals that the evidence and inferential procedures used to judge guilt in such cases don't address the question correctly [08048].

The prosecutor's fallacy is at play in doping cases. For example, Landis's positive test result seemed to be a rare event, but just how rare? In doping cases the odds are dictated by the relative likelihood of a positive test assuming the subject was doping (“sensitivity”) against a positive result assuming no doping (which is one minus “specificity”). Sensitivity and specificity are crucial measures that must be estimated with reasonable accuracy before any conclusion of doping can be made. The studies necessary to obtain good estimates are not easy to do. They require known samples, both positive and negative for doping, tested by blinded technicians who use the same procedures under the same conditions present in actual sporting events. Most often in doping testing, such studies have not been adequately done, leaving the criterion for calling a test positive unvalidated [08048].

Urine samples from cyclists competing in the 2006 Tour de France were analysed at the French national anti-doping laboratory in Châtenay-Malabry. This is one of 34 laboratories accredited by the World Anti-Doping Agency to receive and analyse test samples from athletes. The laboratory flagged Landis's urine sample following race stage 17, which he won, because it showed a high ratio of testosterone to epitestosterone. Based on the initial screening test, the laboratory conducted gas chromatography with mass spectrometry, and isotope ratio mass spectrometry on androgen metabolites in Landis's sample. Such laboratory tests involve a series of highly sophisticated processes that are used to identify the likelihood of abnormal levels of plant-based androgen metabolites (from dietary or pharmaceutical sources) in a urine sample. The goal is to differentiate from endogenous androgen metabolites normally found in urine. Mass spectrometry requires careful sample handling, advanced technician training and precise instrument calibration. The process is unlikely to be error-free. Each of the various steps in handling, labelling and storing an athlete's sample represents opportunity for error. The criteria used to discriminate a positive from a negative result are set by the World Anti-Doping Agency. However, there is no way of knowing which cases are truly positive and which are truly negative. It is proper to establish threshold values such as these, but only to define a hypothesis; a positive test criterion requires further investigation on known samples. However, the method used to establish the criterion for discriminating one group from another has rarely been published, and tests have not been performed to establish sensitivity and specificity. Without further validation in independent experiments, testing is subject to extreme biases [08048].

Landis seemed to have an unusual test result. Because he was among the leaders he provided 8 pairs of urine samples (of the total of approximately 126 sample-pairs in the 2006 Tour de France). So there were 8 opportunities for a true positive - and 8 opportunities for a false positive. If he never doped and assuming a specificity of 95 percent, the probability of all 8 samples being labelled “negative” is the eighth power of 0.95, or 0.66. Therefore, Landis's false-positive rate for the race as a whole would be about 34 percent. Even a very high specificity of 99 percent would mean a false-positive rate of about 8 percent. The single-test specificity would have to be increased to much greater than 99 percent to have an acceptable false-positive rate [08048].

More important than the number of samples from one individual is the total number of samples tested. With 126 samples, assuming 99 percent specificity, the false-positive rate is
72 percent. So, an apparently unusual test result may not be unusual at all when viewed from the perspective of multiple tests. This is well understood by statisticians, who routinely adjust for multiple testing. It is believe that test results much more unusual than the 99th percentile among non-dopers should be required before they can be labelled “positive” [08048].

Other doping tests are subject to the same weak science as testosterone, including tests for naturally occurring substances, and some that claim to detect the presence of a foreign substance. Detecting a banned foreign substance in an athlete's blood or urine would seem to be clear evidence of guilt. But as with testing for synthetic testosterone, such tests may actually be measuring metabolites of the drug that are naturally occurring at variable levels. Whether a substance can be measured directly or not, sports doping laboratories must prospectively define and publicize a standard testing procedure, including unambiguous criteria for concluding positivity, and they must validate that procedure in blinded experiments. Moreover, these experiments should address factors such as substance used (banned and not), dose of the substance, methods of delivery, timing of use relative to testing, and heterogeneity of metabolism among individuals. To various degrees, these same deficiencies exist elsewhere - including in some forensic laboratories. It was stated that all scientists share responsibility for this [08048].

Against this discussion representatives of the World Anti-Doping Agency (WADA) argued that all WADA’s accredited laboratories, including the French national laboratory must meet standards set by the International Standard for Laboratories (ISL) in validation methods, staff competency and chain of custody, for example. Compliance is assessed independently by bodies of the International Laboratory Accreditation Cooperation. It should also be mentioned that the majority of the substances reported by the anti-doping laboratories are exogenous substances not naturally present in human beings. The development of testing procedures for endogenous substances includes samples from normal reference populations and from subjects administered with the substance under investigation, so that test-sample status and positivity criteria can be established. To determine cut-offs for the ratio of testosterone to epitestosterone (T/E), tens of thousands of athlete samples were analysed to establish reference values. To detect exogenous administration of endogenous substances (such as pharmaceutical testosterone) by isotope-ratio mass spectrometry (iRMS), validation is based on a combination of hundreds of known positive and negative samples analysed by many WADA anti-doping laboratories operating under the scrutiny of the ISL and of the International Organization for Standardization (such as ISO 17025) [08049].

Each sample test includes positive and negative quality-control samples to assess the possibility of a false result. If these samples fail, the test must be repeated. An adverse analytical finding is not reported unless the quality criteria are met. All the laboratories participate in at least four rounds of blind and one double-blind proficiency test per year; the results of each round determine the accreditation status of the laboratory. False positives mean immediate revocation of accreditation [08049].

Also, mass-spectrometry identification of exogenous substances relies on at least three diagnostic ions to avoid any interference or misidentification. For immunoassays, antibodies in the initial testing and confirmation procedures must have different antigen-epitope specificity. For analytes that are too small to have two independent antigenic epitopes, two different purification methods or two different analytical methods are used [08049].

Testosterone abuse is conventionally assessed by the urinary testosterone/epitestosterone (T/E) ratio, levels above 4.0 being considered suspicious. A deletion polymorphism in the gene coding for UGT2B17 is strongly associated with reduced testosterone glucuronide (TG)
levels in urine. Many of the individuals devoid of the gene would not reach a T/E ratio of 4.0 after testosterone intake. Future test programs will most likely shift from population based- to individual-based T/E cut-off ratios using Bayesian inference. A longitudinal analysis is dependent on an individual's true negative baseline T/E ratio. The aim of one study was to investigate whether it is possible to increase the sensitivity and specificity of the T/E test by addition of UGT2B17 genotype information in a Bayesian framework. A single intramuscular dose of 500 mg testosterone enanthate was given to 55 healthy male volunteers with either two, one or no allele (ins/ins, ins/del or del/del) of the UGT2B17 gene. Urinary excretion of TG and the T/E ratio was measured during 15 days. The Bayesian analysis was conducted to calculate the individual T/E cut-off ratio. When adding the genotype information, the program returned lower individual cut-off ratios in all del/del subjects increasing the sensitivity of the test considerably. It will be difficult, if not impossible, to discriminate between a true negative baseline T/E value and a false negative one without knowledge of the UGT2B17 genotype. UGT2B17 genotype information is crucial, both to decide which initial cut-off ratio to use for an individual, and for increasing the sensitivity of the Bayesian analysis [08050].

A support vector machine

Due to their performance enhancing properties, use of anabolic steroids (e.g. testosterone, nandrolone, etc.) is banned in elite sports. Therefore, doping control laboratories accredited by the World Anti-Doping Agency (WADA) screen among others for these prohibited substances in urine. It is particularly challenging to detect misuse with naturally occurring anabolic steroids such as testosterone (T), which is a popular ergogenic agent in sports and society. To screen for misuse with these compounds, drug testing laboratories monitor the urinary concentrations of endogenous steroid metabolites and their ratios, which constitute the steroid profile and compare them with reference ranges to detect unnaturally high values. However, the interpretation of the steroid profile is difficult due to large inter-individual variances, various confounding factors and different endogenous steroids marketed that influence the steroid profile in various ways. A support vector machine (SVM) algorithm was developed to statistically evaluate urinary steroid profiles composed of an extended range of steroid profile metabolites. This model makes the interpretation of the analytical data in the quest for deviating steroid profiles feasible and shows its versatility towards different kinds of misused endogenous steroids. The SVM model outperforms the current biomarkers with respect to detection sensitivity and accuracy, particularly when it is coupled to individual data as stored in the Athlete Biological Passport [13019].

Performance profiling

In recent years, antidoping strategies underwent a significant development, from purely biochemical analyses and the detection of substances in urine samples to a biological approach, using blood samples, longitudinal monitoring, and probabilistic techniques. Nowadays, the appropriate timing of testing and the targeting of the athletes to be tested with antidoping tests is a major issue. A new strategy to improve the targeting of suspicious athletes might be the longitudinal monitoring of individual performances. By these means, suspect athletes might be identified, as doping will not only alter their blood or steroid profiles, but ultimately boost their performance, as well. Through the proposed approach, the effectiveness in the fight against doping might be improved considerably [09002].

Controls at random

Substance abuse, particularly among young people, does not seem to have the tendency to
decrease. The knowledges on this phenomenon are manifold and they validly compete to address the actions of contrast. Nevertheless, it would seem profit to be able to have further informations, to place side by side to those already existing, with the aim to improve the surveillance of the phenomenon. For this purpose, in one paper it was proposed a monitoring model based on the results of random controls on road, carried out by the Police (or by the Hospital) in relationship to the road safety. The representativeness of the data that we could get this way appears elevated as a high percentage of the population own the driver's licence. As it is shown, these controls could be both individual and related to drivers' pools of biological samples. This last approach would seem to be more practicable since problems relating to the drivers' privacy would be avoided [09003].

Errors in drug testing

To date, there are estimates for the percentage of unknown cases of doping and illicit drug use in fitness sports, but not for elite sports. This can be attributed to the problem of implementing questionnaires and surveys to get reliable epidemiological estimates of deviant or illicit behaviour. All athletes questioned were subject to doping controls as members or junior members of the national teams. In order to estimate the prevalence of doping and illicit drug abuse, the athletes were either issued an anonymous standardized questionnaire (SQ; n=1394) or were interviewed using randomized response technique (RRT; n=480). Official doping tests only reveal 0.81 percent (n=25,437; 95 % confidence interval 0.70 to 0.92 %) of positive test results, while according to RRT 6.8 percent (n=480; 95 % confidence interval 2.7-10.9 %) of our athletes confessed to having practiced doping. SQ and RRT both revealed a prevalence of about 7 percent for illicit drug use, but SQ failed to indicate a realistic prevalence of doping. It was demonstrate for the first time that data from official doping tests underestimate the true prevalence of doping in elite sports by more than a factor of eight [10011].

Incongruity of data

Antidoping testing is currently exclusively based on hematochemical analysis performed in specialized laboratories accredited by WADA (World Anti-Doping Agency). Many of the analytical methods used for the determination of the parameters considered, such as hematological parameters (hemoglobin, hematocrit and reticulocytes), proteins (soluble transferrin receptor and hepcidin) and hormones (erythropoietin and growth hormone) are often affected by lack of clear standardization and harmonization. The observed incongruity of the data deriving from different laboratories often results in the risk of false positive results in athletes [10012].

Theoretical testing

Currently a large range of pure substance reference materials are available for calibration of doping-control methods. These materials enable traceability to the International System of Units (SI) for the results generated by World Anti-Doping Agency (WADA)-accredited laboratories. Only a small number of prohibited substances have threshold limits for which quantification is highly important. For these analytes only the highest quality reference materials that are available should be used. Many prohibited substances have no threshold limits and reference materials provide essential identity confirmation. For these reference materials the correct identity is critical and the methods used to assess identity in these cases should be critically evaluated. There is still a lack of certified matrix reference materials
to support many aspects of doping analysis. However, in key areas a range of urine matrix materials have been produced for substances with threshold limits, for example 19-norandrosterone and testosterone/epitestosterone (T/E) ratio. These matrix-certified reference materials (CRMs) are an excellent independent means of checking method recovery and bias and will typically be used in method validation and then regularly as quality-control checks. They can be particularly important in the analysis of samples close to threshold limits, in which measurement accuracy becomes critical. Some reference materials for isotope ratio mass spectrometry (IRMS) analysis are available and a matrix material certified for steroid delta values is currently under production. In other new areas, for example the Athlete Biological Passport, peptide hormone testing, designer steroids, and gene doping, reference material needs still need to be thoroughly assessed and prioritized [11029].

Efforts for drug free sport include developing a better understanding of the behavioural determinants that underline doping with an increased interest in developing anti-doping prevention and intervention programmes. Empirical testing of both is dominated by self-report questionnaires, which is the most widely used method in psychological assessments and sociology polls. Disturbingly, the potential distorting effect of socially desirable responding (SD) is seldom considered in doping research, or dismissed based on weak correlation between some SD measure and the variables of interest. The aim of one report was to draw attention to the potential distorting effect of SD and to the limitation of using correlation analysis between a SD measure and the individual measures. Models of doping opinion as a potentially contentious issue was tested using structural equation modeling technique (SEM) with and without the SD variable, on a dataset of 278 athletes, assessing the SD effect both at the indicator and construct levels, as well as testing SD as an independent variable affecting expressed doping opinion. Participants were categorised by their SD score into high- and low SD groups. Based on low correlation coefficients observed in the overall sample, SD effect on the indicator variables could be disregarded. Regression weights between predictors and the outcome variable varied between groups with high and low SD but despite the practically non-existing relationship between SD and predictors in the low SD group, both groups showed improved model fit with SD, independently. The results of this study clearly demonstrate the presence of SD effect and the inadequacy of the commonly used pairwise correlation to assess social desirability at model level. In the absence of direct observation of the target behaviour (i.e. doping use), evaluation of the effectiveness of future anti-doping campaign, along with empirical testing of refined doping behavioural models, will likely to continue to rely on self-reported information. Over and above controlling the effect of socially desirable responding in research that makes inferences based on self-reported information on social cognitive and behavioural measures, it is recommended that SD effect is appropriately assessed during data analysis [11030].

Self-reporting

Social psychology research on doping and outcome based evaluation of primary anti-doping prevention and intervention programmes have been dominated by self-reports. Having confidence in the validity and reliability of such data is vital. Epidemiological and social science research assessing social cognitions linked to doping behaviour has been constrained by the almost exclusive use of self-report methodology. Anti-doping prevention programmes are also evaluated via self-reported changes in attitudes and willingness to use doping substances, anabolic steroids in particular. However, recent research has drawn attention to a potential distorting effect of social desirability observed in self-reported social cognitive measures related to doping. It has been shown, albeit on a small sample, that taking self-reports at face value could lead to misleading conclusions about the social cognitive processes that underlie doping behaviour. Whilst differences in explicit (self-
reported) social cognitive measures between user and non-user groups were observed in the expected direction when groups were created from self-report, generally the reverse was evidenced when the user status was based on hair analysis results (i.e. based on the presence of at least one prohibited performance enhancing drug in hair). Implicit measures were consistent with the grouping based on hair analysis. The outcome of this project suggested that respondents may consistently manipulate their answers on all related measures in order to maintain the image they wish to project, although the possibility that this response bias might stem, at least partially, from self-deception (as opposed to strategic responding for impression management) cannot be ruled out. The strikingly different patterns in self-reports and implicit associations in the context of behavioural data inevitably lead to the question of: Which data should we trust? The self-report methodology has endured a mix of support and criticism in the past. Whilst a plethora of literature suggests that self-report can yield a valid assessment of substance use behaviour, it has also drawn equally strong criticism. Whilst these tests have provided adequate evidence of validity and reliability, other studies using various biomarkers to validate self-reported behaviour data have put convincing evidence forward for a considerable under-reporting of substance use. A systematic review revealed that this bias is not limited to socially undesirable behaviours; it also extends to simple measures such as height and weight with a tendency towards over- and under-reporting, respectively. The effect of gender, race, age and contextual contingencies such as drug type and seriousness of the offence on over- and under-reporting substance use has also been investigated with race as the only factor so far demonstrating an effect on admitting drug taking behaviour. One study utilised a mixed method design to afford triangulation between explicit measures through self-reported questionnaire, implicit associations using a computerised test for latency measures, and bioanalysis via hair specimens. The sample of 82 athletes from 30 sports (52 % female, mean age: 21 years) was split into quasi-experimental groups based on self-admitted previous experience with prohibited performance enhancing drugs (PED) and the presence of at least one prohibited PED in hair covering up to 6 months prior to data collection. Approximately 100 mg untreated head hair, cut at scalp, was screened using ELISA kits for the presence of the most commonly used anabolic steroids (stanozolol, nandrolone and boldenone). Positive samples for anabolic steroids were confirmed and quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods. Erythropoietin (EPO) was detected using quantifiable ELISA. Hair digestion and analytical methods using LC-MS/MS were developed in house to increase sensitivity and to reduce the amount of hair required. Participants responded to questionnaires assessing a range of social cognitive determinants of doping via self-reports; and completed a modified version of the Brief Implicit Association Test (BIAT) assessing implicit attitudes to doping relative to the acceptable nutritional supplements (NS). Social projection regarding NS was used as control. PEDs were detected in hair samples from 10 athletes (12 % prevalence), none of whom admitted doping use. Nutritional supplement use was reported by 60 percent, whereas those having personal experience with doping constituted 13 percent of the athletes. Admitted doping appears to be independent of self-reports on nutritional supplements or social drug use. Eight hair samples were positive for stanozolol and two for EPO, giving a 12 percent prevalence rate for prohibited performance enhancing substance use in the sample. EPO levels were 13 picog/mg and 12.53 picog/mg for the two positives. Interestingly, more males admitted having experience with doping than females (21 % vs 7 %, respectively) with a reversed pattern for positive hair analysis (5 % vs 19 %, respectively). None of the athletes who returned positive hair samples admitted doping use. Conversely, no self-admitted doping was confirmed by current hair sample tests. Social projections for nutritional supplements, doping and social drug use showed a positive, statistically significant but relatively weak relationship between fellow athletes using prohibited performance enhancing drugs and nutritional supplements and social drugs by the general population. Based on self-declared doping behavior, an interaction effect between user group and gender was only found for social projection of
doping and nutritional supplements use by fellow athletes. No gender difference was evidenced for any of the outcome variables except the pressure to use banned substances. A statistically significant difference was found between admitted doping users and non-users in explicit attitude and social projection of doping use, with a borderline significance for pressure to use doping. As expected, those who admitted having personal experience with doping exhibited more of a lenient attitude (shown by higher PEAS score) towards doping and gave higher estimated proportions of doping users among athletes and reported higher perceived pressure to use doping. Athletes who denied doping contrary to the evidence in their hair samples exhibited less lenient attitudes toward doping. These results support the previous observation that athletes who deny their doping use behaviour gave answers on social cognitive measures that are consistent with a typical non-user. In other word, they are “faking good” in a consistent manner. These results are not surprising, considering that the outcome measures were exclusively based on self-declarations. The picture, however, has changed for the implicit associations. Albeit the differences in latency or D-scores did not reach statistical significance, the sample means suggest that performance on the BIAT was revealing for deniers. That is, latency measures and D-scores set deniers apart from clean and self-admitted users, but did not differentiate between the latter two groups. The majority of the athletes (89 %) indicated that they would prefer to compete in a doping free environment. The remaining 11 percent opted for a scenario in which both players use doping. The fact that no athletes opted for a unilateral use of doping suggests that there is a proportion of athletes who might be more motivated about enhancing performance in general (including using prohibited means) than gaining competitive advantage against opponents. Interestingly, 62 percent of all respondents believed that athletes use performance enhancing substances in training and competition. Overall, discrepancies in the relationship between declared doping-related opinion and implicit doping attitudes were observed between the groups, with control measures remaining unaffected. The results suggest, with considerable confidence, that the “denier” group is characterised by a pattern of dissociation between explicit and implicit responding. This dissociation is, in fact, likely to be a cognitive marker for this group, which may lead to a promising application of the combined explicit-implicit cognitive protocol used in this study as a proxy for the less readily available biochemical detection methods for large scale social science research on doping. Athletes’ views on doping were first and foremost influenced by the “legality” of the substance, then on performance. In this, athletes interviewed considered prohibited performance enhancing substances as cheating but acceptable enhancers as essential. The most important contribution that the results can add to drug use research is the observed distinct patterns of explicit and implicit responding among self-declared doping users and deniers which may lead to significant advances in both detection and treatment interventions for these groups. The findings question the validity of self-reports which may have significant implications in interpreting previous and future doping research. A combination of self-report and implicit cognitive measures seems to hold the strongest promise for future doping research. It is this combination that is likely to produce, with attendant methodology refinements, robust cognitive markers of denial. Objective verification using biomarkers or chemical analysis may not be a feasible approach in all social science research. However, our results suggest that triangulating results obtained on the same or related constructs but using different methodologies could be a cost-effective avenue. Hence, further research into the methods of combining self-report methodology, with indirect, implicit methods is warranted. Assuming that social desirability has a root in contextual contingencies, research among different user groups could be beneficial. Doping social science research, particularly quantitative research, is seriously lacking in studies using samples drawn from athletes banned from competition owing to doping offences and longitudinal research. Research in this field would benefit from looking beyond doping and having a greater use of direct and indirect methods from social psychology, particularly those used successfully in substance use and addiction research. Incorporating implicit social cognition is one promising avenue for doping social
Although it is still debated whether implicit social cognitions reveals something about the individual or the individual's environment, implicit social cognition research is among the thriving areas in social psychology. Doping research, owing to the unique nature of doping (i.e. being positioned between illicit behaviour and functional use of ergogenic aids) provides an excellent testing field for developing a better understanding of the explicit and implicit social cognition and the environment. Questionnaire responses thus showed a pattern consistent with self-reported doping use. Following this preliminary work, the study provides further evidence that both self-reports on behaviour and social cognitive measures could be affected by some form of response bias. This can question the validity of self-reports, with reliability remaining unaffected. Triangulation of various assessment methods is recommended [11031].

**Testing as a way of decreasing intent for doping**

To assess the effects of random drug and alcohol testing (DAT) among high school athletes a 2-year prospective randomized controlled study was performed. It was a study of a single cohort among five intervention high schools with a DAT policy and six schools with a deferred policy, serially assessed by voluntary, confidential questionnaires. DAT school athletes were at risk for random testing during the full academic year. Positive test results were reported to parents or guardians, with mandatory counseling. Indices of illicit drug use, with and without alcohol use, were assessed at the beginning and end of each school year for the past month and prior year. Potential mediating variables were evaluated. Student-athletes from intervention and control schools did not differ in past 1-month use of illicit drug or a combination of drug and alcohol use at any of the four follow-up periods. At the end of the initial school year and after 2 full school years, student-athletes at DAT schools reported less drug use during the past year compared to athletes at the deferred policy schools. Combining past year drug and alcohol use together, student-athletes at DAT schools reported less use at the second and third follow-up assessments. Paradoxically, DAT athletes across all assessments reported less athletic competence, less belief authorities were opposed to drug use, and indicated greater risk-taking. At the final assessment, DAT athletes believed less in testing benefits and less that testing was a reason not to use drugs. It was concluded that DAT deterrent effects were evident for past month use during any of the four follow-up periods. Prior-year drug use was reduced in two of four follow-up self-reports, and a combination of drug and alcohol use was reduced at two assessments as well. Overall, drug testing was accompanied by an increase in some risk factors for future substance use. More research is needed before DAT is considered an effective deterrent for school-based athletes [07018].

**Fatigue as a limit for test performance**

Muscle physiologists often describe fatigue simply as a decline of muscle force and infer this causes an athlete to slow down. In contrast, exercise scientists describe fatigue during sport competition more holistically as an exercise-induced impairment of performance. The aim of one review was to reconcile the different views by evaluating the many performance symptoms/measures and mechanisms of fatigue. It was describe how fatigue is assessed with muscle, exercise or competition performance measures. Muscle performance (single muscle test measures) declines due to peripheral fatigue (reduced muscle cell force) and/or central fatigue (reduced motor drive from the CNS). Peak muscle force seldom falls by >30 percent during sport but is often exacerbated during electrical stimulation and laboratory exercise tasks. Exercise performance (whole-body exercise test measures) reveals impaired physical/technical abilities and subjective fatigue sensations. Exercise intensity is initially sustained by recruitment of new motor units and help from synergistic muscles before it
declines. Technique/motor skill execution deviates as exercise proceeds to maintain outcomes before they deteriorate, e.g. reduced accuracy or velocity. The sensation of fatigue incorporates an elevated rating of perceived exertion (RPE) during submaximal tasks, due to a combination of peripheral and higher CNS inputs. Competition performance (sport symptoms) is affected more by decision-making and psychological aspects, since there are opponents and a greater importance on the result. Laboratory based decision making is generally faster or unimpaired. Motivation, self-efficacy and anxiety can change during exercise to modify RPE and, hence, alter physical performance. Symptoms of fatigue during racing, team-game or racquet sports are largely anecdotal, but sometimes assessed with time-motion analysis. Fatigue during brief all-out racing is described biomechanically as a decline of peak velocity, along with altered kinematic components. Longer sport events involve pacing strategies, central and peripheral fatigue contributions and elevated RPE. During match play, the work rate can decline late in a match (or tournament) and/or transiently after intense exercise bursts. Repeated sprint ability, agility and leg strength become slightly impaired. Technique outcomes, such as velocity and accuracy for throwing, passing, hitting and kicking, can deteriorate. Physical and subjective changes are both less severe in real rather than simulated sport activities. Little objective evidence exists to support exercise-induced mental lapses during sport. A model depicting mind-body interactions during sport competition shows that the RPE centre-motor cortex-working muscle sequence drives overall performance levels and, hence, fatigue symptoms. The sporting outputs from this sequence can be modulated by interactions with muscle afferent and circulatory feedback, psychological and decision-making inputs. Importantly, compensatory processes exist at many levels to protect against performance decrements. Small changes of putative fatigue factors can also be protective. We show that individual fatigue factors including diminished carbohydrate availability, elevated serotonin, hypoxia, acidosis, hyperkalaemia, hyperthermia, dehydration and reactive oxygen species, each contribute to several fatigue symptoms. Thus, multiple symptoms of fatigue can occur simultaneously and the underlying mechanisms overlap and interact. Based on this understanding, it was reinforce the proposal that fatigue is best described globally as an exercise-induced decline of performance as this is inclusive of all viewpoints [11032].

Economical aspects

According to the framework legislation promulgated as part of the reform of finance laws in France, quality is a mandatory feature of all governmental actions. In this context, this work was conducted to assess the construction cost of a national health program designed to promote physical and sports activities and prevent doping behaviors. This program was considered to have the characteristic features of a successful governmental health intervention. Four cost categories were evaluated: cost of the activity itself, transportation costs, communication costs and promotion costs. It was found that the program costs for 2002-2007 were 100,000 euros, with 15 percent of the costs in the communication category. This means that economic elements could be associated with factors of successful health service interventions in order to help decision makers responsible for the public interest and the consistency of public health actions [09012].

The overall economic costs of the fight against a doping allegation deserve consideration. WADA is the international independent organisation created in 1999 to promote, coordinate and monitor the fight against doping in sport in all its forms. Composed of and funded equally by the sports movement and governments of the world, WADA received its first 2 years of funding (USD18.3 million) from the Olympic Movement. Since 2002, according to its statutes, WADA's funding is sourced equally from the Olympic Movement and the governments of the world. The total budget for the year 2006 has been estimated at nearly USD 24 million, 60
percent for research, 15 percent for out of competition testing, 15 percent for education and 10 percent for contingencies. The budget of the Australian Sports Anti-Doping Authority (ASADA) for the period 1 July 2005 to 30 June 2006 was 13.681 million Australian dollars. The total revenue from grants and other financial support of the US Anti-Doping Agency (USADA) reached USD 10.9 million in the year 2005. These are only part of the huge economic resources currently devoted to the fight against doping worldwide, which are predicted to reach or exceed USD 10 billion. Therefore, in practice, healthcare systems and national governments worldwide are expected to devote to the fight against doping the same resources that the US government dedicates to prevention and treatment of diseases that cause great morbidity, mortality and economic burden for individuals, families and the entire population. Is this really necessary and morally acceptable? [07030].

Chemoinformatics-based classification of prohibited substances

Representative molecules from 10 classes of prohibited substances were taken from the World Anti-Doping Agency (WADA) list, augmented by molecules from corresponding activity classes found in the MDDR database. Together with some explicitly allowed compounds, these formed a set of 5245 molecules. Five types of fingerprints were calculated for these substances. The random forest classification method was used to predict membership of each prohibited class on the basis of each type of fingerprint, using 5-fold cross-validation. It was also used a k-nearest neighbors (kNN) approach, which worked well for the smallest values of k. The most successful classifiers are based on Unity 2D fingerprints and give very similar Matthews correlation coefficients of 0.836 (kNN) and 0.829 (random forest). The kNN classifiers tend to give a higher recall of positives at the expense of lower precision. A naïve Bayesian classifier, however, lies much further toward the extreme of high recall and low precision. The results suggest that it will be possible to produce a reliable and quantitative assignment of membership or otherwise of each class of prohibited substances. This should aid the fight against the use of bioactive novel compounds as doping agents, while also protecting athletes against unjust disqualification [06034].

Need of excretion studies

The list of prohibited anabolic steroids in sports has grown due to the addition of numerous steroids that have been introduced on the market by non-pharmaceutical companies. Moreover, several designer steroids, specifically developed to circumvent doping control, have also been detected. Because anabolic steroids are most often intensively subjected to phase I metabolism and seldom excreted unchanged, excretion studies need to be performed in order to detect their misuse [06004].

Preanalytical variability

The use of illicit substances and methods contravenes the ethics of sports and may be associated with side effects. Antidoping testing is an essential tool for preventing or limiting the consequences of cheating in sports. As for conventional laboratory testing, major emphasis has been placed on analytical quality, overlooking the inherent risks that may arise from analysis of unsuitable doping samples. The adherence to scrupulous criteria for collection, handling, transportation and storage of samples, especially blood and urine samples, is essential. The leading preanalytical variables that influence doping sample quality include biological variability, sample collection, venous stasis, spurious hemolysis and
presence of other interfering substances, sample manipulation and degradation, and inappropriate conditions for transportation and storage. One article provided a personal overview about the current challenges in preanalytical management of doping samples, as well as potential solutions for preventing the negative impact of preanalytical variables on sample quality and test results [13080].

Chemical and physical manipulations of doping tests

Detection and proof of doping control sample manipulation is a challenging task, and one of the most efficient tools to identifyurine substitution is careful steroid profile evaluation. In 2009/2010, identical steroid profiles of supposedly eight different athletes (from different teams and collection sites) were found and DNA analyses requested, demonstrating that all eight urine specimens were provided from a single donor. This donor was eventually identified as the doping control officer and none of the athletes was actively involved in the sample manipulation. In another case of urine substitution, no natural endogenous steroid was observed in steroid profile analyses, triggering further investigations into the composition of the specimen. Based on findings of hordenine, trace amounts of alcohol, various saccharides and intact proteins including Serpin-Z4, the liquid was identified as non-alcoholic beer. This manipulation however entitled the suspension of the athlete [12017].

Manipulation of urine specimens provided by elite athletes for doping control purposes has been reported several times in the past, and in most of these cases urine substitution was eventually proven. Recent findings of suspected and substantiated manipulation have outlined the complexity and diversity of tampering options, sample appearance alterations resulting from non-manipulative influence, and the analytical challenges arising from these scenarios. Using state-of-the-art mass spectrometric and immunological doping control and forensic chemistry methodologies, four unusual findings were observed. One sports drug testing specimen was found to contain an unusually high content of saccharides accompanied by hordenine and Serpine-Z4, while no endogenous steroid (e.g. testosterone, epitestosterone, androsterone and etiocholanolone) was detected. This specimen was identified as non-alcoholic beer filled into the doping control sample container, constituting an undisputed doping offense. A doping control sample of bright green color was received and found to contain residues of methylene blue, which is not considered relevant for doping controls as no masking or manipulative effect is known. In addition, the number of urine samples of raspberry to crimson red coloration received at doping control laboratories has constantly increased during the last years, attributed to the presence of hemoglobin or betanin/isobetanin. Also here, no doping rule violation was given and an impact on routine analytical results was not observed. Finally, a total of 8 sports drug testing samples collected at different competition sites was shown to contain identical urine specimens as indicated by steroid profile analysis and conclusively proven by DNA-STR (short tandem repeat) analysis. Here, the athletes in question were not involved in the urine substitution act but the doping control officer was convicted of sample manipulation [12088].

The case of seven urine samples collected for anti-doping purposes during a cycling stage race with moderately elevated testosterone and epitestosterone ratio (T/E) is reported. The very low probability of having all seven urine samples with such similar elevated T/E ratio (from 3.2 to 4.7) was very suspicious. Different pattern classification tools were tested to categorize the most similar steroid profiles, but none of the models enabled a clear classification of the different urine samples. Subsequently, genetic profiling of all urine samples was performed and demonstrated that three of the seven samples were collected from the same cyclist. Finally, the International Federation confirmed DNA profiling results.
This suggests that urinary steroid data using several methodologies are not appropriate for identification purposes and to an extent not unique to individuals [06033].

**Designer drugs in Japan**

In recent years, many analogs of narcotics have been widely distributed as easily available psychotropic substances and have become a serious problem in Japan. To counter the spread of these non-controlled substances, the Pharmaceutical Affairs Law in Japan was amended in 2006 to establish a new category; Designated Substances in order to more strictly control these substances. In April 2007, 31 compounds and 1 plant were first controlled as Designated Substances. Before 2007, the major compounds distributed in the Japanese illegal drug market were tryptamines, phenethylamines and piperazines. Alkyl nitrates, such as isobutyl nitrite and isopentyl nitrite, were also widely distributed. After they were listed as Narcotics or Designated Substances in 2007, these compounds, especially the tryptamines, quickly disappeared from the market. In their place, cathinone derivatives have been widely distributed, as well as different phenethylamines and piperazines. Additionally, in recent years, new herbal products containing synthetic cannabinoids have appeared globally. As at July 2012, 78 substances (including 1 plant; Salvia divinorum) were listed in the category of Designated Substances. They were 13 tryptamines, 17 phenethylamines, 11 cathinones, 4 piperazines, 23 synthetic cannabinoids, 6 alkyl nitrates, 3 other compounds and 1 plant. In this review, we show our survey of the spread of new designer drugs in Japan, focusing especially on synthetic cannabinoids and cathinone derivatives. Also, the prevalence and legal status of these substances in other countries will be presented [13057].

**Testing efficiency**

In the early 2000s, United States track and field athlete Kelly White passed 17 drug tests while on steroids (tetrahydrogestrinone, THG, testosterone), stimulants (modafinil), and EPO before she was caught on modafinil, then confessed to having used the whole regimen. THG was not found in her samples because laboratories were still blind to this designer steroid (used only to beat the test). Testosterone use was not detected because she masked it by taking epitestosterone as well; because her T/E never exceeded the cut-off, it never triggered IRMS analysis, which would have detected exogenous testosterone. Modafinil was first targeted and found by the French WADA-accredited laboratory; her EPO use was not detected because sprinters’ samples were not tested for EPO yet [07011].

**Anti-doping research opportunities**

The key areas of antidoping research include detection of compounds and methods for increasing the absorption, transportation, and delivery of oxygen from the lung to the muscle; detection of compounds or methods that increase the efficiency of conversion of oxygen to intracellular energy; detection of compounds and methods that enhance muscle growth and recovery; detection of genetic modifications applied to sport performance; identification of additional matrices (oral fluid, dried blood spots, etc) to detect doping; and identification and detection of masking techniques. The challenge of developing and validating methods for the long list of prohibited substances and methods is daunting, requiring analytical skills, a thorough understanding of drug metabolism and pharmacokinetics, and an appreciation of human physiology and endocrinology. Antidoping science would benefit from the expertise of scientists working on proteomics collaborating with scientists interested in changes in the red
blood cell during storage – hopefully resulting in a test for autologous blood transfusions. Other key areas of research in the future will involve recombinant protein and glycoprotein characterization and quantification using mass spectrometry and other techniques. The potential for genetic modification for performance enhancement is of great concern, and methods will need to be developed to detect gene doping. New methods of drug administration may impact detection methods. Alternative testing matrices, such as oral fluid, dried blood spots, and hair, may become more important [12006].

**Economy**

According to the WADA website, they have distributed over USD 50 million in research funding since 2001. Between 2002 and 2010, USADA distributed an additional USD 9 million in research funding. In 2008, the USADA, the U.S. Olympic Committee, the National Football League, and Major League Baseball joined forces to create the Partnership for Clean Competition (PCC) to support research in antidoping science. PCC has now funded 28 proposals totaling USD 6 million. In addition to research proposals, PCC will continue to establish collaborative working groups to focus the efforts of the best researchers on the science of antidoping. While significant advances have been made in improving detection strategies and methods, increased collaboration with the basic research community should enhance the progress of drug abuse deterrence. Translation of those findings to routine testing will also require funding and effort [12006].

**Placebo**

In the perpetual quest for better performance, athletes are using an increasingly diverse range of ergogenic aids. Some are permitted; however, this “drug” use is often seen as an ethically questionable behavior. A variety of research suggests that much of the impact of such aids may be due to expectancy—the belief that the substance will aid performance. It would be useful to demonstrate this to athletes considering such usage, especially as a pillar of antidrug education. Accordingly, this investigation used sodium bicarbonate and placebo additives in a double dissociation design, with athletes completing a series of 1,000-m time trials. Results showed that believing one had taken the substance resulted in times almost as fast as those associated with consuming the drug itself. In contrast, taking the drug without knowledge yielded no significant performance increment. Results are discussed against the backdrop of applying expectancy effects in high-performance sport, including dissuading athletes from using illegal aids [07026].

One article described a study examining placebo effects associated with the administration of a hypothetical ergogenic aid in sport. Forty-two team-sport athletes were randomly assigned to 2 groups. All subjects completed 3 x 30-m baseline sprint trials after which they were administered what was described to them as an ergogenic aid but was in fact 200 mg of cornstarch in a gelatin capsule. Group 1 was provided with positive information about the likely effects on performance of the substance, whereas Group 2 was provided with negative information about the same substance. The sprint protocol was repeated 20 min later. Although for Group 1 mean speed did not differ significantly between baseline and experimental trials, a significant linear trend of greater speed with successive experimental trials suggested that positive belief exerted a positive effect on performance. Group 2 ran 1.6 percent slower than at baseline (95 % confidence intervals 0.32 to 2.82 %), suggesting that negative belief exerted a negative effect on performance. Collectively, data suggest that subjects’ belief in the efficacy or otherwise of a placebo treatment might significantly influence findings in experimental research [07027].
The recent advances in the neurobiology of the placebo effect have shown that the administration of a placebo (inert substance), along with verbal suggestions of clinical benefit, activates different neurotransmitters in the brain, like endogenous opioids and dopamine and is associated to neural changes at both the cortical and subcortical level. Powerful placebo responses can be obtained after pharmacological preconditioning, whereby the repeated administration of a drug is replaced with an inert substance. For example, the morphine-like effects of placebos after morphine preconditioning have been shown in the context of pain management. Although these drug-like effects of placebos represent an interesting phenomenon in the clinical setting, they also have implications that have been ignored so far. One of these has to do with the use of drugs in sport competitions to boost physical performance. Among performance-boosting drugs, morphine is known to be a powerful analgesic that increases tolerance to pain, thereby improving physical performance. The importance of opioid-mediated placebo responses consists in the fact that they can be exploited when one wants morphine-like effects without giving morphine. For example, in the context of pain management, it has been shown that morphine administration for 2 days in a row may induce robust placebo analgesic responses when morphine is replaced with a placebo on the third day. This raises the important question whether two morphine administrations separated several days or weeks from each other have similar powerful effects on subsequent placebo responses. The neurobiological investigation of the placebo effect has thus shown that placebos can activate the endogenous opioid systems in some conditions. So far, the impact of this finding has been within the context of the clinical setting. Here it was present an experiment that simulates a sport competition, a situation in which opioids are considered to be illegal drugs. The subjects were healthy males who agreed to participate in one of the experimental groups after they signed an informed consent form in which the details of the experiment, including the drugs to be administered, were explained. In particular, the subjects were told that either morphine or naloxone would be administered at a given time, depending on the experimental group. None of them were training as a competitive athlete, but all the subjects engaged in recreational fitness training. After repeated administrations of morphine in the precompetition training phase, its replacement with a placebo on the day of competition induced an opioid-mediated increase of pain endurance and physical performance, although no illegal drug was administered. The placebo analgesic responses were obtained after two morphine administrations that were separated as long as 1 week from each other. These long time intervals indicate that the pharmacological conditioning procedure has long-lasting effects and that opioid-mediated placebo responses may have practical implications and applications. For example, in the context of the present sport simulation, athletes can be preconditioned with morphine and then a placebo can be given just before competition, thus avoiding administration of the illegal drug on the competition day. However, these morphine-like effects of placebos raise the important question whether opioid-mediated placebo responses are ethically acceptable in sport competitions or whether they have to be considered a doping procedure in all respects. The present study demonstrates that a pharmacological preconditioning, with morphine given twice at intervals as long as 1 week, can induce robust placebo analgesic responses when morphine is replaced with a placebo. It should also be noted that placebo administration without previous morphine conditioning induced a small but significant increase in pain endurance, which indicates smaller effects when a placebo is given for the first time compared with its administration after pharmacological conditioning. The study shows that long time lags between two consecutive administrations of morphine and the administration of the placebo are not very different from short time lags, at least in the range of days/weeks. This indicates that the pharmacological conditioning procedure has long-lasting effects. In addition to the mechanisms of placebo responsiveness and the preconditioning effects of morphine, this study raises important ethical questions: do opioid-mediated placebo effects during competitions have to be considered a doping procedure?
Should we consider morphine conditioning in the training phase ethical and legal? This issue is not easy to be resolved and will need both an ethical and legal discussion. Although one must be aware that the experimental conditions of the present study do not represent a real competitive event, but a pain challenge paradigm, the increase in pain endurance after the placebo is real and robust and has key attributes relevant to situations encountered in sport competitions. For example, our model of tonic ischemic arm pain represents a long-lasting painful stimulation that is likely to be encountered in real long-lasting sport activities. Therefore, if the conditioned subjects of this study engaged in a real sport activity, they would tolerate pain for a longer time [07202].

**Medical history of placebo**

Over the last 200 years, the placebo effect has cast a large and persuasive shadow over the medical field. In that time it has been by turn; harmless charade, charlatan's ruse, therapeutic device, methodological tool, ethical dilemma, research theme and source of controversy. Despite popular recognition, pervasive problems underlie conceptualisation of placebos and placebo effects. With medicine now firmly entrenched in the age of evidence based practice, there is a question as to whether it is time to leave the old placebo behind us. The idea of a magical black box from which unexplained therapeutic effects spring up is archaic and also unhelpful from a scientific point of view. If there really is an effect, surely we are best served by directly investigating what is responsible for it. This knowledge can be used in the clinic to improve treatment effectiveness and in research to inform study design. The view and role of placebos have developed over time. Modern medical understanding of placebos dates back to the late 1700s, and since then, placebo interventions appear to have been used fairly commonly in medical practice alongside other treatments. Up until midway through the 20th century, the prevailing opinion appears to have been that placebo interventions had no effect on pathophysiology. Placebos were used only to bring comfort to the patient, “a camouflage behind which to watch nature takes its course.” From the 1950s onwards, there was a shift towards regarding placebos as having genuine effects of their own. This period also saw the start of rapid growth in the use of placebos as a control intervention for testing the efficacy of other treatments. Thus, as research generally, and randomised controlled trials (RCTs) in particular, grew in stature and volume, placebo interventions also gained a more prominent face in the medical literature [13029].

**Powerless placebo**

The view that placebos have a powerful therapeutic effect remained largely unchallenged until the 1990s. Around this time, authors began to question the scientific basis upon which the claims of large therapeutic placebo effects were made. Critically, it was pointed out that longitudinal changes in a group of patients who have received a placebo intervention are not only due to placebo effects. From the point of onset (or clinical contact), many conditions improve with the passage of time independent of clinical management, due to the natural history of the condition. Improvements over time are likely to be further enhanced by regression to the mean which is a statistical artifact whereby scores that are high at one point in time, i.e. at study entry, are always more likely to be lower at a later time. Thus, when a group of patients receive a placebo intervention the changes afterwards may be due to placebo effects plus changes due to natural history plus the effects of regression to the mean. Experimental pain researchers pointed out the apparent discrepancy between the very small placebo effect sizes reported in clinical studies and the much larger effects in studies investigating placebo mechanisms [13029].

**Lack of effect of flavor**
One study investigated whether a change in beverage flavor during endurance cycling improves subsequent performance. Eight trained male athletes (age 24 years) undertook 3 trials, with training and diet being controlled. Trials consisted of 120 min of steady-state (SS) cycling at approximately 70 percent VO$_2$peak, immediately followed by a 7-kJ/kg time trial (TT). During exercise subjects were provided with fluids every 20 min. After 80 min of SS cycling subjects either continued drinking the same-flavor sports drink or changed to an alternate flavor—in either a alternate-flavor sports drink (AFSD) or cola. All beverages were carbohydrate and volume matched. Changing drink flavor caused no significant change in TT time. The various flavors produced no treatment effects on heart rate, blood glucose, or rating of perceived exertion throughout the SS exercise protocol. The influence of other taste variables such as palatability, bitterness, or timing of flavor change on endurance-exercise performance requires more rigorous investigation [07028].

**Ethical issues**

Along with the developing view of the role of placebos, the last half century has seen a shift towards acceptance of the evidence-based medicine paradigm. These two factors have led to increasing recognition of ethical and legal concerns associated with the administration of placebos in practice. The issues are difficult and consensus as to the appropriateness of placebos in clinical practice has not been achieved. At the heart of the matter lies the question of whether deliberate deception of the patient is acceptable in the course of their treatment. Implicit in the definition of placebo effects is that they are psychologically mediated. Whether the placebo is an ingested agent such as a sugar or vitamin pill, or a procedural intervention such as non-penetrating acupuncture, sham electrotherapy or an inert topical formulation, it must be physically inert. However, there follows the issue of disentangling inert from non-inert psychological interventions. The problem is, if there is a (psychological) mechanism, can the psychotherapeutic part of the intervention still be said to be inert? Along a similar line, researchers have proposed the term “nocebo” to describe undesirable effects of placebos. For example, studies have described immunosuppressive effects of placebo interventions which may be undesirable (a nocebo) for one patient group, for example general surgery patients but desirable (a placebo) for another group, for example, organ transplant recipients. Further, an intervention assumed to have an effect at one point in time could subsequently be shown to have no physiological effect and be used as a placebo. In some ways, it is therefore not surprising a definition for the placebo effect has proven so elusive. Conceivably, the same agent might be a placebo, a nocebo or an active intervention depending on who gives it, who gets it or when it is given [13029].

**Concluding remarks on placebo**

The conception of placebos and placebo effects has developed significantly over the past 200 years in concert with changes in the nature and practice of healthcare as a whole. In the past 20 years, questions have arisen as to the clinical significance of placebo effects and perhaps more importantly, the logical basis of the concept itself. As things stand, enquiry into placebo effects faces the nonsensical situation of attempting to explain an effect that has no mechanism. Abandoning the placebo black box in favour of theory-directed research that specifically targets and investigates the cause of therapeutic effects offers several advantages. Effective techniques can be manipulated and incorporated in clinical practice to produce better outcomes for patients, and better control interventions can be designed too [13029].
Measurement uncertainty in anti-doping quantitative analysis

The standards of laboratory performance of the World Anti-Doping Agency (WADA)-accredited laboratories are defined in the WADA International Standard for Laboratories and its associated Technical Documents. These sets of rules aim to harmonize the production of valid laboratory test results and evidentiary data as well as the reporting of laboratory analytical findings. The determination of anti-doping rule violations in sport made on the basis of analytical quantitative confirmatory analyses for the presence of prohibited threshold substances, in particular, requires the application of specific compliance decision rules, which are established in the WADA Technical Document on Decision Limits. In one article, the use of measurement uncertainty information in the establishment of compliance Decision Limits and in evaluating the performance of a laboratory's quantitative analytical procedures over time and in relation to other laboratories through WADA's External Quality Assessment Scheme program is reviewed and discussed. Furthermore, a perspective is provided on the emerging challenges associated with the harmonization of the quantitative measurement of large-molecular weight biomolecules [12053].

Statistical aspects

The detection of growth hormone (GH) abuse by athletes raises statistical problems as well as biochemical ones. It was outlined the statistical approaches to the various issues which have arisen; in particular, it considers the need to develop a test which detects GH abuse in any elite athlete “beyond reasonable doubt”. The test needs to be robust enough to withstand legal challenge, while minimising the risk of false accusation. Since GH is a naturally occurring hormone whose concentration varies substantially, its abuse cannot be detected by direct measurement. The methodology considered here made use of markers whose levels are more stable but are influenced by GH. The statistical methods employed aimed to make the best use of these markers, taking account of all factors contributing to errors in measurement. There were two key steps in the statistical investigation undertaken to develop the GH detection algorithm. The first was the requirement to identify GH-dependent biomarkers which would identify GH doping reliably and robustly for a significant length of time. The second was to calibrate the GH detection method in the elite athlete population, so that the method would be applicable to all athletes, regardless of age, sex and ethnicity, and regardless of whether they had recently sustained an injury. In practice, further work was needed to ensure that the methodology met the WADA testing protocol rules, but also that the proposed method can be used by any WADA accredited lab without placing any athlete at an unfair disadvantage and ensuring a high level of confidence in any result produced [09004].

The comparison among different modelling techniques, such as multiple linear regression, partial least squares and artificial neural networks, has been performed in order to construct and evaluate models for prediction of gas chromatographic relative retention times of trimethylsilylated anabolic androgenic steroids. The performance of the quantitative structure-retention relationship study, using the multiple linear regression and partial least squares techniques, has been previously conducted. In the present study, artificial neural networks models were constructed and used for the prediction of relative retention times of anabolic androgenic steroids, while their efficiency is compared with that of the models derived from the multiple linear regression and partial least squares techniques. For overall ranking of the models, a novel procedure [Trends Anal Chem 2010; 29: 101-9] based on sum of ranking differences was applied, which permits the best model to be selected. The
suggested models are considered useful for the estimation of relative retention times of designer steroids for which no analytical data are available [12052].

**Wald test**

One article derived the power curves for a Wald test that can be applied to randomized response models when small prevalence rates must be assessed (e.g., detecting doping behavior among elite athletes). These curves enable the assessment of the statistical power that is associated with each model (e.g., Warner's model, crosswise model, unrelated question model, forced-choice models, item count model, cheater detection model). This power analysis can help in choosing the optimal model and sample size and in setting model parameters in survey studies. The general framework can be applied to all existing randomized response model versions. The Appendix of the article contains worked-out numerical examples to demonstrate the power analysis for each specific model. (PsycINFO Database Record) [12051].

**Bayesian statistics**

Research on biological markers is a fast-growing field for assessing evidence in biomedical toxicology. In forensic toxicology in particular, major goals are to develop and validate measurements of endogenous substances that may reveal the presence of toxic substances, drugs of abuse, and/or doping agents. The development and validation of biomarkers of response are either based on the statistical description of endogenous substances measured on a population or on a longitudinal evaluation of a series of repeated tests performed on the same individual. Longitudinal studies are particularly interesting in forensic toxicology when the biomarker has a significantly smaller intraindividual variability than interindividual variability. This is the case for several biomarkers currently used in antidoping investigations, such as indirect markers of blood doping and the testosterone over epitestosterone (T/E) ratio for the detection of the abuse of testosterone and its precursors. These biomarkers are all characterized by a small ratio of intra- to interindivdual variability. In contrast to the abundant number of statistical models that analyze serial biomarkers of disease, the development of reliable methods for the detection of abnormal variations of a longitudinal biomarker has remained astonishingly limited in forensic toxicology. Current methods employ either population-derived limits – to detect “absolute” abnormal values of the biomarker – or individual-based thresholds – to detect abnormal deviations relative to an individual baseline. There is no reason why a test cannot combine formally population-based information with individual-based data for better decision making. Failure to combine these two types of information may lead to a low sensitivity/specificity relation of the biomarker. For example, when a biological product such as the carbohydrate-deficient transferrine or a transaminase (alanine transaminase (ALAT) and/or aspartate transaminase (ASAT)) is used as an indirect marker of chronic alcohol abuse no effective method integrates previous readings for better decision making. In the antidoping world, at least four readings are currently required by the World Anti Doping Agency (WADA) for the detection of abnormal variations of the T/E ratio, with no knowledge of the rate of false positives. Note that a precise valuation of the specificity is important in forensic toxicology, because a very low false-positive rate is demanded in order to prevent the accusation of an innocent individual. Finally, with biomarkers of blood doping, there is currently no procedure that has a specificity that does not depend on the number of test results. It is believed that “at least six samples for the baseline reading and possibly considerably more” are needed to derive cutoff thresholds that take into account the intraindividual variability of the biomarker. It was developed a test that compares sequential measurements of a biomarker against previous readings performed on the same individual.
A probability mass function expresses prior information on interindividual variations of intraindividual parameters. Then, the model progressively integrates new readings to more accurately quantify the characteristics of the individual. The idea is to use prior knowledge on interindividual variations of intraindividual parameters, and to progressively integrate previous readings to more accurately quantify the characteristics of the individual. Each new measurement of the biomarker is compared to a critical range. Before the first measurement on the individual, the critical range is derived from the population only. Then, this range progressively adapts itself as the number of readings performed on the same subject increases to finally characterize a particular individual only as the number of readings becomes very large. The rate of false positives does not vary with the number of previous test results. The specificity is independent of the number n of previous test results, with a model that gradually evolves from population-derived limits when n = 0 to individual-based cutoff thresholds when n is large. We applied this model to detect abnormal values in an athlete's steroid profile characterized by the testosterone over epitestosterone (T/E) marker. A cross-validation procedure was used for the estimation of prior densities as well as model validation. The heightened sensitivity/specificity relation obtained on a large data set shows that longitudinal monitoring of an athlete's steroid profile may be used efficiently to detect the abuse of testosterone and its precursors in sports. Mild assumptions make the model interesting for other areas of forensic toxicology [06032].

**Decision limit (CCalpha) and detection capability (CCbeta)**

Initially in the Decision 2002/657/EC the criteria for the calculation of the decision limit (CCalpha) and the detection capability (CCbeta) have been estimated as purely quantitative (alpha-error is 1 % and beta-error is 5 %). In 2004, the European Commission has issued a document to provide guidance for the interpretation of the 2002/657/EC. In this document it is mentioned that also qualitative criteria should be fulfilled. Therefore, the calculated CCalpha and CCbeta must be verified by using fortified samples. The method should be able to detect/identify the target component in 50 percent of the cases at CCalpha and in 95 percent of the cases at CCbeta. Analytical methods for the analysis of nitroimidazoles, nitrofurans and corticosteroids with LC-MS/MS have been validated by fortifying blank samples below and above the MRPL. CCalpha and CCbeta were calculated using the ISO 11843 approach. In addition, the frequency of methodical compliance for the qualitative criteria was determined at each concentration level. It was observed that at the calculated CCalpha and CCbeta levels the qualitative criteria were not fulfilled. It was concluded that the detection capability of the analytical method should be calculated by using decreasing fortification levels at and below the MRPL. A protocol validating methods for banned substances by limiting the number of samples is presented and the qualitative criteria for the assessment of CCalpha and CCbeta were verified based on the same set of data without the need of performing additional validation experiments [06035].

**Prediction of future doping**

The World Anti-Doping Agency (WADA) publishes the Prohibited List, a manually compiled international standard of substances and methods prohibited in-competition, out-of-competition and in particular sports. It would be ideal to be able to identify all substances that have one or more performance-enhancing pharmacological actions in an automated, fast and cost effective way. Here, it was used experimental data derived from the ChEMBL database (7,000,000 activity records for 1,300,000 compounds) to build a database model that takes into account both structure and experimental information, and use this database to predict both on-target and off-target interactions between these molecules and targets.
relevant to doping in sport. The ChEMBL database was screened and eight well populated categories of activities (Ki, Kd, EC50, ED50, activity, potency, inhibition and IC50) were used for a rule-based filtering process to define the labels "active" or "inactive". The "active" compounds for each of the ChEMBL families were thereby defined and these populated our bioactivity-based filtered families. A structure-based clustering step was subsequently performed in order to split families with more than one distinct chemical scaffold. This produced refined families, whose members share both a common chemical scaffold and bioactivity against a common target in ChEMBL. It was thus used the Parzen-Rosenblatt machine learning approach to test whether compounds in ChEMBL can be correctly predicted to belong to their appropriate refined families. Validation tests using the refined families gave a significant increase in predictivity compared with the filtered or with the original families. Out of 61,660 queries in an Monte Carlo cross-validation, belonging to 19,639 refined families, 41,300 (67 %) had the parent family as the top prediction and 53,797 (87 %) had the parent family in the top four hits. Having thus validated our approach, we used it to identify the protein targets associated with the WADA prohibited classes. For compounds where we do not have experimental data, we use their computed patterns of interaction with protein targets to make predictions of bioactivity. It was hoped that other groups will test these predictions experimentally in the future [13081].
ATHLETE BIOLOGICAL PASSPORT (ABP)

Overviews and background

The illicit routes to enhanced oxygen transfer capacities in athletes are manifold and the provision of evidence has been a considerable challenge for doping control laboratories. Comprehensive reviews on accomplishments as well as unsolved issues were reviewed in several recent articles. A central aspect of contemporary efforts towards the determination of autologous blood doping in particular is the Athlete Biological Passport (ABP), which has been employed as an anti-doping tool since 2009 and enabled various convictions of doped athletes during the last three years. The ABP’s principle relies on the intra-individual stability of selected blood parameters such as % reticulocytes (%Ret) and hemoglobin concentration ([Hb]), the long-term variation of which was tested over 4 consecutive competition seasons in elite triathletes. Both parameters were found stable and thus suitable for sports drug testing purposes, although significant variations among female athletes were detected concerning %Ret. Since ABP results must allow for comparison of data with other doping control laboratories, harmonized protocols are important. In that context, the influence of pre-analytical mixing strategies (manual, mechanical mixing, and automated mixing in the analyzer autosampler) on full blood counts was assessed, demonstrating that no significant difference was observed and that 15 min of mechanical shaking as commonly conducted are more than sufficient [13072].

In the last few years, the increasing number of published studies on variations in hematological parameters in elite athletes reflects the growing interest in the athlete’s physiological response to strenuous metabolic effort, especially in the context of the current debate on blood doping and the ABP. Current knowledge of sports physiology and doping biomarkers is formalized in the Athlete Biological Passport (ABP) program: an algorithm tracking the longitudinal record of hematological parameters as a means to define an individual’s hematological profile and thereby identify potential deviations. The central concept of the ABP is that a better appreciation of the physiological changes in the hematological profile related to training, competition, and altitude will allow discrimination of variations induced by illicit practices from those due to homeostatic response to physical activity [13072].

During the last four decades, the main instrument at the disposal of anti-doping authorities has been the detection of prohibited substances in biological samples collected from athletes. However, the availability of substances identical to those produced by the human body, such as EPO, testosterone and GH, necessitated a new drug-testing paradigm. From the early 2000’s, the Athlete Biological Passport (ABP) was proposed as an alternative means to drug testing. Doping leaves a characteristic fingerprint on the biology of the athlete and the ABP is used to prove the act of doping from the detection of that fingerprint. Once a biomarker of doping is implemented in the ABP, it will continue to remain valid and should be able to detect the physiological changes brought on by performance-enhancing drugs that have not yet been invented. However, the sensitivity of the ABP to detect doping is limited if the physiological result of a low level of doping remains within the individual's own reference range. Recent advances in proteomics and metabolomics show the huge potential of the ABP [12040].

Expert evaluation of biological data is a key component of the Athlete Biological Passport approach in the fight against doping. The evaluation consists of a longitudinal assessment of biological variables to determine the probability of the data being physiological on the basis
of the athlete’s on own previous values (performed by an automated software system using a Bayesian model) and a subjective evaluation of the results in view of possible causes (performed by experts). The role of the expert is therefore a key component in the process. Experts should be qualified to evaluate the data regarding possible explanations related to the influence of doping products and methods, analytical issues, and the influence of exercise or pathological conditions. The evaluation provides a scientific basis for the decision taken by a disciplinary panel. This evaluation should therefore encompass and balance all possible causes for a given blood profile and provide a likelihood for potential scenarios (pathology, normal variation, doping) that might have caused the pattern. It should comply with the standards for the evaluation of scientific evidence in forensics. On the basis of their evaluation of profiles, experts might provide assistance in planning appropriate target testing schemes [12041].

The increase of the body’s capacity to transport oxygen is a prime target for doping athletes in all endurance sports. For this purpose, blood transfusions or erythropoiesis stimulating agents (ESA), such as erythropoietin, NESP, and CERA are used. As direct detection of such manipulations is difficult, biomarkers that are connected to the haematopoietic system (haemoglobin concentration, reticulocytes) are monitored over time (Athlete Biological Passport, ABP) and analyzed using mathematical models to identify patterns suspicious of doping. With this information, athletes can either be sanctioned directly based on their profile or targeted with conventional doping tests. Key issues for the appropriate use of the ABP are correct targeting and use of all available information (e.g. whereabouts, cross sectional population data) in a forensic manner. Future developments of the passport include the correction of all concentration-based variables for shifts in plasma volume, which might considerably increase sensitivity. New passport markers from the genomic, proteomic, and metabolomic level might add further information, but need to be validated before integration into the passport procedure. A first assessment of blood data of federations that have implemented the passport show encouraging signs of a decreased blood-doping prevalence in their athletes, which adds scientific credibility to this innovative concept in the fight against ESA- and blood doping [12042].

Steroid profile analyses represent an important resource of information concerning both the administration of natural (endogenous) steroids as well as those of xenobiotic origin. Steroid profiling has been utilized in sports drug testing for more than three decades and still much effort is invested in elaborating and improving this valuable tool, particularly to increase its screening efficiency and to allow for consideration of more recently clarified (genetically or pharmacologically induced) variations influencing the steroid profile interpretation. A central aspect of contemporary efforts towards the determination of autologous blood doping in particular is the Athlete Biological Passport (ABP), which has been employed as an anti-doping tool since 2009 and enabled various convictions of doped athletes during the last three years. The ABP’s principle relies on the intra-individual stability of selected blood parameters such as %reticulocytes (%Ret) and hemoglobin concentration ([Hb]), the long-term variation of which was tested over 4 consecutive competition seasons in elite triathletes. Both parameters were found stable and thus suitable for sports drug testing purposes, although significant variations among female athletes were detected concerning %Ret. Since ABP results must allow for comparison of data with other doping control laboratories, harmonized protocols are important. In that context, the influence of pre-analytical mixing strategies (manual, mechanical mixing, and automated mixing in the analyzer autosampler) on full blood counts was assessed, demonstrating that no significant difference was observed and that 15 min of mechanical shaking as commonly conducted are more than sufficient [12017].

The Athlete Biological Passport (ABP), is a new testing paradigm with immense potential
value in the current climate of rapid advancement in biomarker discovery. It offers the enormous advantage of being independent of this endless pharmaceutical race. In addition to its original aim of providing proof of a doping offense, the ABP can also serve as a platform for a Rule of Sport, with the presentation before competition of the ABP to objectively demonstrate that the athlete will participate in a healthy physiological condition that is unaltered by performance-enhancing drugs. Finally, the decision-support system used today for the biological monitoring of world top-level athletes can also be advantageously transferred to other areas of clinical practice to reach the goal of personalized medicine. The promotion of ethical values and the protection of health in and throughout sports are the primary objectives of the sport movement. In that context, the abuse of substance doping represents the most serious threat to the integrity of modern sports. The World Anti-Doping Code, the reference document that provides the framework for harmonized antidoping rules within sports organizations, has been written to preserve the core values of natural performance, protection of health, and the spirit of the sport. Accordingly, a substance or method is considered for prohibition if it violates at least 2 of these 3 values. Doping triggers physiological changes that provide physiological enhancements. In the same way that disease-related biomarkers are invaluable tools that assist physicians in the diagnosis of pathology, specifically selected biomarkers can be used to detect doping. The primary tool used by sports authorities to ensure a doping-free sport has been the detection of prohibited substances in the biological fluids of athletes, specifically urine and blood. This drug-testing paradigm was introduced in the 1960s and has since been remarkably successful in the detection of substances that are not naturally produced by the body, such as stimulants, narcotics, beta2-agonists, and diuretics. This success is largely attributed to the use of chromatography coupled to mass spectrometry techniques that have revolutionized the detection of a large number of compounds. As a result of advances in biotechnology, the pharmaceutical industry continues to market new drugs at a remarkable pace. A substantial number of these new substances are recombinant proteins or peptides that are strikingly similar in structure, and in some instances absolutely identical, to those naturally produced by the human body. The identification of these substances in biological fluids can be difficult or virtually impossible in some cases. In modern sports, doped athletes are in a constant race with antidoping researchers, who must employ great ingenuity to develop toxicology tests capable of distinguishing exogenous substances from their endogenous counterparts. In addition, detection is further complicated by the medical supervision and increased sophistication of doping protocols. Contemporary protocols are shifting towards long cycles of small microdoses taken repeatedly that are difficult to detect by using conventional drug tests. Worse, designer drugs are currently being produced by black-market laboratories to get around existing drug tests. Consequently, the drug-testing paradigm established in the 1960s cannot prevent elite athletes from doping with impunity when using many potent doping substances such as designer recombinant erythropoietin (rEPO) and designer testosterone. For these reasons, alternative strategies that are independent of this endless pharmaceutical race must be developed to maintain fairness in elite sports [11426]. Biological fluids, such as blood and urine, contain a treasure trove of potential doping markers that can be discovered by today’s omics techniques, such as proteomics and metabolomics. The usefulness of this gold mine for diagnostic purposes has been recognized, and the same is true for doping biomarkers. By definition, any deviation in a biomarker from what is expected in a healthy physiological condition according to well-defined protocols can be attributable only to doping or a medical condition. Interestingly, these two possible causes are the exact targets of any antidoping program; hence, the criteria that are used to introduce new biomarkers into the ABP are the same as those that are used to define a banned substance, more specifically, the criteria of performance and health. In addition, an eligibility rule becomes a logical consequence of this assumption, wherein athletes present their passports at the beginning of a competition and individuals are
allowed to participate only if their passport indicates that they are in a healthy and unaltered physiological condition. Therefore, in addition to proving a doping offense under the World Anti-Doping Code, the ABP can be a platform for a Rule of Sport enforced by the sport authorities to prevent athletes from manipulating their physiology to an extent that would significantly impact their performance and health. It may be foreseen the implementation of a Rule of Sport in which the athletes who have demonstrated unnatural deviations in physiology would be temporarily withdrawn from competition to allow a period for return to normal physiological levels or initiation of appropriate medical controls or treatments. This short period of debarment could also be used by a panel of experts to determine the cause of the abnormality and may lead to sanctioning the athlete for a longer period if doping is the cause [11426].

Although drug tests have been remarkably successful in the detection of synthetic doping substances, the recent availability of doping substances identical to those naturally produced by the human body demonstrates the limits of this testing paradigm to ensure fairness and health protection in elite sports. In that context, the ABP represents the new paradigm in detection of doping-triggered physiological changes in elite sports. Doping biomarkers provide a means to deter the athlete from using performance-enhancing drugs that will lead to deviation from natural baseline values. In contrast to a drug test that returns a result for a precise moment in time and does not have any memory or perspective, the presentation at the beginning of competitions of an ABP that demonstrates normal longitudinal profiles will allow athletes to objectively demonstrate that they will participate in an unaltered physiological condition clear of any doping suspicion. Scientists are developing methods that provide an unequalled opportunity to ensure fairness and the protection of health in elite sports; worldwide ABP implementation is now at the discretion of antidoping organizations. The same paradigm can be used in the clinics so that personalized medicine will not only be centered on the deeper molecular makeup of each patient, but also on an interpretation of existing biomarkers tailored to each individual [11426].

The gatekeepers of fair sport have a whole new way to identify doping: the “athlete biological passport.” An approach that has evolved over the last several years, it is an electronic record of test results of the lingering effects of banned substances in the body, rather than the substances themselves. Like a valid passport required for entry into foreign countries, a valid (clean) biological passport is now required for many athletes to gain entry into elite competitions. Hopes are high among sports federations and antidoping agencies that the biological passport will close some of the biggest loopholes that have let cheaters slip into the Olympics, the Tour de France, and other major events [10008].

One gaping loophole in such tests is that some drugs, like erythropoietin, can only be detected in the body for a few days. But the effects of the drugs last for a week or more, increasing the odds that users will get caught. Another loophole is that banned substances, for a variety of reasons, are sometimes impossible to detect: they can be designed to elude specific tests, new substances can be made for which there are no tests, and a genetic trait—missing copies of a gene called UGT2B17, which makes testosterone soluble in urine—renders testosterone doping invisible to conventional urine tests (the absence of this gene is strongly related to ethnicity: 81 percent of Asians, 22 percent of Africans, and 10 percent of Caucasians are missing both copies). Looking for the physiological footprints left by such drugs, rather than the particular culprits, should reduce these problems [10008].

Few reliable estimates of the prevalence of doping in elite sports have been published. Since 2001, the international governing body for athletics has implemented a blood-testing program to detect altered hematological profiles in the world's top-level athletes. A total of 7289 blood samples were collected from 2737 athletes out of and during international athletic
competitions. Data were collected in parallel on each sample, including the age, gender, nationality, and birth date of the athlete; testing date; sport; venue; and instrument technology. Period prevalence of blood-doping in samples was estimated by comparing empirical cumulative distribution functions of the abnormal blood profile score computed for subpopulations with stratified reference cumulative distribution functions. In addition to an expected difference between endurance and nonendurance athletes, it was found nationality to be the major factor of heterogeneity. Estimates of the prevalence of blood doping ranged from 1 to 48 percent for subpopulations of samples and a mean of 14 percent for the entire study population. Extreme cases of secondary polycythemia highlighted the health risks associated with blood manipulations. It was concluded that when applied at a population level, in this case the population of samples, hematological data can be used to estimate period prevalence of blood doping in elite sports. It was found that the world’s top-level athletes are not only heterogeneous in physiological and anthropometric factors but also in their doping behavior, with contrasting attitudes toward doping between countries. When applied at the individual level, the same biomarkers, as formalized in the Athlete Biological Passport paradigm, can be used in analysis of the observed different physiological characteristics and behavioral heterogeneities [11130].

In 1996 several sports federations introduced an upper limit for hemoglobin and hematocrit. Violation did not lead to a doping conviction, but a temporary suspension for health reasons. By recording the hematological parameters over time and analyzing the data with a scoring system based on Bayesian probability theory it is possible to determine whether or not the changes are physiological. This model is called the Athlete's Biological Passport (ABP). Thus, the Athlete Biological Passport (ABP) is principally founded on monitoring an athlete's biological variables over time, to identify abnormal biases on a longitudinal basis. Several factors are known to influence the results of these markers. However, the manner in which the altitude factor is taken into account still needs to be standardized. Causal relationships between haematological variables should be correctly integrated into ABP software. In particular, modifications of haematological parameters during and after exposure to different altitudes/hypoxic protocols need to be properly included within detection models [13072].

Individualized statistics

The relevance of indirect biomarkers persisted even after the introduction of a direct test for rhEPO abuse because the time frame of direct rhEPO detection was short and other forms of blood doping such as transfusions are believed to have been revived by fraudulent athletes in that time. Even with the availability of a direct test for homologous transfusions biomarkers may be used to reveal manipulations induced by blood transfusions regardless of the origin. It is clear now that the biomarker approach bears another advantage. These markers are already sensitive to any future compound that elicits a similar physiological response, such as the increase of the oxygen carrying capacity. Therefore, the concept of a hematologic passport was developed further and is presented in detail in this section. Early subject-specific reference ranges were defined for Hb and Hct to encourage the hematologic passport in a global strategy to deter blood doping. The main theory behind this concept is that each athlete provides individual reference values that allow a longitudinal analysis by applying various algorithms [13006].

Owing to high individuality, the use of absolute reference ranges for hematological parameters is not really useful for monitoring athletes. However, as the analytical and intra-individual biological variability of most hematological parameters are both contained, the definition of a type of “hematological passport” would allow a longitudinal comparison of data
for individual patients, accomplished with major transferability among clinical and antidoping laboratories. So far, this appears suitable for detecting a variety of blood doping practices, and would also be acceptable in practice, considering that the individuality index of most hematological parameters (ratio between intra- and inter-individual biological variability) is always -0.6. The current availability of fully automated hematological systems can easily provide the traditional parameters of the hematological profile, along with a wide range of additional parameters for reticulocytes and erythrocytes, increasing the potential use of laboratory testing in clinical and sports medicine. Repeated evaluation over a period of time of several of these parameters, including hemoglobin, hematocrit, reticulocyte count and indexes, would define a highly specific hematological profile, which is supposed to remain relatively stable over time. At least five sequential determinations should be obtained to define a reliable subject-specific reference range; substantial variations from the baseline, or any value exceeding the allowable variation, could highlight either pathologies or unfair practices, in both cases providing a good reason for an athlete’s withdrawal from competition. Same major drawbacks of this approach, including collecting samples at altitude, sample manipulation or the use of plasma expanders, and the type of instrument used to measure reticulocytes, can be prevented by implementation of standardized analytical protocols, exclusion of values obtained from samples collected at altitude, specific instrument calibration, etc. A basic problem is that competitive athletes display significant differences in hemoglobin, erythrocytes, hematocrit and mean corpuscular volume compared to the sedentary population, whereas other erythrocyte indexes, reticulocyte counts and reticulocyte parameters appear to be less influenced by lifestyle [06005].

Personalized monitoring of biomarkers for doping

The Athlete Biological Passport (ABP) is an individual electronic document that collects data regarding a specific athlete that is useful in differentiating between natural physiologic variations of selected biomarkers and deviations caused by artificial manipulations. A subsidiary of the endocrine module of the ABP that which here is called Athlete Steroidal Passport (ASP), collects data on markers of an altered metabolism of endogenous steroidal hormones measured in urine samples. The ASP aims to identify not only doping with anabolic-androgenic steroids, but also most indirect steroid doping strategies such as doping with estrogen receptor antagonists and aromatase inhibitors. Development of specific markers of steroid doping, use of the athlete’s previous measurements to define individual limits, with the athlete becoming his or her own reference, the inclusion of heterogeneous factors such as the UDPglucuronosyltransferase B17 genotype of the athlete, the knowledge of potentially confounding effects such as heavy alcohol consumption, the development of an external quality control system to control analytical uncertainty, and finally the use of Bayesian inferential methods to evaluate the value of indirect evidence have made the ASP a valuable alternative to deter steroid doping in elite sports. The ASP can be used to target athletes for gas chromatography/combustion/ isotope ratio mass spectrometry (GC/C/IRMS) testing, to withdraw temporarily the athlete from competing when an abnormality has been detected, and ultimately to lead to an antidoping infraction if that abnormality cannot be explained by a medical condition. Although the ASP has been developed primarily to ensure fairness in elite sports, its application in endocrinology for clinical purposes is straightforward in an evidence-based medicine paradigm [10007].

In elite sports, the growing availability of doping substances identical to those naturally produced by the human body seriously limits the ability of drug-testing regimes to ensure fairness and protection of health. The Athlete Biological Passport (ABP), the new paradigm in testing based on the personalized monitoring of biomarkers of doping, offers the enormous
advantage of being independent of this endless pharmaceutical race. Doping triggers physiological changes that provide physiological enhancements. In the same way that disease-related biomarkers are invaluable tools that assist physicians in the diagnosis of pathology, specifically selected biomarkers can be used to detect doping. The ABP is a new testing paradigm with immense potential value in the current climate of rapid advancement in biomarker discovery. In addition to its original aim of providing proof of a doping offense, the ABP can also serve as a platform for a Rule of Sport, with the presentation before competition of the ABP to objectively demonstrate that the athlete will participate in a healthy physiological condition that is unaltered by performance-enhancing drugs. Finally, the decision-support system used today for the biological monitoring of world top-level athletes can also be advantageously transferred to other areas of clinical practice to reach the goal of personalized medicine [11126].

**Technical specifications necessary**

The prerequisite of valid biomarker data for objective interpretation and legal use is a strict process for sample collection, transport, and analysis that has been put into place for the ABP. For instance, sample collection must not occur within 2 h after training or competition and must be carried out after the athlete has remained in a seated upright position for 10 min with feet on the floor in order to allow the vascular volumes to equilibrate. In addition, important minimum information should be included on the ABP control form to review blood data in an individual context such as and among others blood loss in the 3 months preceding each sample collection, use of simulated hypoxic conditions (e.g. altitude house, tents), or exposure to altitude above 1,000 m above sea level. Furthermore, technical documents of the ABP guideline define the transport and storage conditions, e.g., the type of storage device, the necessity of a storage temperature data logger, and the rapid transport so that analysis can ideally be performed within 36 h of sample collection, although there is growing evidence that this time frame might be extended. Samples should only be analyzed in WADA-accredited or WADA-approved laboratories or their satellite facilities which must all be subject to strict regular internal and external quality control procedures and follow the WADA international standard of laboratories [13006].

Current ABP guidelines describe the particular importance of an athlete passport management unit (APMU) that is responsible for the administrative management of the ABP. This includes advising anti-doping organizations about intelligent, targeted testing, liaising with the expert panel, compiling an ABP documentation package, and reporting adverse analytical findings to anti-doping organizations and WADA. It seems essential that APMU personnel has profound knowledge of the ABP concept and indirect doping detection methods to ensure the intelligent, targeted approach that helps to avoid a waste of organizational and financial resources. It can be postulated that a main goal is to avoid parallel passport data collected by different stakeholders such as national anti-doping organizations (NADO) or governing bodies of each sport. Therefore, harmonization of data storage with mutual sharing of results that were analyzed with strict adherence to the WADA protocol is important to use the ABP in its full capacities. The ADAMS (anti-doping administration and management system) platform could provide a system for all stakeholders for the planning, collection, and evaluation of all ABP data although the exact responsibility of each stakeholder remains to be defined. For instance, NADOs could emphasize the testing of a young and/or nationally active athlete but as the athlete moves on to a regular international competition setting, testing could be taken over by the international sporting federation [13006].

*Still an interpretation*
Furthermore, it is prudent to consider that blood values may always be subject to biological variability (e.g. gender, age) – heterogeneous factors – and confounding factors such as physical activity (e.g. type of sport, competition vs training, or exposure to altitude). Some of these factors may change over time, such as altitude or the type of instrument used, and then influence longitudinal monitoring. Yet, time-independent or fixed factors such as ethnic origin or gender are specific for a given athlete. It has also been considered heterogenous factors and proposed a model based on a global Bayesian inference approach for the detection of abnormal blood values over time. In such a Bayesian network, the causal relationship between a doping activity and the induced alterations of blood markers is represented as probabilities where every causal relationship is itself a model represented by a conditional probability density function. WADA and other sports federations observed the growing scientific knowledge of longitudinal subject-specific monitoring of blood values until, in 2008, UCI was the first sports organization to introduce the hematological module of the ABP to detect blood doping. WADA followed in December 2009 and approved the first version of the WADA ABP operating guidelines which have since been revised. Nowadays, the hematological module of the ABP includes the longitudinal monitoring of eight hematological markers (Hct, Hb, RBC, reticulocyte count (#retics), %retics, MCV, MCH, and MCHC) to identify abnormal patterns with a subject becoming her or his own reference. From some of the hematological markers additional models are calculated: OFF-hr and ABPS (containing 7 markers). According to the WADA ABP guidelines the application of the “adaptive model” predicts an expected range for an individual within which a series of marker values falls assuming a normal physiological condition. Outliers correspond to values out of the 99.9 percent range (0.05-99.95 percentiles) and warrant further attention and review. Anti-doping organizations may select a value lower than 99.9 percent to identify atypical samples and/or profiles that warrant further investigation. An expert chosen by the responsible anti-doping organization shall initially review atypical values or an atypical longitudinal profile. This expert shall evaluate the anonymous passport data and respond on the basis of four hypotheses that will trigger the indicted further action:

- the measured values can be considered natural variation and thus normal: normal testing pattern is continued
- the measured values are suspicious: further data are required and target tests shall be performed
- the measured values may be the result of the use of a prohibited substance or prohibited method: two further experts review the passport
- the measured values are indicative of a pathological condition: information of the athlete or involvement of two further experts.

As the role of scientific experts in doping cases based on indirect evidence will considerably gain in importance, an emphasis should be made in regular scientific exchange and continuous education of the experts [13006].

**An international tool**

The ABP introduces a new form of doping evidence and, as such, paves the way for a more global and integrated fight against doping. In particular, we foresee a global forensic approach in which multiple pieces of evidence, not restricted to those in the current testing paradigm, are used to demonstrate the culpability of a suspect. For example, the drug enforcement agencies and customs departments of many countries seize large quantities of doping substances with investigations that target illicit drugs, manufacturing companies, and
trafficking networks. Until recently, the lack of collaboration between governmental, public, and sports authorities has hindered the combination of analytical and nonanalytical evidence in many countries. In the current fight against doping, a customs agency can learn that a top-level athlete has received some rEPO by mail before an important competition, but this information is not shared with sports authorities, so that the athlete is still permitted to participate in that competition. Interestingly, the methodology developed for the ABP provides the necessary framework to combine evidence gathered by sports-testing organizations with nonanalytical evidence gathered by public law enforcement agencies. For example, knowledge that an athlete received some rEPO by mail can be combined with the information stored in the ABP to evaluate whether the athlete used that substance before competition. As such, we do not foresee any scientific limitation for a global fight against doping that is based on various sources of evidence [11426].

A handful of sports federations have used the biological passport on a trial basis, but it is becoming more widespread because the World Anti-Doping Agency, which leads the international effort against banned sports enhancement, just released guidelines on its use. The U.S. Anti-Doping Agency and similar groups in the United Kingdom and Norway have adopted the passport for elite athletes in those countries. To prevent false positives, information is collected about legitimate activities that can affect the readings, such as blood donation during the previous six months, medications or supplements taken, and training [10008].

Three complementary items of ABP

Three distinct modules can be distinguished in the ABP: the hematological, steroidal, and endocrinological modules. The hematological module of the ABP aims to detect any form of blood doping. As part of a full blood count, 8 hematological variables are considered today in this module. In 2008, the Union Cycliste Internationale was the first sports organization to implement the hematological module of the ABP to deter blood doping in elite cycling, and subsequently, several riders have been prosecuted and sanctioned on the sole basis of their abnormal hematological profiles. Currently, hematological tracing is performed by several antidoping organizations for several thousand athletes worldwide. The steroidal module of the ABP, which aims to detect direct and indirect forms of doping with anabolic agents, is presently being finalized for implementation in the near future. The endocrinological module of the ABP aims to detect doping with growth factors, such as growth hormone and insulin growth factor-1. Despite abundant scientific publications on growth hormone-dependent markers, the implementation of the endocrinological module in the ABP in the network of WADA-accredited laboratories requires further validation to fulfill forensic standards [11426].

Aiming for three complementary items, the ABP will consist of a hematological, a steroidal, and an endocrinological module with the hematological module aiming to detect any form of blood manipulation. The fact that the ABP relies on indirect evidence for an anti-doping rule violation, i.e. changes in parameters that are not consistent with natural variations as determined in extensive validation studies rather than the detection of a prohibited substance, raised concerns about the validity of the approach and led to several (scientific) retorts and discussions [12016].

Hematological parameters
The longitudinal tracking and recording of eight hematological parameters should provide a fingerprint for both the doped and the non-doped athletes, thus representing an approach that anti-doping organizations have supported in its pioneering endeavour to demonstrate the use (rather than the presence) of a prohibited substance or method of doping. Limitations were reported concerning the sensitivity of the ABP analytical approach in case of EPO microdosing, where a 12-week intravenous EPO injection intervention remained undetected in ten subjects. The necessity to improve the current approaches was stressed (particularly in light of the required logistics and costs concerning the ABP) and anecdotal evidence that athletes might have adapted to the “new situation” was presented. Complementary to the existing algorithms employing predominantly the blood parameters hemoglobin concentration ([Hb], g/l), hematocrit (Hct), and %reticulocytes (e.g. in the OFF-hr score model) the utility of haemoglobin mass particularly in combination with %reticulocytes was evaluated in an original mathematical model. In addition to the subject-based interpretation of blood parameters collected in the course of ABP programs between 2001 and 2009, the utility of the available data concerning the estimation of the blood doping prevalence among elite track and field athletes has been described. Evaluating the empirical cumulative distribution functions of the abnormal blood profile scores in comparison to stratified reference cumulative distribution functions differences in blood doping prevalence were observed to be connected to the type of sports (endurance vs. non-endurance) and, to the athletes' nationality. That study revealed that the average prevalence of blood doping in the investigated population is to be estimated at 14 percent [12016].

Regarding blood doping, the traditional analyses based on the detection of a substance in biologic fluids have major limitations. Presently, only the misuse of allogeneic blood can be directly detected, whereas retransfused autologous blood is not detectable. There is a plethora of novel ESAs that are difficult to uncover. To overcome the detection problems, the “Athletes Biologic Passport” has been developed, which is based on the monitoring of selected RBC parameters. Blood doping may be suspected, when these parameters change in a nonphysiologic way. There are methodologic problems because of the lack of clear standardization and harmonization in antidoping testing. The longitudinal evaluation of several hematologic variables needs high comparability among various analytical technologies used by the different accredited laboratories. Although some parameters (i.e. concentration of hemoglobin and hematocrit) are comparable when measured on different instrumentations, others (i.e. percentage of macrocytes or reticulocytes parameters) are peculiar. This bears the risk of false-positive results in athletes. On the other hand, when 400 blood samples obtained from 24 subjects receiving rhEpo injections were screened by the passport parameters, 42 percent of the subjects were not identified as rhEpo doped. The statistical approach for evaluating the passport data are focused on the biologic variation of hematologic values. Critical experts in the analysis of laboratory data have argued that antidoping tests are based on fraud statistics. Traditional antidoping analyses are based on the detection of a substance in biologic fluids (“Adverse analytical finding”). This approach has major limitations in regard to blood doping. As outlined in the previous section, autologous blood cannot be detected, there is a plethora of ESAs, the detection window is limited, and there is urine manipulation. Some sports federations earlier introduced upper concentration of hemoglobin and Hct limits to escape from this dilemma. Athletes tested above the limits were declared unfit for competition (“No-start rule”). However, concentration of hemoglobin and hematocrit are influenced by external factors, such as body posture, exercise, or residence at altitude. In addition, “clean” athletes can have naturally high hemoglobin and hematocrit values. A large retrospective study on male blood donors in Denmark revealed that 3.9 percent of nonathletes and 10.4 percent of elite rowers had hematocrit values more than 0.51 (i.e. above the recommended limits for athletic competition). Hematologic parameters depend on ethnicity, age, and gender. Some blood parameters, such as the concentration of Epo and reticulocytes (Ret), increase on
administration of ESAs (ON-score), whereas they decrease after RBC transfusion or after the cessation of ESA administration (OFF-score). The “Abnormal Blood Profile Score” (not presently used for the assessment of abnormal blood profiles based on the passport data) regards additional red cell parameters, including the mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular hemoglobin mass (MCH), Ret counts, serum Epo, and soluble transferrin receptor (sTfR).95 Algorithms have been used that are sensitive during one of the two phases, with ON-score being sensitive during ESA treatment and OFF-score during the cessation phase. Having become effective in December 2009, the “Athlete Biologic Passport Operating Guidelines” equip Anti-Doping Organizations with a framework in which to pursue antidoping rule violations in accordance with Article 2.2. of the World Anti-Doping (WAD) Code (“Use or At tempted Use by an Athlete of a Prohibited Substance or a Prohibited Method”). The guidelines include mandatory requirements for collection, transportation, analysis of blood samples, and results management. The following markers are considered in the Athlete Biologic Passport hematologic module: Hct, Hb, RBC count, reticulocyte percentage, reticulocyte number, MCV, MCH, MCHC, and OFF-hr score (Index of stimulation derived from a formula. In addition, parameters of interest can be the mean Ret cell volume (MCVr), Ret Hb concentration (MCHCr) and Ret Hb content (MCHr), as measured by flow cytometry as in clinical routine. The results reported to the WADA are processed by an “Adaptive Model” that identifies abnormal blood parameter changes related to the athlete’s individual profile. In particular, concentration of hemoglobin or OFF-hr score abnormalities with a 99.9 percentage probability or more will be reviewed by experts [11428].

The development and validation of blood-doping biomarkers have also greatly evolved since the introduction of hematological variables by some international sports federations in the mid-1990s. With the advent of automated blood analyzers, blood variables can be quantitatively measured to yield a complete hemogram, either in an accredited laboratory or directly at the location of the competition, in less than a minute after blood collection. Several approaches have contributed in recent years to make the use of biomarkers of altered erythropoiesis an efficient approach to deterring any form of blood doping in sports:

- the introduction of multiparametric blood-doping markers
- the inclusion of heterogeneous factors, such as sex and age, as recommended by the WHO in the diagnosis of anemia as well as other factors specific to sports
- the add-on of potentially confounding factors, such as the athlete’s exposure to altitude
- the record of the athlete’s own previous measurements, with the underlying concept being the use the athlete as his or her own reference
- the adoption of standardized protocols for sample collection and analysis, in addition to the extensive use of external QC systems to control analytical uncertainty
- the development and validation of probabilistic inference techniques to evaluate the value of doping evidence

All of the knowledge that has been acquired in recent decades concerning doping biomarkers has been formalized in the athlete biological passport (ABP) program. The term passport was first proposed in the early 2000s when the preservation and tracking of a longitudinal record of hematological variable measurements were planned to be used as a means to define an individual’s hematological profile. Large disparities between an athlete’s historical values and the values obtained in a recent test indicate that either doping has taken place or that the athlete has a potential medical condition that requires closer examination. The concept of the ABP has been discussed and then further elaborated for antidoping application by the World Anti Doping Agency (WADA) beginning in 2002. Since the 2006 Torino Winter Olympic Games, several international sports federations have agreed that the WADA should
harmonize the development and validation of the ABP program. As a result, in 2009, the WADA published the Athlete Biological Passport Operating Guidelines, which can be used as a reference for any antidoping organizations that are interested in developing a concordant biological monitoring program [11426].

The World Anti-Doping Agency has implemented the Blood Passport in attempt to detect blood doping in athletes. The Blood Passport looks for uncommon changes overtime in reticulocytes percentage (Ret %), as a variable of the OFF-hr score, and hemoglobin concentration reflecting potential doping violations. Few studies, however, have actually investigated the concurrent stability of Ret % and hemoglobin concentration in athletes over extended periods of time, none of which were measured in athletes who undergo strenuous and prolonged physical exercise. Measurements of Ret % and hemoglobin were assessed over the course of four competitive seasons in elite triathletes (10 males and seven female). Blood was obtained at the start of the season, precompetitive period, competitive period and at the end of the competitive period. Significant differences were observed in both hemoglobin concentration and Ret % between genders and there was a high variability between subjects. Neither males nor females exhibited differences in hemoglobin across all periods within one season. Within gender, analysis revealed that Ret % varied significantly between periods only in female athletes. It was thus concluded that Ret % and hemoglobin concentration remain stable over four consecutive seasons in elite triathletes, confirming that both parameters are valid for antidoping purposes based on the Blood Passport. In addition, Ret % fluctuations within one season require further investigation in females [11127].

The Athlete’s Biological Passport (ABP) is an evaluation of hematological parameters, hemoglobin (Hb), reticulocytes (Ret), and their combination in the OFF-score. Recently, the Court of Arbitration for Sport accepted it as a suitable indirect method for detecting blood doping. There are various topics which are not defined and scientifically completely explained in ABP, limiting its effectiveness as evidence and as suspect of blood manipulation. The data source the ABP used for designing a profile is unclear. The variance used for cyclists is not correct. The covariables which should be calculated together with the measures of Hb and Ret are not always considered in the statistical program. The pre-analytical warnings for correct and valid collection, transport, and storage of the specimens are not assured. Quality control of the instruments is not completely assured. Analytical variability is not appropriately considered in the program. The seasonal changes of the hematological parameters, due to training and competitions, are not calculated. Statistical analysis, based on a Bayesian-like program, not available to the scientific community, does not follow the classical decision-making approach of medicine and science. Therefore, the ABP needs of additional evidences and of scientific debate [11128].

The Athlete Blood Passport is the most recent tool adopted by anti-doping authorities to detect athletes using performance-enhancing drugs such as recombinant human erythropoietin (rhEPO). This strategy relies on detecting abnormal variations in haematological variables caused by doping, against a background of biological and analytical variability. Ten subjects were given twice weekly intravenous injections of rhEPO for up to 12 weeks. Full blood counts were measured using a Sysmex XE-2100 automated hematology analyser, and total hemoglobin mass via a carbon monoxide rebreathing test. The sensitivity of the passport to flag abnormal deviations in blood values was evaluated using dedicated Athlete Blood Passport software. Our treatment regimen elicited a 10% increase in total haemoglobin mass equivalent to approximately two bags of reinfused blood. The passport software did not flag any subjects as being suspicious of doping whilst they were receiving rhEPO. It was conclude that it is possible for athletes to use rhEPO without eliciting abnormal changes in the blood variables currently monitored by the Athlete Blood Passport [11131].
Manipulation of the blood's oxygen carrying capacity (CaO$_2$) through reinfusion of red blood cells, injections of recombinant erythropoietin or by other means results in an increased maximal oxygen uptake and concomitantly enhanced endurance performance. Therefore, there is a need to establish a system – "A Blood Pass" – through which such illegal and unethical methods can be detected. Venous blood samples were taken under standardized conditions from 47 male and female Swedish national and international elite endurance athletes four times during the athletic year of the individual sport (beginning and end of the preparation period and at the beginning and during peak performance in the competition period). In these samples, different hematological values were determined. ON(hes) and OFF(hre) values were calculated according to the formula of Gore et al. A questionnaire regarding training at altitude, alcohol use and other important factors for hematological status was answered by the athletes. There were some individual variations comparing hematological values obtained at different times of the athletic year or at the same time in the athletic year but in different years. However, the median values of all individual hematological, ON(hes) and OFF(hre), values taken at the beginning and the end of the preparation or at the beginning and the end of the competition period, respectively, as well as median values for the preparation and competition periods in the respective sport, were all within the 95% confidence limit (CI) of each comparison. It must be mentioned that there was no gender difference in this respect. This study shows that even if there are some individual variations in different hematological values between different sampling times in the athletic year, median values of important hematological factors are stable over time. It must be emphasized that for each blood sample, the 95% CI in each athlete will be increasingly narrower. The conclusion is that there is a physiological basis for establishing an individual-based "Blood Pass" system, mainly for athletes competing at the international level. On indications of manipulations of hemoglobin concentration and red cell mass by deviations from established "Blood Pass" data, more specific methods can be applied [07023].

Indirect markers of altered erythropoiesis can provide enough evidence to differentiate between natural variations and blood doping. Forensic techniques for the evaluation of the evidence, and more particularly Bayesian networks, allow antidoping authorities to take into account firstly the natural variations of indirect markers – through a mathematical formalism based on probabilities – and secondly the complexity due to the multiplicity of causes and confounding effects, through a distributed and flexible graphical representation. The information stored in an athlete's biological passport may be then sufficient to launch a disciplinary procedure against the athlete. The strength of the passport is that it relies on a statistical approach based on sound empirical testing on large populations and justifiable protocols. Interestingly, its introduction coincides with the paradigm shift that is materializing today in forensic identification science, from archaic assumptions of absolute certainty and perfection to a more defensible empirical and probabilistic foundation [10010].

Following the doping scandals at the World Championships in cross-country skiing in 2001, the International Ski Federation decided to generate individual blood profiles. From 2001 to 2007, 7081 blood samples from 1074 male and female elite cross-country skiers were collected and analyzed for hemoglobin concentration and % reticulocytes. Data were applied to blood algorithms wherefrom blood model scores were calculated. From 1997-1999 to 2001-2002, the mean hemoglobin concentration was reduced by 0.9 g/dL to 15.3 g/dL in male skiers and by 0.4 g/dL to 13.8 in female skiers. From 2002-2003 to 2006-2007, the combination of increases in hemoglobin concentration and decreases in % reticulocytes led to pronounced increases in mean OFF-model scores. Hemoglobin concentration was 0.2 g/dL higher at Olympic Games/World Championships (WOCs) than at World Cups competitions <4 weeks before and after WOCs. Hemoglobin concentration and % reticulocytes increased with altitude in both genders. Since the introduction of an enlarged
blood testing program, the mean hemoglobin concentration values were lowered to close to normal levels, but over the last 2-3 years there has been a small elevation and an increase in OFF-model scores, which may indicate a change in the manipulations used to elevate the hemoglobin concentration [08211].

The aim of one study was to investigate an indirect method based on a determination of absolute norms of variation in biological markers that could be used to identify autologous blood transfusion within the framework of the fight against doping. The selection of markers was made from experimental variations obtained during different phases including an increase in training volume at sea level, high altitude training, blood withdrawal and autologous blood reinfusion. The global statistical method was then developed in order to fix absolute norms of variation for each selected marker. The markers selected were haematocrit (Hct), haemoglobin concentration (Hb), stimulation index (Off-hr) and the absolute norms of variation (normDelta) established for a maximal 15 days period were normDeltaHct(0-15) >6%, normDeltaHb(0-15) >4% and normDeltaOff-hr(0-15) >20 percent. From analyses between two blood samples spaced at an interval of maximum 15 days, this method allows to show "abnormal" variation when a variation for one of the selected markers is strictly superior to the absolute norms of variation established. The legal framework for an immediate application of this method could be that of the internal regulations implemented by each international federation in accordance with the health policy in vigour [08212].

An efficient antidoping test designed to obtain direct proof of allogeneic blood transfusion was developed and validated. This test, based on flow cytometry analysis of red blood cell (RBCs) phenotypes, was used to determine the absence or the presence of numerous RBCs populations in a blood sample. A such, it may constitute a direct proof of an abnormal blood population resulting from homologous transfusion. Single-blind and single-site studies were carried out to validate this method as a forensic quality standard analysis and to allow objective interpretation of real cases. The analysis of 140 blood samples containing different percentages (0-5 %) of a minor RBCs population were carried on by four independent analysts. Robustness, sensitivity, specificity, precision and stability were assessed. ISO-accredited controls samples were used to demonstrate that the method was robust, stable and precise. No false positive results were observed, resulting in a 100 percent specificity of the method. Most samples containing a 1.5 percent minor RBCs population were unambiguously detected, yielding a 78 percent sensitivity. These samples mimicked blood collected from an athlete 3 months after a homologous blood transfusion event where 10 percent of the total RBCs present in the recipient originated in the donor. The observed false negative results could be explained by differences in antigen expression between the donor and the recipient. False negatives were more numerous with smaller minor RBCs populations. The method described here fulfils the ISO-17025 accreditation and validation requirements. The controls and the methodology are solid enough to determine with certainty whether a sample contains one or more RBCs populations. This variable is currently the best indicator for homologous blood transfusion doping [08213].

The aim of one study was to evaluate physical performance loss and underlying mechanisms following voluntary blood donation. Eleven voluntary subjects (four female) completed a symptom-limiting cardio-pulmonary exercise test before and after blood donation (500 mL blood). The haemoglobin value significantly decreased by 1.2 mg/dL (9 %), maximal oxygen uptake by 9 percent, maximal work rate by 13 percent and duration of exercise fell from 663 down to 607 seconds. Anaerobic transition occurred at 81 percent and 72 percent of maximal oxygen uptake before and after blood donation, respectively, which was a significant difference. Subjects who practise recreational endurance sports appear to be more effected by endurance loss. The haemoglobin value was the only significant predictor of maximal oxygen uptake in regression analysis. It was concluded that maximal physical performance is
impaired after blood donation. Haemoglobin decline accounts for the decreased oxygen uptake. As a consequence thereof the anaerobic transition occurs earlier. Subjects not engaged in regular sports activity did not experience a decline in their capacity [08214].

Allogeneic transfusions are normally mismatched at one or more minor blood group antigens. The most sensitive and accurate method known to detect this form of blood doping is flow cytometry. Low percentages of antigen-positive and antigen-negative red blood cells (RBCs) can be quantitated using suitable specific alloantibodies and careful analysis. By testing blood samples taken at various times, a reduction in the percentage of a minor population of RBCs will indicate transfusion has occurred [08215].

Hemoglobin (Hb) and hematocrit (Hct) are measured as indirect markers of doping in athletes. We studied the effect of posture on these parameters in a typical antidoping setting. Venous blood samples were obtained from nine endurance athletes (six males, three females) and nine control subjects (six males, three females) immediately and after 5, 10, 15, 20 and 30 min after having adopted a seated position from normal daily activity. Hb (CV 0.72 %) and Hct (CV 0.87%) were determined using an automated cell counter, plasma volume changes were calculated. Differences between the time points, gender and groups were calculated using a mixed-model procedure. Significant changes were observed in the first 10 min after sitting down but no further changes were noted between 10 and 30 min. Mean directional change for Hb and Hct between 0 min and the average of the period from 10 to 30 min was -2.4% (-0.35 g/dL) for Hb and -2.7% (-1.2%) for Hct. Plasma volume increased accordingly. Neither group nor gender had significant effects. Under typical conditions encountered during blood testing in doping control, a period of 10 min in a seated position is sufficient for the vascular volumes to re-equilibrate and to adapt to the new posture [10465].

Hemoglobin mass

Coinciding with the agreement on the ABP, it was evaluated three passport approaches for their sensitivity and specificity for the detection of autologous blood transfusion. The best possible marker for lower dosages of transfused blood (e.g. one bag) was OFF-hr. Interestingly, a new score (Hbmr) was introduced and showed the best performance for larger amounts (e.g. three bags), but requires the determination of total hemoglobin mass (Hb mass) as a further measure. Hb mass determined by the optimized CO rebreathing method was first suggested as a potential biomarker in the context of blood doping in 2007 mainly because it is independent of plasma volume fluctuations. The usefulness and applicability in several circumstances were evaluated in several studies thereafter. Hb mass was also evaluated as a marker in the adaptive model of the ABP in a longitudinal blinded study, in which a new score (OFFmass including %retics) was likewise published and yielded a sensitivity of 73 percent without false-positives at the 99.9 percent specificity level. Hb mass was also evaluated for the potential to detect rhEPO misuse. Various efforts have been made to improve the problems associated with the CO rebreathing method such as the administration of a potentially toxic substance and lack of a quality control system. Nevertheless, it seems that these problems limit the applicability of the method in antidoping. Therefore, the search is on for an alternative to Hb mass determination which is compatible with today's standards of testing because of its potential to improve the detection rate of autologous blood transfusion. On such approach could be the indirect modeling of Hb mass from indirect markers [13006].

Animal studies

The extent to which hematocrit is regulated and the impact of altered hematocrit on blood
oxygen transport in avian embryos are largely unknown. Consequently, it was investigated how acute blood removal or Ringer solution injection modified hematocrit in day 15 embryos, and how “blood doping” with erythrocyte-enriched whole blood influenced O\textsubscript{2} consumption in day 15-17 chicken embryos. Mean hematocrit (± s.e.m.) at day 15, 16 and 17 was 27 ± 1 percent, 28 ± 0 percent, and 31 ± 1 percent, respectively. Blood withdrawal (19 increments of 125 ml each, separated by 30 min) caused a progressive fall in hematocrit to approximately 12 percent at day 15. Hematocrit decline was strictly proportional to the extent of blood withdrawal. Incremental Ringer solution injection over an 8 h period, transiently increasing blood volume up to 85 percent over initial values, did not decrease hematocrit, indicating that injected Ringer solution rapidly left the circulating blood compartment. Blood doping with erythrocyte-enriched whole blood artificially elevated hematocrit from 27 percent to 38 percent, but caused no significant change in routine O\textsubscript{2} consumption (0.35-0.39 ml O\textsubscript{2} per min per egg) at any point over the subsequent 6 h period in day 15-17 embryos. It was conclude that hematocrit is not protected acutely in day 15 chicken embryos, with no evidence of erythrocyte sequestration or release. Additionally, at day 15-17, hematocrit increases of approximately 10 percent do not enhance embryonic oxygen consumption, suggesting that blood oxygen carrying capacity per se is not limiting to oxygen consumption [08216].

**Biking**

During the Tour de France 2007, 7 riders were randomly tested on 3 different occasions; the day before the prologue, and 12 and 19 days after the prologue. Blood was drawn into 3 mL EDTA covered tubes and kept at 4 degrees Celsius. They were analyzed within 24 hours. The concentration of hemoglobin and hematocrit were significantly lower on day 12 and day 19 compared to baseline. All 7 riders had lower hemoglobin and hematocrit on day 19 compared to baseline, while this was the case in 6 out of 7 riders already on day 12. The concentration of hemoglobin and hematocrit were 11.5 percent and 12.1 percent lower on day 19 compared to baseline. Whether or not this low value is due to decrease in hemoglobin mass or hemodilution, or the latter solely, increases in hemoglobin and hematocrit during prolonged stage racing seem unphysiological and should therefore lead to further examination of the rider [08217].

**Possible variables**

Blood passport has been suggested as an indirect tool to detect various kinds of blood manipulations. Autologous blood transfusions are currently undetectable, and the objective of this study was to examine the sensitivities of different blood markers and blood passport approaches in order to determine the best approach to detect autologous blood transfusions. Twenty-nine subjects were transfused with either one (n=8) or three (n=21) bags of autologous blood. Hemoglobin concentration ([Hb]), percentage of reticulocytes (%ret) and hemoglobin mass (Hb\textsubscript{mass}) were measured 1 day before and 6 times after reinfusion. The sensitivity and specificity of a novel marker, Hb\textsub{nr} (based on Hb\textsub{mass} and %ret), was evaluated together with [Hb], Hb\textsub{mass} and OFF-hr by different passport methods. The novel Hb\textsub{nr} marker showed superior sensitivity in detecting the highest dosage of transfused blood, with OFF-hr showing equal or superior sensitivities at lower dosages. Hb\textsub{nr} and OFF-hr showed superior but equal sensitivities from 1 to 4 weeks after transfusion compared with [Hb] and Hb\textsub{mass}, with Hb\textsub{mass} being the only tenable prospect to detect acute transfusions. Because autologous blood transfusions can be an acute practice with blood withdrawal and reinfusion within a few days, Hb\textsub{mass} seems to be the only option for revealing this practice [11132].
Erythropoietin

The so-called third-generation tests or the z-score were introduced to distinguish the effect of rhEPO abuse from natural biological fluctuations with longitudinal observations of Hb or OFF-hr. Assuming a universal within-subject variation only two values from one athlete could be used for the calculation of the z-score. In a new approach, it was concluded that longitudinal data might not only be used to ban participation in competition but also to establish target testing of suspicious athletes. This was important as only positive direct test results could be used for sanctioning of manipulating athletes. Progress of the passport concept prevailed as Sottas et al. combined all data contained in a single blood profile of an athlete in a universal multiparametric score (abnormal blood profile score, ABPS) that was initially presented in various versions using between three and 12 different biomarkers responding to either rhEPO administration or blood transfusion. In the current version of the WADA ABP operating guidelines, ABPS calculated from Hct, Hb, RBC count, reticulocyte percentage (RET%), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) is recommended as a marker in addition to OFF-hr. In a universal marker such as ABPS various doped states might equally lead to a high score, e.g., the donation and reinfusion of autologous blood both will elevate the score, whereas this will lead to a one-directional change only from a low to a high value for the non-universal marker OFF-hr [13006].

Steroid profile for athlete biological passport

The athlete's steroid profile analysis represents a powerful tool to identify the administration of AAS (including those of endogenous as well as xenobiotic nature). This is achievable by monitoring urinary concentrations of selected steroids and comparing their levels and specific steroid ratios to inter-individual and intra-individual reference ranges. Traditionally, population-based reference ranges are applied as defined in respective technical documents issued by WADA. The five-year data review from a single laboratory demonstrated a rather limited gain in sensitivity for identifying testosterone abuse along with a de facto considerably increased workload concerning the confirmatory analyses required in cases of T/EpiT ratios > 4. Alternatively, subject-based reference ranges were favoured and the consideration of additional hormone concentrations (e.g. of luteinizing hormone, LH) was recommended. In another study, the threshold values for testosterone (200 ng/mL), epitestosterone (200 ng/mL), androsterone (10,000 ng/mL) etiocholanolone (10,000 ng/mL), dehydroepiandrosterone (DHEA, 100 ng/mL), 5alpha-androstane-3alpha,17beta-diol (200 ng/mL) and 5beta-androstane-3alpha,17beta-diol (200 ng/mL) were evaluated. The consideration of subject-based (intra-individual) reference ranges in particular has gained much attention and several initiatives pursuing this route for complementing modern anti-doping efforts have been initiated. In a recent pilot study, new potential biomarkers to support the identification of testosterone misuse in sports were described. Besides the established testosterone/epitestosterone ratio (T/EpiT), other additional steroid ratios have been found to possess the potential to support the detection of testosterone abuse, namely 6alpha-OH-androstenedione/16alpha-OH-dehydroepiandrosterone, 4-OH-androstenedione/16alpha-OH-androstenedione, 7alpha-OH-testosterone/7beta-OH-dehydroepiandrosterone, and dihydrotestosterone/5beta-androstane-3alpha,17beta-diol. Since these findings represent results of a pilot study, the variability and susceptibility of the identified marker ratios will require follow-up studies under different conditions to demonstrate their long-term stability and influences caused for example by acute ethanol consumption or bacterial activity. The same approach was also used to screen for additional biomarker ratios supporting the identification of
dihydrotestosterone (DHT) or DHEA misuse in sports. Besides the detection of steroid abuse, steroid profiles have also been shown to provide valuable information concerning possible degradation processes occurring in urine specimens due to the non-sterile collection conditions. The measurement of free testosterone and/or epitestosterone (the percentage of which should be below 5 % of the glucuronic acid-bound counterpart) and free 5alpha- and 5beta-androstane-3,17-dione (elevated concentrations of which are considered as indicative for urine degradation) is used to test for the validity of doping control samples regarding the steroid profile interpretation [12016].

The Athlete Biological Passport (ABP) is an individual electronic document that collects data regarding a specific athlete that is useful in differentiating between natural physiologic variations of selected biomarkers and deviations caused by artificial manipulations. A subsidiary of the endocrine module of the ABP that which here is called Athlete Steroidal Passport (ASP), collects data on markers of an altered metabolism of endogenous steroidal hormones measured in urine samples. The ASP aims to identify not only doping with anabolic-androgenic steroids, but also most indirect steroid doping strategies such as doping with estrogen receptor antagonists and aromatase inhibitors. Development of specific markers of steroid doping, use of the athlete’s previous measurements to define individual limits, with the athlete becoming his or her own reference, the inclusion of heterogeneous factors such as the UDPglucuronosyltransferase B17 genotype of the athlete, the knowledge of potentially confounding effects such as heavy alcohol consumption, the development of an external quality control system to control analytical uncertainty, and finally the use of Bayesian inferential methods to evaluate the value of indirect evidence have made the ASP a valuable alternative to deter steroid doping in elite sports. The ASP can be used to target athletes for gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) testing, to withdraw temporarily the athlete from competing when an abnormality has been detected, and ultimately to lead to an antidoping infraction if that abnormality cannot be explained by a medical condition. Although the ASP has been developed primarily to ensure fairness in elite sports, its application in endocrinology for clinical purposes is straightforward in an evidence-based medicine paradigm [10009].

Confounding factors

Physical exertion, training periodization, time of year, dehydration and altitude may all affect different hematological parameters and lead to false positives. Even though the ABP protocol is designed to account for these factors, the addition of an expert panel minimizes this risk [13006].

Although the ABP had the initial exclusive intent of biological monitoring, today, the ABP contains more than a simple series of individual biomarker values. Heterogeneous factors, such as age, gender, and genotype; confounding factors, such as exposure to higher altitudes for the hematological module; and some information regarding the conditions of sample collection, transport, and analysis are also stored in the passport for improved decision making. As such, the ABP becomes a platform for the evaluation of multiple pieces of scientific evidence, which is similar to a forensic approach. Similarly to forensic identification science, the strength of the ABP is that it relies on sound empirical testing in large populations by use of justifiable protocols. The decision support system that is used routinely to interpret the biomarker data stored in an ABP heavily relies on Bayesian inference techniques. Every element of information that constitutes doping evidence can be incorporated into other elements and/or corroborated by additional evidence. For example, the result of traditional drug tests, such as the detection of rEPO in urine; some
characteristics of athletes, such as a particular genotype; and the longitudinal monitoring of individual performance are evidentiary values that can be incorporated into the ABP decision support system for improved detection of doping [11426].

**Plasma osmolality**

One investigation quantified the effect of changes in plasma osmolality on the measurement of hematocrit (Hct) and the implications for the subsequent use of these data to calculate changes in plasma volume and application to the WADA Athlete Biological Passport. Two groups of eight male volunteers visited the laboratory after an overnight fast. In study 1, a 20 mL blood sample was collected and aliquoted into collection tubes containing saline of varying concentrations to alter the sample osmolality. In study 2, plasma osmolality was manipulated in vivo through prolonged exercise. Samples were analysed for hemoglobin concentration and Hct using manual methods and using an automated hematology analyser (AHA). Changes in blood, plasma and red cell volumes were calculated. While AHA Hct values did not change spun plasma volume changes (PCV) fell progressively as the osmolality of the sample increased. Consequently, there was a significant increase in apparent plasma volume as osmolality increased: regression analysis revealed that a 10 mOsmol/kg change in plasma osmolality produced a difference of 0.8 Hct units and a 1.6 percent change in plasma volume. In study 2, exercise produced a 12 ± 3 mOsmol/kg increase in plasma osmolality. No difference in Hct was observed at rest, but spun PCV was 1.0 ± 0.9 Hct units lower during exercise compared to AHA data. There was a difference in the degree of plasma volume change calculated, with a reduction of 8.7 ± 3.4 percent and 11.3 ± 3.5 percent reported with the manual and AHA methods respectively. It was concluded that conditions or interventions which result in a marked change to plasma osmolality produce a discrepancy in Hct measured using an AHA, consequently introducing errors into any calculation of changes in plasma volume using these data. These findings may also have implications for the measurement of Hct by WADA-accredited laboratories [13074].

**Stability of athlete blood passport parameters during air freight**

Fluctuations in ambient temperature and pressure, as well as physical jostling, may affect the stability of whole blood samples transported by air freight. The aim of one study was to characterize the stability of key blood variables during air freight and to investigate whether vibration or reduced pressure alone affected results. Over a 72-h interval, it was evaluated the stability of full blood count indices (plus reticulocytes) in tubes that were air-freighted a total of 2, 10 and 28 h. It was also examined the impact of 24 h of reduced atmospheric pressure (750 hpa or approximately 2500 m.a.s.l) and vibration (5 Hz). Samples were measured on a Sysmex XT-2000i instrument. The two key variables in the context of antidoping (haemoglobin concentration, reticulocytes) remained stable over a 72-h period regardless of the duration of air freight. Atmospheric pressure and vibration had no discernible effect. It was concluded that whole blood samples stored in NanoCool devices can be relied upon to remain stable for at least 72 h despite interim air freight [13075].

**Gastroenteritis**

The hematological module of the "Athletes Biological Passport" (ABP) is used to detect blood doping through the longitudinal variation of blood variables, such as hemoglobin concentration (Hb). Sporting federations have opened disciplinary procedures against athletes based on ABP results. Suspicious athletes try to explain the variations in their blood values with dehydration caused by gastrointestinal (GI) problems. The aim of one report was to describe hemoglobin concentration, a key variable of the ABP, during acute gastroenteritis
in athletes. 5 athletes with severe gastroenteritis were studied in retrospective. Blood test results (Hb, white blood cell count (WBC) and differential, CRP) obtained on hospital admission for GI problems were compared to data obtained from the same athletes in states of good health on previous occasions. During GI problems, athletes displayed marked inflammatory constellations with increased CRP and typical WBC shifts. Hb was not affected and remained mostly unchanged. This is in line with basic physiologic fluid regulation, where plasma volume is kept constant, even under conditions of severe dehydration. It is therefore unlikely that fluid loss associated with gastroenteritis will cause athletes blood data to reach levels of abnormality that will be suspicious of blood doping [11133].

Also for research

Marion Jones, who were tested for years and were never found to be cheating, admitted in 2007 to using “the clear,” a steroid formulated by the Bay Area Laboratory Cooperative, known as BALCO, to elude steroid testing. It is probable that the passport approach will identify athletes like Jones. If the athletes consented to have their test samples used for research, it would improve scientific understanding of the range of readings that are normal. But support for a voluntary program has not caught on. But culture change takes time, and the need to secure the borders of fair sport is urgent [10008].

Law issues

The Athletes Biological Passport (ABP) has received both criticisms and support during this year. In an issue of The Lancet, Michael Wozny considered that the use of the ABP makes it more difficult to take banned substances and that it was successfully used against the Italian elite cyclist Franco Pellizotti. After that, Italy's anti-doping tribunal considered that there was not enough evidence to prove manipulation of his own blood profile in Pellizotti's case. However, the UCI appealed to the Court of Arbitration for Sport (CAS) that sanctioned Pellizotti with a suspension of 2 years. Since its implementation, some problems have emerged. From 2010 to date, a large number of reports regarding the stability of the blood variables used to determine the ABP have been published, showing mixed results. One study considered that there is a risk of misinterpreting the physiological variations of the hematological parameters determined by the anti-doping authorities in the ABP. The analytical variability due to exercise training and competitions and/or to different metabolic energy demands, hypoxia treatments, etc. could lead to an increase in false-positives when using the ABP with the dramatic consequences that they might cause in major sports events like the forthcoming London Olympic Games. Moreover, the ABP characteristics, procedures, thresholds, or individual determination of reference ranges, abnormal out-comes, strikes, "how the profile differs from what is expected in clean athletes" should be clearly stated and explained in a new public technical document to avoid misunderstandings and to promote transparency [11129].

Software

Substances and methods used to increase oxygen blood transport and physical performance can be detected in the blood, but the screening of the athletes to be tested remains a critical issue for the International Federations. One project, AR.I.E.T.T.A., aimed to develop a software capable of analysing athletes’ hematological and performance profiles to detect abnormal patterns. One-hundred eighty athletes belonging to the International Biathlon Union
gave written informed consent to have their hematological data, previously collected according to anti-doping rules, used to develop the AR.I.E.T.T.A. software. Software was developed with the included sections: 1) log-in; 2) data-entry: where data are loaded, stored and grouped; 3) analysis: where data are analysed, validated scores are calculated, and parameters are simultaneously displayed as statistics, tables and graphs, and individual or subpopulation profiles; 4) screening: where an immediate evaluation of the risk score of the present sample and/or the athlete under study is obtained. The sample risk score or AR.I.E.T.T.A. score is calculated by a simple computational system combining different parameters (absolute values and intra-individual variations) considered concurrently. The AR.I.E.T.T.A. score is obtained by the sum of the deviation units derived from each parameter, considering the shift of the present value from the reference values, based on the number of standard deviations. Future studies aiming to validate the AR.I.E.T.T.A. score and improve the diagnostic accuracy will improve the system [11134].

Passports in practice

The 2011 International Association of Athletics Federation (IAAF) World Championships took place in Daegu, Korea. For the first time, all athletes were blood tested prior to the competition in order to give a clear signal to the world athletic community of the wish to enter into the era of the Athlete Biological Passport and fight against doping in their sport. The hematological parameters were measured on site. Thus, a mobile-accredited laboratory for blood testing was created in Daegu. Two serum tubes were collected for clinical chemistry and hormonal analyses in order to build the bases of the endocrine and the androgen (steroid) modules of the Athlete Biological Passport in blood. One paper described some of the main challenges the project faced with regard to the large number of athletes, competing in different disciplines, and the logistic problems that had to be solved for smart implementation of one of the most complex operations organized in the last decade in the fight against doping [12043].

The most promising attempt to reveal otherwise undetectable autologous blood doping is the Athlete Biological Passport enabling a longitudinal monitoring of hematological measures. Recently, the determination of hemoglobin mass (tHb) was suggested to be incorporated in the adaptive model of the Athlete Biological Passport. The purpose therefore was to evaluate the performance of tHb as part of the adaptive model for the detection of autologous blood transfusions in a longitudinal blinded study. Twenty-one subjects were divided into a doped group (n=11) and a control group (n=10). During the time course of a simulated cycling season (42 weeks) including three major competitions (Classics, Grand Tour, World Championships), multiple autologous transfusions of erythrocyte concentrates were assigned in the doped group. A blinded investigator ordered up to 10 tHb measurements (carbon monoxide rebreathing) per subject, mimicking an intelligent doping testing approach in obtaining hematological data (tHb, OFFmass (novel marker including reticulocytes), and respective sequences) for the adaptive model. The final analysis included 199 of 206 overall tHb measurements. The use of tHb, OFFmass, and their sequences as markers of the adaptive model at the 99 percent specificity level allowed identification of 10 of 11 doped subjects (91 % sensitivity) including one false positive in the control group. At the 99.9 percent specificity level, 8 of 11 subjects were identified without false positives (73 % sensitivity). It seems that the problems of tHb determination by carbon monoxide rebreathing limit the application of this method in antidoping. Because of its potential to detect individual abnormalities associated with autologous blood transfusions shown in this study, a method for tHb determination that is compatible with today's standards of testing should be the focus of future research [12044].
Erythropoiesis stimulating agents

Blood doping, through the increase of red cells, induces changes of hematological parameters. The aim of the Biological Passport is first to analyse individual longitudinal profiles in order to identify, through variations of the specific parameters, doping manipulations. Additionally, on the basis of abnormal values or profiles, athletes can be targeted for traditional anti-doping tests in order to detect forbidden substances or methods. We report the experience of the International Cycling Union in applying the Biological Passport to target athletes for the presence of erythropoiesis stimulating agents. All positive results which have been reported between 2008 and 2010 concerning athletes enrolled in the Biological Passport program are presented. Four cases are discussed more in details. To conclude, it was propose possible ways of using the Biological Passport in order to better understand athletes' doping modalities, so that testing programs efficiency can be improved [12045].

Testing during cycle tournament

Cycling stage races are among the most strenuous of endurance events. The exercise-induced variations observed in hematological parameters appear to be consistent with the rider's physiological response to maintain and improve highly demanding performances day-after-day. During training and competition, an essential part of evaluating the health and performance of professional and recreational athletes is periodic assessment of the hematological profile. Together with evaluation of iron metabolism, serial blood chemistry analysis can point to whether an out-of-range shift in blood parameters may be attributable to the response to physical effort or to an index of abnormal response. A starting point for determining irregular and suspect behavior in athletes is a better appreciation of the hematological response to vigorous physical activity. This is of particular interest in the context of the Athlete's Biological Passport (ABP), which was devised to detect abnormal variation(s), even at a single time-point, versus a subject-specific physiological range deduced from the athlete's own previous data. Because the variations during a competitive season affect the behavior of hematological parameters over a season, knowing their variability could help to define the physiological ranges in an athlete. The GiroBio, held in mid-June every year in northern Italy, is the "under-27s amateur Giro d'Italia", a surrogate for the Giro d'Italia and other international road races (Tour de France, Vuelta a Espana) for young cyclists. It attracts more than 150 professional cyclists from all over the world annually. Since 2005 it has been included in the Union Cycliste Internationale (UCI) Europe Tour circuit, category 2.2. About half the duration of its major counterpart, the GiroBio format is 10 stages over 11 days. The GiroBio race represents the entry step to a fully professional career for most cyclists. The race aims to promote the values of sport and fair play in healthy competition, counteracting the doping culture, through the adoption of innovative organizational aspects, as discussed below. The aims of one study were to determine the hematological response to middle-term strenuous endurance and to determine whether a relationship exists between the athlete-specific hematological profile and final placement in a cycling stage race. The study population was male professional cyclists (n=253) competing in the 2010 (n=144) and 2012 (n=109) GiroBio 10-day stage races. Blood draws taken before the start of the race, at mid-race, and at end-race were performed in strict compliance with academic and anti-doping pre-analytical warnings. Blood chemistry included white blood cell, red blood cell, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean hemoglobin content (MCH), mean corpuscular hemoglobin content (MCHC), platelets, and reticulocyte relative and absolute counts. Compared to baseline values, erythrocyte, hemoglobin, hematocrit, MCHC, platelet and reticulocyte counts were all consistently lower.
at mid-race, but returned to normal by race-end, while leukocytes were increased in the final phase. MCV increased during both events. MCH increased in the first part to then return to baseline in the 2012 race. The calculated OFF-score consistently decreased in the first half of the race before increasing, but remained lower than the baseline value. The trends of variation in hematological parameters were substantially similar in both events. There was an inverse, albeit weak, relationship between placement and erythrocyte, platelet, hemoglobin, hematocrit and OFF-score values in the 2010, but not in the 2012 race. In conclusion, the data confirm that, in this large series of elite road cyclists, the strenuous effort a rider sustains during a stage race induces appreciable changes in the hematological profile.

Positive cases in biking

Blood doping, through the increase of red cells, induces changes of hematological parameters. The aim of the Biological Passport is first to analyse individual longitudinal profiles in order to identify, through variations of the specific parameters, doping manipulations. Additionally, on the basis of abnormal values or profiles, athletes can be targeted for traditional anti-doping tests in order to detect forbidden substances or methods. We report the experience of the International Cycling Union in applying the Biological Passport to target athletes for the presence of erythropoiesis stimulating agents. All positive results which have been reported between 2008 and 2010 concerning athletes enrolled in the Biological Passport program are presented. Four cases were discussed more in details. To conclude, it was proposed possible ways of using the Biological Passport in order to better understand athletes’ doping modalities, so that testing programs efficiency can be improved.

Continuous evaluation of the ABP

Further studies evaluated the strength and possible limitations of the ABP after introduction of the hematological module by UCI and WADA which will be presented in the subsequent paragraph. As the ABP depends on analytically flawless data, the pre-analytical conditions and the laboratory testing were put into question. Thus, it seemed of great importance whether the adherence to the ABP guidelines will allow high quality results respecting forensic standards and thus assure their applicability in a juridical context. It was confirmed that the rigorous adherence to WADA’s guidelines yields excellent results required in the anti-doping context. According to previous studies on the topic, the limit of 36 h from blood collection to analysis is reasonable to guarantee analytical quality, when the samples are transported at 4 °C which seemed to improve the stability of hematological parameters independently from the analytical methodology. Further studies on the stability of blood parameters used within an anti-doping setting were available before the ABP was officially introduced. In addition, pursuing the goal to provide a sound foundation for the interpretation of blood profiles in athletes, more data were published on various confounding factors and analytical aspects which may help to refine existing guidelines. For instance, from a practical point of view, it was reported that long-haul air travel leads to normal diurnal variations of Hb without any indication that travel will affect the hematological measures in a way that might be interpreted as blood doping. Despite the general appreciation of the concept, it also became clear that the ABP has some limitations. It has also been discussed that “the sensitivity of the ABP to detect doping is limited if the physiological result of a low level of doping remains within the individual’s own reference range”. It fits in this context that microdosing of rhEPO does not lead to abnormal changes in the current ABP markers during rhEPO administration. Nevertheless, for example, blood counts were measured only during
the titration and following maintenance phase, but not longitudinally after rhEPO was discontinued for which OFF-hr was originally described [13006].

In order to evaluate the best possible realistic performance of the ABP adaptive model for a blood doping technique (autologous blood doping), a longitudinal blinded study design included 21 subjects that were divided into two groups of which one was transfused with 1-2 bags of blood at 2-3 time points during a simulated time course of a professional cycling season. A blinded investigator who was well trained in the use of the ABP was requested to apply an intelligent testing approach which allowed a sensitivity of 82 percent at the 99 percent threshold of the adaptive model as 9 of 11 subjects in the doped group had at least one value outside of the individual limits for Hb and OFF-hr or a sequence (Hb and OFF-hr) above the 99.9 percent probability threshold. The specificity was 90 percent for Hb (one false-positive value in 1 of 11 athletes) and 100 % for OFF-hr, sequence Hb as well as sequence OFF-hr [13006].

Several aspects allude to the effectiveness of the ABP hematological module. First, individual non-physiological data from the ABP have been used to sanction athletes and were recognized by the Court of Arbitration in Sport (CAS) in a final hearing. Second, these precedents might have had a deterrent effect on future behavior of athletes as the value of %retics has significantly changed and normalized since the introduction of the ABP in 2008, which may reflect a decreased prevalence of blood manipulations in the professional cycling peloton. From a practical and more measurable perspective, the introduction of the ABP hematological module and dedicated target testing based on the ABP data led to a 250 percent increase in the number of positive rhEPO cases in 2008 and 2009. In 2011 the number of cases was still about 300 percent higher than it was before the introduction of the ABP (data available from www.wada-ama.org) [13006].
SOCIO-MEDICAL ASPECTS ON DOPING

Doping in sport has been a focus of medical, physiology and social science research in recent years. Whereas medical and physiology researchers focus on improving methods (e.g. blood, urine and gene tests) for detecting the use of prohibited substances and to deter athletes from their use, social science researchers strive to better understand the psychosocial factors (e.g. attitudes, environment and beliefs) that may offer targets for educational programs aimed at preventing this behaviour [13017].

Costs of antidoping programs are high, and programs are increasingly complex and difficult to manage. As the bureaucratic burden imposed on athletes, physicians and sport organizations grows, concerns about sustainability abound. But the resolve of athletes and sport organizations to maintain integrity and eradicate cheating is strong [014]. However, taken together it is obvious that the fight against doping not only is played on a morality ground but also have economical implications.

Although difficult to document, it has been postulated that more steroid use takes place outside organized sport, within body-building and other subcultures, where no attempt is made to address drug-taking behaviour. Adolescent athletes, often eager to gain weight and muscle mass, are ripe targets for those who offer easy approaches that promise startling results in just a few weeks. To some, performance and size are paramount; they are seen to be the keys to success and stardom. And therein lies a special challenge for physicians interested and involved in sport: one must ensure that those athletes and, perhaps more importantly those who coach them, develop approaches to sporting success that emphasize excellence in every domain. Pharmacologic approaches to enhanced performance represent a witches' brew of distorted values and maligned perspectives with potentially devastating health risks [08014]. This means that doping is not a problem only for the athletic movements.

Semantics in doping

Several studies have looked at the unsupervised drug habits of AAS users, but these are clearly subject to different types of bias. Nevertheless, these field studies should not be overlooked. According to the studies, drug regimens follow typical patterns. Different oral and injectable compounds are generally combined (“stacked”), creating large dose regimens usually self-administered during periods (“cycles”) lasting 4-12 weeks. “Stacking” is based on the idea that smaller dosages of multiple drugs might reduce the chance of complications than larger dosages of a single drug. This may also facilitate the administration of multiple AASs (necessary to achieve supraphysiologrphical doses) for longer periods, and so minimizing the plateauing effect. The aim of “stacking” is to rationally combine different characteristics, avoiding overlap of benefits or side effects. Combinations of testosterone and nandrolone (or similar drugs) are the basis of the “massbuilding stacks” used to maximize muscular and strength gains. Combinations containing potent androgens are preferred for dieting and body definition, because of their lack of estrogenic activity (less water, salt and fat retention). These are the “cutting stacks”. Heavy users may combine a “mass-building cycle” with a subsequent “cutting cycle”, finishing with a ‘post-cycle therapy’, with anti-estrogens or hCG, in an attempt to restart androgen production. Frequent users may also combine AASs with other “performance drugs”, such as painkillers (including opioids), diuretics, insulin, growth hormone, stimulants, aromatase inhibitors and thyroxine [11028].
Political and economical aspects of anti-doping

One article examines the processes by which the Anabolic Steroid Control Act of 2004, an act that added steroid precursors such as androstenedione to the list of Schedule III Controlled Substances in the United States, came to pass in both the House of Representatives and the Senate. Grounded theoretically in political economy, the article addressed how the interplay of political pressures and economic influences stands to affect the actions of public officials, and how "tougher" drug policies-those touted to be more substantive and efficacious than existing regulations-often fail to effect change. The article concludes with implications for those involved in the regulation of anabolic steroids and steroid precursors [06011].

Social and socio-medical issues

Social psychological determinants

The aim of one study was to identify the social psychological determinants of the use of performance-enhancing drugs by gym users who practice bodybuilding, fitness, powerlifting or combat sports. In this questionnaire-based study, 144 respondents answered questions on their actual use and intention to use such drugs and also on their background characteristics and beliefs, such as their attitudes, social influences and self-efficacy. While all social psychological determinants correlated with intention to use these drugs, the most important predictors were personal norms, beliefs about performance outcomes and the perceived behavior of others. Non-users held more restrictive norms about using performance-enhancing drugs, were less optimistic about the performance-enhancing outcomes and believed that fewer significant others used performance-enhancing drugs than users and ex-users. The results of this study indicate that users attribute advantages to performance-enhancing drugs and are inclined to overlook the risks of using them. Preventive interventions should focus on influencing personal norms and social processes [07019].

Risk-taking individuals, such as those who abuse alcohol or drugs, engage in criminality or have eating disorders, have been shown to have an increased predisposition for doping use. Despite this evidence, questions remain about the relationship between risk behaviours such as abuse of alcohol and narcotics and doping. For example, in a study on 15,000 sports active high school students in the US, there was no correlation between doping use and risk behaviour [Dodge & Jaccard, 2006]. Coakley and Pike [2009] argue that such results reflect young people’s use of alcohol and drugs to escape from reality being a completely different phenomenon to athletes doping to improve athletic performance. Coakley and Pike argue doping is not about violating social and legal norms, typically seen when abusing alcohol and drugs, but rather a manifestation of an “over conformity” in relation to basic norms in sport – the “sport ethic” (which not should be confused with norms of fair play). The “sport ethic” is characterised as how the athlete should prioritise sport and their team before everything else in life, continually striving to improve sporting performances, struggle for victories. Doping becomes a tool in this endeavour. One article investigated doping use in relation to substance abuse, health risks, risks of being caught in a doping control, and other risk behaviours, in a qualitative interview study of 11 Swedish elite athletes sanctioned for using anabolic androgenic steroids (AAS) during their sports careers. Most respondents grew up in secure, intact families with siblings. Virtually all respondents were from working class homes.
where the father typically worked in industry and the mother a housewife who worked part time in retail when the children become older. The majority of respondents were also workers, including business owners, clerks and managers. Most attended a vocational program at high school, although some have no secondary education. No respondent had a university degree. Respondents reported a positive school experience, with good friends and mediocre or good school performances. Most respondents had excellent results in junior and/or senior national championships. Several respondents had a good international record, participating in national teams, with some winning national and international championships. Ten of the respondents were convicted 1990–1996 and one in the mid-2000s. AAS was the only doping agent used by the athletes in this study. None of the respondents reported using other doping agents; some reported amphetamine and cocaine use for social rather than sporting reasons. Respondents in this study were not using AAS in cycles, and were instead using preparations on a daily basis. However, respondents did interrupt use before competitions to avoid being caught in a doping control. Some respondents mentioned that they stopped using AAS when they were injured and unable to carry out their training sessions. Taking AAS without training was typically described as a complete waste of time, effort and drugs. The “gateway effect” seen in other substance use behaviour (where abusing one substance is a risk factor for developing other forms of more harmful drug behaviours) has also been established in doping. Patterns of other substance abuse were investigated in this study. Overall the respondents were very restricted in their use of alcohol and drugs. Substance abuse was unusual among the respondents, and in that sense they could not be regarded as typical risk takers. Their AAS use could characterise them as risk-takers with regards to doping control and health. Overall, the respondents perceived the health risks as very limited. Even though respondents did not perceive any serious health risks due to their use of AAS, they would arguably have been better off if they refrained entirely. However, this depends on what benefits the AAS provide. If athletes have a lot to gain in terms of medals, money, less pain or making exercise easier, and the risks are moderate or small – then doping could be considered a rational choice of action instead of an irrational risk behavior. Only two respondents were uncertain about the positive effects of the steroids. Comparing the positive and negative effects of AAS use, most respondents perceived distinct positive effects from using. It was easier to work-out and they achieved better results. The negative effects of doping were, except for one respondent, very small. Another characteristic of risk-taking behaviour, in addition to the lack rationality, is the failure to analyse the consequences of different opportunities before action. This was also investigated among the respondents by probing how they reasoned in relation to the risk of being caught in doping control, and in connection with possible health risks. Almost all respondents said that they were cautious and used moderate doses. Respondents were asked to self-assess their propensity to take different risks in life using a broad understanding of the concept of risk-taking. To do this, respondents were asked how they see themselves in relation to risk-taking using examples like drug use, crime, gambling, speeding, driving without seat belt, skydiving and bungee jumping. Not one of the respondents describes themselves as risk-takers. It is clear that results focusing on risk behaviour to explain doping use are not relevant to explain AAS use among most of the respondents in this study. The respondent who was the heaviest user went on regular medical surveillance to insure against health risks. Finally, none of the respondents in the study assessed themselves as risk-takers; they described themselves as cautious, calculating, planning and cowardly. The findings around risk and doping may also be a result of the methodological approach taken by the study. Most studies on this topic have a quantitative design. Evaluating different methodological approaches is an important but complex question for doping related research [13034].

Psychological background
Drugs and methods to improve physical performance among athletes have been used since the beginning of sport history, but the use of performance enhancing drugs has not always been regarded as cheating. In short, the motives for doping are improving and maintaining physical functioning, coping with the social/psychological pressures and striving for social and psychological goals, including economic benefits. Factors such as, "doping dilemma", "win at all costs", cost versus benefit, and the specificity of some specific doping agents, also play major roles. It seems that action on the athletes' attitude about the achievement of physical improvement and creating effective methods to reveal the drug abuse, are two main ways in winning the struggle against doping [09005].

One of the major justifications for the ban on the use of performance-enhancing drugs in sport has been that relating to the protection of the health of athletes. One paper subjected this argument to critical analysis by putting it in the context of the broader relationship between sport and health. More particularly, the paper sought to unravel some of the complexities of this relationship by an examination of some aspects of sports sponsorship, particularly with alcohol and tobacco companies; (the health risks associated with elite level sport; and the widespread and legal use within the sporting context of drugs that can have dangerous side effects. The paper concluded with an examination of some aspects of anti-doping policies within sport and it is suggested that a more imaginative approach to athlete education is needed to prevent the misuse of drugs [09006].

A total of 40 talented male and female athletes (mean average age 20 years) from 13 different sports attended 12 focus groups held over the UK intended to investigate athletes' attitudes toward doping. Athletes in general did not report a significant national doping problem in their sport, but exhibited sporting xenophobia with regard to both doping practices and the stringency of testing procedures outside of the UK. Athletes often viewed doping as "unnatural" and considered the shame associated with doping to be a significant deterrent. Athletes perceived no external pressure to use performance enhancing drugs. In response to hypothetical questions, however, various factors were acknowledged as potential pressure points: most notably injury recovery and the economic pressures of elite sport. Finally, a significant minority of athletes entertained the possibility of taking a banned hypothetical performance enhancing drug under conditions of guaranteed success and undetectability. It was concluded that the athletes in this study generally embraced those values promoted in anti-doping educational programmes, although there were some notable exceptions. That the social emotion of shame was considered a significant deterrent suggests anti-doping efforts that cultivate a shared sense of responsibility to remain "clean" and emphasise the social sanctions associated with being deemed a 'drugs cheat', resonate with this atypical social group [10013].

**Athletes' interpersonal antisocial behaviour**

The link between fear of failure and students' antisocial behaviour has received scant research attention despite associations between fear of failure, hostility, and aggression. Also, the effect of sport experience on antisocial behaviour has not been considered outside of the sport context in adult populations. Further, to date, gender differences have not been considered in fear of failure research. To examine whether fear of failure and sport experience predict antisocial behaviour in the university and sport contexts in student athletes, and whether this prediction is the same in males and females; and gender differences exist in antisocial behaviour and fear of failure. British university student athletes (n=176 male; n=155 female; median age 20 year) completed questionnaires assessing fear of failure, sport experience, and antisocial behaviour in both contexts. Fear of failure and sport experience positively predicted antisocial behaviour in university and sport and the strength of these predictions did not differ between males and females; females reported higher levels of fear of devaluing one's self-estimate than males whereas males reported
higher levels of fear of important others losing interest than females. Males engaged more frequently than females in antisocial behaviour in both contexts. It was concluded that fear of failure and sport experience may be important considerations when trying to understand antisocial behaviour in student athletes in education and sport; moreover, the potential effect of overall fear of failure and of sport experience on this frequency does not differ by sex. The findings make an important contribution to the fear of failure and morality literatures [11421].

Subjective effects

The use of anabolic androgenic steroids (AAS) to increase muscle size and strength is widespread. Information regarding self-administered AAS used nonmedically to enhance athletic performance or improve physical appearance is sparse and poorly documented. The purpose of one study was to identify current trends in the drug-taking habits of AAS users. An anonymous self-administered questionnaire was posted on the message boards of Internet Web sites popular among AAS users. Of the 500 AAS users who participated in the survey, 78 percent (392/500) were noncompetitive bodybuilders and nonathletes; 60 percent (298/500) of the respondents reported using at least 1000 mg of testosterone or its equivalent per week. The majority (99%) of AAS users (496/500) self-administer injectable AAS formulations, and up to 13 percent (65/500) report unsafe injection practices such as reusing needles, sharing needles, and sharing multidose vials. In addition to using AAS, 25% of users admitted to the adjuvant use of growth hormone and insulin for anabolic effect, and 99 percent (496/500) of users reported subjective side effects from AAS use. The survey reveals several trends in the nonmedical use of AAS. Nearly four out of five AAS users are nonathletes who take these drugs for cosmetic reasons. AAS users in this sample are taking larger doses than previously recorded, with more than half of the respondents using a weekly AAS dose in excess of 1000 mg. The majority of steroid users self-administer AAS by intramuscular injection, and approximately 1 in 10 users report hazardous injection techniques. Polypharmacy is practiced by more than 95 percent of AAS users, with one in four users taking growth hormone and insulin. Nearly 100% of AAS users reported subjective side effects [06020].

Expectation of the doped

Long-term use of anabolic-androgenic steroids (AASs) is associated with both positive and negative effects. The authors examined possible mechanisms by which these effects contribute to AAS satisfaction and predict intentions for future AAS use. Five hundred male AAS users completed an interactive Web-based instrument assessing the psychological and physical effects of AAS use. Covariance structure modeling was used to evaluate both direct and indirect effects of AAS consequences on satisfaction with AASs and intentions for future AAS use. Results suggest that gain in muscle mass and psychological benefits from AAS use uniquely contributed to both AAS satisfaction and intentions for future use. Side effects from AAS use also uniquely contributed to AAS satisfaction, but ancillary drug use was found to partially mediate this relationship, suggesting that the satisfaction of experienced AAS users is enhanced by their mastery of side effects through the use of ancillary drugs. The final model explained 29 percent of the variance in intentions for future AAS use. Mechanisms for sustained AAS use and implications for intervention and prevention strategies are discussed [06013].

Muscle dissatisfaction in young adult men

Appearance concerns are of increasing importance in young men’s lives. It was investigated whether muscle dissatisfaction is associated with psychological symptoms, dietary supplement or anabolic steroid use, or physical activity in young men. As a part of a
questionnaire assessment of health-related behaviors in the population-based FinnTwin16 study, we assessed factors associated with muscle dissatisfaction in 1245 men aged 22-27 using logistic regression models. Of men, 30 percent experienced high muscle dissatisfaction, while 12 percent used supplements/steroids. Of highly muscle-dissatisfied men, 22 percent used supplements/steroids. Mean body mass index, waist circumference, or leisure aerobic activity index did not differ between individuals with high/low muscle dissatisfaction. Muscle dissatisfaction was significantly associated with a psychological and psychosomatic problems, alcohol and drug use, lower height satisfaction, sedentary lifestyle, poor subjective physical fitness, and lower life satisfaction. It was concluded that muscle dissatisfaction and supplement/steroid use are relatively common, and are associated with psychological distress and markers of sedentary lifestyle [06014].

Beliefs about the causes of success in sports and susceptibility for doping
One study set out to assess the impact of attributional beliefs about success on the susceptibility for doping use in adolescent athletes. The sample consisted of 309 adolescent athletes participating in both team and individual sports. Participants completed a battery of questionnaires including Beliefs about the Causes of Success in Sport Questionnaire (BACSSQ), current and past doping use, and measures of attitudes, norms, situational temptation and social desirability. Variance reduction rate analysis revealed that social desirability did not act as a confounder in the relationship between doping susceptibility and its predictors. With regard to beliefs about the causes of success dimensions, only deception emerged as a significant predictor of doping use susceptibility over and above the effects of well-established social-cognitive predictors of doping intentions and use. These findings imply that beliefs about the causes of success in youth sports may comprise another dimension of risk factors for doping susceptibility and use [13051].

Rejuvenation
A search for a hormonal fountain of youth has been hotly pursued over the last century, predominately by those who wish to market hormones to a gullible public. There is little or no benefit of hormone replacement in persons who do not have a hormone deficiency. Overall, the present state of the art suggests that the findings have been disappointing. In persons who fail to get adequate sunlight, and therefore have low vitamin D levels, vitamin D replacement appears to have positive effects, including decreasing mortality. Testosterone in hypogonadal males has a number of positive effects such as improving libido and erectile capacity, increasing strength and bone mineral density, and perhaps having a small effect on cognition. These effects need to be balanced against long-term side effects, the evidence for which studies are lacking. There is little evidence to recommend DHEA, pregnenolone, growth hormone, ghrelin, or melatonin to older persons. Overall, exercise, adequate exposure to sunlight, and adequate dietary protein appear to have at least as positive an effect as any of the hormones being used to rejuvenate older persons [13052].

Interaction between athletes and coaches
The sport nutrition and doping are known to be important issues in sports, but there is evident lack of studies which investigated those issues in swimming, especially with regard to parallel analysis of coaches and athletes. The first aim of one study was to compare knowledge of swimming coaches and their athletes about nutrition and doping. Also, it was identified interrelationships between studied sociodemographic-, sport-; nutrition- and doping-related-factors. The sample of subjects comprised 55 athletes (20 years of age; 24
females) and 22 coaches (mean age 37 years; 4 females) from Croatia (98% of respondents). In the first phase of the investigation we have validated specific questionnaires to determine the knowledge of sport nutrition (KSN), and knowledge on doping (KD). The test-retest correlation and percentage of equally responded queries revealed both questionnaires as reliable. The discriminative validity was proven also since coaches scored better than their athletes on both questionnaires. Athletes declared their coaches as the primary sources of knowledge about nutrition and doping. Among coaches, formal and self-education are equally important sources of information about doping and nutrition. The age is negatively, while the formal education is positively correlated to KD and KSN scores among coaches. Consequently, permanent educational programs about nutrition and doping are emphasized, especially among older coaches and younger athletes [13053].

Athlete support personnel (ASP) failing to meet responsibilities under the World Anti-Doping Code risk sanction. It is unclear whether the poor knowledge of responsibilities seen in sports physicians and coaches applies to other ASP (e.g. administrators, chiropractors, family, nutritionists, physiotherapists, psychologists, and trainers). A purposive sample of Australian ASP (n=292) responded to a survey on knowledge of anti-doping rules (35 true/false questions), ethical beliefs and practice, and attitudes toward performance enhancement. Some ASP declined to participate, claiming doping was irrelevant to their practice. Physicians were most knowledgeable (31/35), with family and trainers the least (26/35). ASP reported that improvements were needed to support anti-doping education (e.g., basis for anti-doping) and practice (e.g. rules). ASP also had a slightly negative attitude toward performance enhancement. Linear regression showed that being a sports physician, providing support at the elite level, and 15 years of experience influenced knowledge. The results confirm gaps in knowledge, suggesting that stronger engagement with ASP anti-doping education and practice is needed. Applying the principles of andragogy could help foster active engagement through emphasis on active inquiry, rather than passive reception of content [13054].

The purpose of one study was to examine whether the relationships between contextual factors (i.e. autonomy-supportive vs. controlling coaching style) and person factors (i.e. autonomous vs. controlled motivation) outlined in self-determination theory (SDT) were related to prosocial and antisocial behaviors in sport. We also investigated moral disengagement as a mediator of these relationships. Athletes’ (n=292, median 19 years) responses largely supported our SDT-derived hypotheses. Results indicated that an autonomy-supportive coaching style was associated with prosocial behavior toward teammates; this relationship was mediated by autonomous motivation. Controlled motivation was associated with antisocial behavior toward teammates and antisocial behavior toward opponents, and these two relationships were mediated by moral disengagement. The results provide support for research investigating the effect of autonomy-supportive coaching interventions on athletes' prosocial and antisocial behavior [11419].

Sports addiction

Socially valorised, sport like other forms of behaviour, can take on an addictive aspect. A review of the English and French literatures from 1979 to 2012 was conducted, using PubMed, Google Scholar, EMBASE, and PsycInfo, using the following key words alone or combined: sport, dependence, exercise, addiction. Exercise dependence is defined as a craving for physical activity that leads to extreme exercise intensity and generates physiological and psychological symptoms. Measurement scales have been proposed to make the diagnosis. No epidemiological studies have examined the prevalence of exercise dependence in the general population, although some studies suggest a frequency ranging
from 10 to 80 percent. Disorders begin with a search for pleasure in physical effort, which then gives way to an obsession for sport resulting in a need to practice a sport more and more frequently and intensely. This addiction is more common among alcohol and illicit drug addicts than among the general population, while the rate of eating disorders can reach 40 percent. Personality traits most often associated are perfectionism, extraversion, and sensation seeking, while possible links between sporting activity and intensive doping will be discussed [13039].

**Motivational and social cognitive predictors of doping**

Doping use is an important issue in both competitive and non-competitive sports, and poses potentially irreversible health consequences to users. Scholars increasingly call for theory-driven studies on the psychosocial processes underlying doping use that will inform subsequent policy-making and prevention interventions. The aim of the study was to implement an integrative theoretical model to assess the direct and indirect effects of motivational variables, moral orientations, and social cognitions on doping intentions. A randomly selected and representative sample of 750 elite athletes anonymously completed a battery of questionnaires on motivational and moral constructs, and social cognitions related to doping. Hierarchical linear regression analysis and multiple mediation modeling were used. The effects of achievement goals and moral orientations were significantly mediated by attitudinal, normative, and self-efficacy beliefs, in both lifetime ever and never doping users. Moral orientations indirectly predicted the doping intentions of never users, but did not predict ever users’ doping intentions. Achievement goals and sportspersonship orientations influence doping intentions indirectly, through the effects of attitudes and self-efficacy beliefs. Sportspersonship (moral) orientations were relevant to doping intentions among athletes with no prior experiences with doping, while achievement goals and situational temptation were relevant to both lifetime never and ever dopers [13040].

**Motives for use**

Several factors that are unique to our current society may contribute to young people’s using drugs to succeed in sports. First is the message that is being sent by many sports idols today. The steroid investigations in baseball and books by former players make it clear that steroids and other drugs have played a part in many record-breaking performances. However, the fame and the respect that still is garnered by these athletes sends the message that ergogenic drugs are accepted, if not necessary, to reach such success. As young athletes begin to model themselves after sport icons, heartbreaking stories are beginning to unfold. One young athlete in a tear-filled confrontation with his father only months before committing a now highly publicized suicide linked to his steroid use confessed, “I'm on steroids, what do you think? Who do you think I am? I'm a baseball player, baseball players take steroids. How do you think Bonds hits all his home runs? How do you think all these guys do all this stuff? You think they do it from just working out normal?” Second, society today places a huge emphasis on sports with collegiate football stadiums seating nearly 100000 people and countless events featured on national television. From high school basketball all-star games to the Little League World Series, our youths are placed under the national spotlight at exceedingly young ages. With professional scouts now following high school sports and collegiate coaches eyeing even younger talent, the pressure to succeed is placed now on younger, more impressionable shoulders than ever before. Finally, several economic factors encourage drug use to gain an edge in sports. One is the resultant money and social stature that accompany athletic success when worthy of garnering professional contracts. Another more subtle but increasingly wide-reaching monetary influence is the rising cost of collegiate education. This has been shown to be a
self-reported factor for high school girls to use performance-enhancing drugs while competing for prestigious and now very valuable athletic scholarships to college. Whether they turn to ergogenic drugs for the competitive edge or as a means of keeping up with fellow students who are already using these substances, it is clear that the pressure to do so is significant today [06003].

The use of anabolic androgenic steroids (AAS) has been associated with the use of illegal drugs. Earlier observations suggested that users of illegal drugs may use AAS for reasons other than increasing muscle strength or size. The aim of one study was to investigate the motives for AAS use among outpatients at a substance abuse center in Stockholm, Sweden. All male patients under the age of 50 were asked whether they had used AAS during a 2-month period. An AAS survey was administered to those who reported AAS experiences in the admittance interview. Twenty of the 175 respondents (11%) reported using AAS. The most frequently reported motives were related to anabolic effects (i.e. for a good-looking body, to become stronger, or to perform better in sports). However, some users reported other motives; for example, to conceal concomitant drug use, to alleviate insecurity or low self-esteem, to become brave, or in preparation of committing a crime. Furthermore, many respondents reported side effects that were associated with AAS; most notably, irritability and depression/suicidal ideation. It was concluded that some users of illicit drugs also use AAS for reasons other than the anabolic properties of these compounds. Therefore, considering that AAS may cause or contribute to diverse morbidity, it is important to ask users of illicit drugs about AAS use, even when obvious external signs of AAS use are lacking [10014].

One study presents an opportunistic examination of the theoretical tenets outlined in the Australian Sport Drug Control Model using questionnaire items from a survey of 643 elite Australian athletes. Items in the questionnaire that related to the concepts in the model were identified and structural equation modelling was employed to test the hypothesised model. Morality (cheating), benefit appraisal (performance), and threat appraisal (enforcement) evidenced the strongest relationships with attitude to doping, which in turn was positively associated with doping susceptibility. Self-esteem, perceptions of legitimacy and reference group opinions showed small non-significant associations with attitude to doping. The hypothesised model accounted for 30 percent and 11 percent of the variance in attitudes to doping and doping susceptibility, respectively. These present findings provide support for the model even though the questionnaire items were not constructed to specifically measure concepts contained in it. Thus, the model appears useful for understanding influences on doping. Nevertheless, there is a need to further explore individual and social factors that may influence athletes' use of performance enhancing drugs [11003].

One study aimed to construct a hierarchy of motives linked to doping behaviors. Between 2000 and 2005, calls to a national antidoping phone-help service by 115 cyclists, 203 bodybuilders, and 40 footballers were analyzed. The results showed that the main motives were preserving health for cyclists, increasing muscular strength for bodybuilders, and personal recreation for footballers. However, in contrast to the literature, group influence was low and health preoccupations were high for cyclists; the influence of body image was relatively low for bodybuilders; and footballers cited muscular strength enhancement as a motive. The study's limitations are noted. The prevention campaigns therefore need to be specific [11004].

Anti-ageing and body building substances became widely accepted in the 21st century due to modified social and economical attitudes. Such requirements are characteristic of a visually oriented consumer society. The body is considered to be a marker of social prestige and in the striving for a nicer appearance is becoming a part of šperaon's identity. Medical
and pseudo-medical approaches have been developed to fulfill these desires. In the general perception of a modern society such efforts are considered as positive, but some of such procedures are potentially risky, while others seem to be not efficient [07013].

One paper addressed a gap in the literature of empirically derived models of performance enhancing supplement use by developing a demographic and psychosocially based model of athlete supplement use. Selected questions were used from a larger survey conducted by UK Sport into British athletes’ experiences, knowledge, attitudes and opinions in relation to anti-doping activities. Forward conditional step wise logistic regression was employed on data from 757 athletes to develop a model that discriminated supplement non-users from users. The model identified that British athletes most likely to use supplements were younger (under 23) males who were more likely to see doping as a problem in their sport and were more knowledgeable about testing procedures than their non-user counterparts. Post hoc analysis reinforced that non-users saw doping as less of a problem in their sport and were less knowledgeable about drug testing procedures relative to those using supplements. The pattern of results indicated gender-specific interventions on supplement use for young male athletes may yield significant benefits. The relationship between supplement use and perception of a doping problem suggested more work is needed to understand supplement use culture within sports. The relationship between knowledge of testing procedures and supplement use suggested further research is needed to see whether this is a positive or negative effect of detection-based doping deterrence activity [07014].

The objective of this study was to use self-determination theory to analyze the relationships of several motivational variables with exercise dependence. The study involved 531 exercisers, ranging in age from 16 to 60 years old, who responded to different questionnaires assessing perception of motivational climate, satisfaction of basic psychological needs, motivation types, and exercise dependence. The results of multiple mediation analysis revealed that ego-involving climate and perceived competence positively predicted exercise dependence in a directed and mediated manner through introjected and external regulation. Gender and age did not moderate the analyzed relationships. These results allow us to better understand the motivational process explaining exercise dependence, demonstrating the negative influence of the ego-involving climate in the context of exercise [12020].

**Specificity of motives for doping**

Most of the data to rank doping motives have been gathered from epidemiological with the use of questionnaires. However, questionnaires are known to systematically underestimate the reality (regarding the nature and relative proportion of motives) of addictive behaviors because of common social representations of doping. Qualitative studies based on retrospective interviews or information from champions’ confessions tend to emphasize extrinsic reasons for doping and thereby limit personal responsibility and protect self-esteem. Moreover, the “true story” may be modified to limit the risks of sanction. Whatever the protocol design, researchers are confronted with the approach to doping in sport, in which it is never admitted to any substance abuse. Furthermore, the motives for using prohibited substances seem to concern a minority of athletes: generally those of a high level and those who have tested positive in doping controls. The motives offered by these athletes, such as enhancing performance, increasing financial gain, and making a sporting name for one-self, are associated with common social representations. The studies to determine and rank the motives for secretive doping behaviors have met with methodological difficulties. Few sporting figures have a personal interest in breaking the secrecy of doping, not individual athletes or teammates, staff or federation members, journalists, or sponsors. Deliberately damaging the popular myth of athletic perfection is anathema to virtually all members of the world sporting community. Thus, the power of common social representations limits access to the real thoughts and behaviors concerning doping.
Researchers can obtain cold cognitions, with memory biases (deliberate or not), reconstructions to protect self-esteem, and external causal attributions. But classical interviews with athletes who have tested positive cannot deliver “the whole story.” These athletes are under intense social pressure and at risk of condemnation; media scrutiny has a powerful impact, and any inquiry is retrospective, with responses subject to deformation and omission. Doping thus remains a taboo, and the biases inherent to standard retrospective studies are too great to obtain a model with quantitative ranking of doping motives in athletes. Moreover, many studies have focused their investigations on adolescents, sportspersons of a low level (local, regional), or retired elite athletes. Nevertheless, the evidence shows that the risk of doping is highest among men, young adults (20-25 years old), and high-level practitioners [10303].

One study aimed to construct a hierarchy of motives linked to doping behaviors. Between 2000 and 2005, calls to a national antidoping phone-help service by 115 cyclists, 203 bodybuilders, and 40 footballers were analyzed. The results showed that the main motives were preserving health for cyclists, increasing muscular strength for bodybuilders, and personal recreation for footballers. The cyclists first cited health concerns to justify the use of several illicit substances (rank 1). They reported trying to reduce health problems associated with their sport. Other motives in the same vein were limiting pain during effort (rank 6), reducing fatigue (rank 7), reducing pain in general (rank 10), treating acute injury (rank 14), and enhancing fatigue recovery (rank 14). It was thus logical that glucocorticosteroids were the most cited substance (35%). Therefore, the priority of the cyclists appeared to be a strategy to preserve fitness and prevent injuries and symptoms due to intensive training. Their health concerns were due to the training intensity and sensitivity to pain and fatigue. Associated with this motive, it was also observed that sport doctors were a real support in justifying consumption (rank 4). Cyclists have been shown to be predisposed to collaborate with team sport doctors to treat and medicate for any health concerns that may affect their performance. It is also interesting to note that concern for health was the argument put forth by the east Germans to defend their use of illegal substances: They argued that these substances were used not as performance enhancers but as performance enablers. From their viewpoint, these substances were required to maintain homeostasis during heavy and prolonged training. This conception (i.e. medical use of substances to maintain health) is also a current topic in American horse racing, another example that emphasizes the complexity of understanding the “doping” activity. The second motive was performance enhancement (rank 2). The third motive was the social norms associated with the sport. The cycling subculture has a powerful impact on its members. Cycling is ruled by implicit principles that create high team cohesion. Doping is one of the principles of this cohesion, but no one refers to it. The risks are minimized, considered as banal, so that they become normal. The first motive for the bodybuilders was strength enhancement. Thus, the frequent phone discussions about steroids were unsurprising (78%), as noted by several researchers and as it has been shown that 38 percent of amateur bodybuilders used anabolic steroids, but body image disturbance was not the main reason. First of all, these athletes hoped to increase their muscular strength and seemed to be in good shape. The doping substance might not be considered by the bodybuilders to be dangerous. The substance is perceived as a “natural” addition of exercise to rapidly increase muscular volume. The second motive was the sport's norms. Bodybuilders justify doping as a natural means to increase muscular volume, improve attractiveness, and decrease fatigue. Because nearly all bodybuilders consume these substances, their use is legitimized. Moreover, the bodybuilders reported receiving considerable advice from proximal circles such as other athletes (rank 7), family (rank 11), dealers (rank 12), others (rank 13), and friends (rank 15). This finding supports the observation that bodybuilders have higher scores of social dependence than other athletes. They easily follow the lead of others in denying any inappropriate behavior and controlling their use of anabolic substances. They underestimate their own consumption compared with
other athletes who use steroids dangerously in intensive sessions, such as cyclists. The third motive was body image. The high value that society gives to physical attractiveness encourages bodybuilders to try to conform to an ideal image. They are fascinated by their own image, and the walls of mirrors in fitness centers reinforce this feeling. This motive was associated with the need to affirm masculine identity. The bodybuilders wanted to become more “male” and attractive. They hoped to dominate and use their bodies as a power vehicle. However, in this particular sport culture, a pervasive belief is that power must be acquired by pain and effort, enhancing virility and courage, with bodybuilders seen as modern gladiators. The first motive of the footballers was conformity to the social norms of their general culture and not their sport. This motive was associated with the influence of friends (rank 4) and suggests that football exerts a pressure on players to do as others do. The substances massively cited were cannabinoids (52 %) as well as stimulants (13 %). Creating and maintaining a festive atmosphere thus emerged as a predominant motivation are widely used at social events among friends and are characterized by relaxation and a decrease in inhibition. They are found in all social classes. Although the players reported using cannabis for reasons other than performance enhancement, the substance is still prohibited by football governing bodies and the WADA. The group enhances a collective dynamic to use this substance. The second motive was the control of anxiety, probably due to the uncertainty of the game results. The game of football is highly unpredictable because it is a team sport that depends on the performances of many players, the performances of opponents, the weather, the partiality/impartiality of referees, coaches’ choices, and injuries, among other. The high number of factors that footballers cannot control generates considerable anxiety. The efficiency of cannabis to decrease anxiety has been well demonstrated. Use of this substance can also be a self-handicapping strategy to limit the impact of poor sporting results and explain defeat. The third motive was strength enhancement. Football requires leg speed, and anabolic steroid substances can enhance individual performance during matches. The results showed that doping motives differed between cyclists, bodybuilders, and footballers. The main motives for doping were specific: preserving health for cyclists, increasing muscular strength for bodybuilders, and enjoyment for footballers. The callers all knew the best substances to obtain the desired effect. Compared with the literature, the surprising results of this study were the weak group influence and the impact of health preoccupations for the cyclists, the relatively low influence of body image for the bodybuilders, and the motivation to increase muscular strength in the footballers. Thus, prevention campaigns need to be specific. Comparing motives for prohibited substance use can lead to confusion if the individual sport is not taken into consideration. The first motive cited by cyclists indicates the paradoxical strategy of current antidoping campaigns. The campaigns focus on the dangers of illicit substances to athletes’ health. Yet these athletes were using appropriate and efficient substances to preserve their health. Thus, a prevention message targeting health maintenance misses the point because their main doping motive is to combat the very real (and subjective) risk of falling ill. The real risk instead seems to be self-medication, and a more effective message to cyclists should thus emphasize means to maintain a good balance between high performance and optimal health. Issues of domination and male identity need to be addressed in campaigns for bodybuilders via better health education. Prevention should be more focused on body self-acceptance. The footballers hoped to strengthen their team relationships. Prevention campaigns must insist on limiting use of recreational drugs and supplements [10303].

The use of anabolic androgenic steroids (AAS) has been associated with the use of illegal drugs. Earlier observations suggested that users of illegal drugs may use AAS for reasons other than increasing muscle strength or size. The aim of the present study was to investigate the motives for AAS use among outpatients at a substance abuse center in Stockholm, Sweden. All male patients under the age of 50 were asked whether they had used AAS during a 2-month period. An AAS survey was administered to those who reported
AAS experiences in the admittance interview. Twenty of the 175 respondents (11%) reported using AAS. The most frequently reported motives were related to anabolic effects (i.e. for a good-looking body, to become stronger, or to perform better in sports). However, some users reported other motives; for example, to conceal concomitant drug use, to alleviate insecurity or low self-esteem, to become brave, or in preparation of committing a crime. Furthermore, many respondents reported side effects that were associated with AAS; most notably, irritability and depression/suicidal ideation. It was thus concluded that some users of illicit drugs also use AAS for reasons other than the anabolic properties of these compounds. Therefore, considering that AAS may cause or contribute to diverse morbidity, it is important to ask users of illicit drugs about AAS use, even when obvious external signs of AAS use are lacking [10316].

Population heterogeneity
Appearance- and performance-enhancing drugs (APEDs) constitute a wide range of substances, including anabolic-androgenic steroids, nonsteroidal anabolics, and licit and illicit ergo/thermogenics. A great deal of heterogeneity exists in APED use patterns among weight-lifting men, and, consequently, little is known about how these patterns are related to side effect profiles or risk potential. In the current study, a sample of 400 adult men who were regular APED users completed an interactive Web-based instrument detailing information about APED use, side effects, and related indicators of risk. To explore the heterogeneity of APED use patterns, the authors subjected data on use patterns to latent class analysis (LCA), latent trait analysis (LTA), and factor mixture analysis to determine the best model of APED use. Results indicated that a 4-class factor mixture model provided a better fit than LCA and LTA models. The authors also found that severity and latent class were uniquely associated with negative outcomes. Each of the 4 classes was associated with unique side effects, motivations, and participant use patterns. Implications for identifying pathological forms of APED use are discussed [07016].

An age factor
It was attempted to qualitatively investigate why men of two age categories have chosen not to use androgenic-anabolic steroids (AASs). Twelve men (22 years [group I] and 53 years [group II]) were selected on the basis of specific inclusion criteria, including age and fitness levels (i.e. "do you weight train?"). Subjects were classified in 1 of 2 categories-younger or older precluders-and were asked to complete two survey instruments before their participation. The Drive for Muscularity Scale (reliability 0.85) and Body Image Questionnaire were used to gain a better understanding of perceptions and motivations regarding health, fitness, and body image. A series of semistructured questions were used to enhance focus group discussion regarding attitudes. Questions were validated by a panel of experts in qualitative methods. Member checks were conducted to enhance trustworthiness of the data. Data were transcribed verbatim and analyzed with thematic open-coding techniques. Various behaviors were reported regarding body image. Emerging themes showed a clear demarcation between age categories. Younger subjects cited power, control, body image, and narcissism, whereas older subjects viewed AAS use as more of an athletic-based phenomenon, such as with performance enhancement, when asked about steroids. Groups were in agreement that media trends and perceptions of the ideal male body are becoming "superhuman" and unattainable without chemical means. Understanding attitudinal perspectives might help complement national data on AAS trends. Future investigations could help coaches and allied health professionals collaborate with each other, as well as with national groups and foundations, to devise more appropriate strategies in addressing this growing athletic and public health concern [07017].

Elite athletes’ attitudes, beliefs, and knowledge
In the absence of objective information on the use of performance-enhancing drugs (PED), attitudes are often used as a proxy for doping behaviour, assuming that those who use banned substances show greater leniency towards doping than those who stay clear of doping. Consequently, researchers have identified the need to develop more sophisticated and bespoke interventions to support the athletes with attitudes that increase their likelihood of using banned substances, and the need to develop empirically tested models. Doping has evolved greatly in recent years, and greater understanding of it is essential for developing efficient prevention programmes. In the psychosocial approach, attitudes are considered an index of doping behaviour, relating the use of banned substances to greater leniency towards doping. The aim of one review was to gather and critically analyse the most recent publications describing elite athletes' attitudes, beliefs and knowledge of doping in sport, to better understand the foundations provided by the previous work, and to help develop practical strategies to efficiently combat doping. For this purpose, we performed a literature search using combinations of the terms "doping", "sport", "elite athletes", "attitudes", "beliefs", "knowledge", "drugs", and "performance-enhancing substances" (PES). A total of 33 studies were subjected to comprehensive assessment using articles published between 2000 and 2011. All of the reports focused on elite athletes and described their attitudes, beliefs and knowledge of doping in sport. It has been emphasized that athletes who use banned substances mainly do so to improve their performance, even though most athletes acknowledge that doping is dishonest, unhealthy and risky because of the impact of sanctions. The "false consensus effect" seems to play a key role in legitimizing the use of banned substances. Anti-doping programmes are generally considered to be ineffective and inefficient, and the way tests are performed is often criticized, particularly WADA's location reporting system. Athletes consider the severity of punishment to be appropriate or not severe enough, although there are some differences between sports. In this sense, the advisors and stakeholders who can influence athletes should also be educated and punished if they are found guilty of supporting doping. In this way, all interested parties would be aware of the magnitude of the problem. The current generation of athletes is more familiar with anti-doping rules than earlier generations, but there is still a lack of knowledge that should be improved using well designed educational programmes. There is also a distinct lack of information around dietary supplements and the possible side effects of PES. There is a general belief about the inefficacy of anti-doping programmes, and athletes criticise the way tests are carried out. Most athletes consider the severity of punishment to be appropriate or not severe enough. There are some differences between sports, as team-based sports and sports requiring motor skills could be less influenced by doping practices than individual self-paced sports. However, anti-doping controls are less exhaustive in team sports. The use of banned substance also differs according to the demand of the specific sport. Coaches appear to be the main influence and source of information for athletes, whereas doctors and other specialists do not seem to act as principal advisors. Athletes are becoming increasingly familiar with anti-doping rules, but there is still a lack of knowledge that should be remedied using appropriate educational programmes. There is also a lack of information on dietary supplements and the side effects of PES. Therefore, information and prevention are necessary, and should cater to the athletes and associated stakeholders. This will allow us to establish and maintain correct attitudes towards doping. Psychosocial programmes must be carefully planned and developed, and should include middle- to long-term objectives (e.g. changing attitudes towards doping and the doping culture). Some institutions have developed or started prevention or educational programmes without the necessary resources, while the majority of the budget is spent on anti-doping testing. To minimize the phenomenon of doping, information and prevention programmes, starting with athletes at a young age, and involving other stakeholders (e.g. the athletes’ doctors, coaches or family), are necessary to establish and maintain correct attitudes and behaviours. It is also very important that the sport institutions at all levels (from WADA to regional governments) provide more resources to psychosocial projects in relation to the biomedical approach (i.e. anti-doping controls),
which have been the main priority of anti-doping programmes currently in use. Also, event organizers and federations should check that sporting rules do not favour the possible advantages of using banned substances in competitions (i.e. by reducing the distance covered in competitions, allowing longer recovery between stages and encouraging, where possible, the importance of technical/tactical aspects rather than physical aspects). The programmes targeting athletes and those around them must be carefully planned and developed as a middle- to long-term objective and, ultimately, change attitudes towards doping and the doping culture. Current research methods are weak, especially questionnaires. A combination of qualitative and quantitative measurements are recommended, using interviews, questionnaires and, ideally, biomedical tests. Studies should also examine possible geographical and cultural differences in attitudes towards doping [13017].

Reports of illicit substance use by college athletes have become commonplace in recent years, yet comparatively little effort has been put forth by the research community to understand these behaviors. Data for this study came from a large, national dataset collected by the National Collegiate Athletic Association (NCAA). This study compared substance use behaviors of male undergraduate student athletes who reported using ergogenic performance enhancing substances (e.g., anabolic steroids and peptide hormones) during college (PES users) to those who did not (PES non-users). A consistent pattern of higher substance use rates was observed among PES users compared to non-users, including heavier drinking, higher prevalence rates of cigarettes, marijuana, amphetamines, narcotics, and a variety of permissible and impermissible dietary supplements. An unexpected finding was that there were large discrepancies in reported prevalence rates between similar or overlapping survey items (e.g., past year use of "narcotics" versus "I have taken Vicodin, Oxycontin or Percocet with/without a prescription"). These findings suggest that male college athletes who use PES while in college demonstrate a general tendency to engage in alcohol and drug use behaviors, regardless of whether these behaviors improve or impede athletic performance. The results further suggest that college athletes may not fully appreciate drug categorizations that are commonly employed to gauge substance use behaviors. Changes to drug education and prevention programs may be needed to enhance understanding of drug properties and actions [13041].

It was examined whether constructs outlined in self-determination theory [Deci & Ryan, 2002], namely, autonomy-supportive and controlling motivational climates and autonomous and controlled motivation, were related to attitudes toward performance-enhancing drugs (PEDs) in sport and drug-taking susceptibility. It was also investigated moral disengagement as a potential mediator. It was surveyed a sample of 224 competitive athletes (59 % female; median age 20 years; median 10 years of experience participating in their sport), including 81 elite athletes. Using structural equation modeling analyses, the hypothesis proposing positive relationships with controlling climates, controlled motivation, and PEDs attitudes and susceptibility was largely supported, whereas our hypothesis proposing negative relationships among autonomous climate, autonomous motivation, and PEDs attitudes and susceptibility was not supported. Moral disengagement was a strong predictor of positive attitudes toward PEDs, which, in turn, was a strong predictor of PEDs susceptibility. These findings are discussed from both motivational and moral disengagement viewpoints [13797].

Although nutrition and doping are important factors in sports, neither is often investigated in synchronized swimming (Synchro). One study aimed to define and compare Synchro athletes and their coaches on their knowledge of sports nutrition (KSN) and knowledge of doping (KD); and to study factors related to KSN and KD in each of these groups. Additionally, the KSN and KD questionnaires were evaluated for their reliability and validity. Altogether, 82 athletes (17 ± 2 years of age) and 28 coaches (31 ± 5 years of age) from
Croatia and Serbia were included in the study, with a 99 percent response rate. The test and retest correlations were 0.94 and 0.90 for the KD and KSN, respectively. Subjects responded equally to 91 percent queries of the KD and 89 percent queries of the KSN. Although most of the coaches are highly educated, they declared self-education as the primary source of information about doping and sport-nutrition. Coaches scored higher than their athletes on both questionnaires which defined appropriate discriminative validity of the questionnaires. Variables such as age, sports experience and formal education are positively correlated to KSN and KD scores among athletes. The athletes who scored better on the KD are less prone to doping behavior in the future. These data reinforce the need for systematic educational programs on doping and sports nutrition in synchronized swimming. Special attention should be placed on younger athletes. Although most of the synchro coaches are highly educated, self-education is declared as the primary source of information about doping and sport-nutrition. The knowledge of doping and doping-health hazards are negatively related to potential doping behavior in the future among synchronized swimmers. The data reinforce the need for systematic educational programs on doping and sports nutrition in synchronized swimming. It was advocated improving the knowledge of sports nutrition among older coaches and the knowledge of doping among younger coaches, while among athletes, younger swimmers should be targeted [13042].

Although athletes' beliefs and values are known to influence whether or not an athlete will use banned drugs, little is known about the athletes' beliefs and attitudes in different sports. The aim of this study was to clarify the beliefs and attitudes of elite athletes towards banned substances and methods in sports. A total of 446 athletes (response rate 90 %; 446/494) financially supported by the National Finnish Olympic Committee completed a structured questionnaire during their national team camps in 2002. More than 90 percent of the athletes reported to believe that banned substances and methods have performance enhancing effects, and 30 percent reported that they personally know an athlete who uses banned substances. Of the male athletes 35 percent, and 23 percent of females reported they personally know an athlete using banned substances. A total of 15 percent of the athletes reported that they had been offered banned substances: 21 percent of the speed and power athletes, 14 percent of the team sport athletes and of the athletes in motor skills demanding events, and 10 % of the endurance athletes. Stimulants were the most often offered substance group (to 7 % of all the athletes) followed by anabolic steroids (4 %). Subjects who regarded doping as a minor health risk seemed to be more often associated with doping users than those regarding doping as a significant health risk. Athletes in different sports have a different approach to doping. Risk of doping appears to be highest in speed and power sports and lowest in motor skills demanding sports. Males are at higher risk than females. Controlling doping only by tests is not sufficient. A profound change in the attitudes is needed, which should be monitored repeatedly [06015].

One study aimed to investigate, among a sample of elite Australian athletes, the extent to which this group supports drug testing as a deterrent to drug use. Data was collected from a convenience sample of (n=974) elite Australian athletes who self-completed a questionnaire, and semi-structured telephone surveys with key experts. The athletes surveyed endorsed testing for banned substances as an effective way of deterring drug use; believed that the current punishments for being caught using a banned substance was of the appropriate severity; and indicated that there should be separate policies regarding illicit drug and performance-enhancing drug use. A large proportion of elite athletes in Australia endorse drug testing as an effective means of deterring drug use. However, they perceive a difference between being detected using a performance-enhancing drug and an illicit drug and believe that penalties should reflect this difference. Future research may wish to investigate attitudes towards newer methods employed to detection drug use [10315].
Information-seeking behaviours among elite athletes

Many sporting organisations conduct drug information seminars for their athletes; however, it is uncertain whether these programs provide athletes with pertinent drug information in formats that are conducive to information retention. The aims one study were to investigate self-reported confidence in knowledge of illicit drugs and information seeking behaviours among elite athletes. Data were collected from two sources: quantitative surveys with elite athletes; and qualitative interviews with key experts who come into contact with elite athletes. Athletes were confident in their knowledge of the effects of illicit drugs such as cannabis and meth/amphetamine, but less confident in their knowledge of the effects of illicit drugs such as GHB and ketamine. A substantial proportion felt that athletes in their sport would benefit from more information concerning illicit drugs. It was concluded that both athletes and key expert believed that information on illicit drugs should be delivered to athletes in a specific and relevant manner. There may be stigma attached to information seeking within a sports club or organisation. Accordingly, improving the accessibility to creditable information via the Internet may prove to be an effective means by which to educate athletes on the effects of illicit drugs [11417].

Attitude of elite cyclists on doping

The protection of the health of athletes is one of the three criteria taken into account when registering a substance in the World Anti-Doping Agency prohibited list. Nevertheless, in elite-level cycling, banned substance use is widespread. On study adopted a psychological approach to examine how or whether perceived health risks influence elite-level cyclists' decisions to use banned substances. Sixteen semi-structured interviews were conducted with cyclists hoping to join a professional team (n=6), neo-professional cyclists (n=2), and former professional cyclists (n=8). Although an evolution was observed in the organization of doping and perceptions of doping over the last decade, the perceived health hazards did not influence, most of the time, decisions to use banned substances among the sample of cyclists. There was a systematization of exogenous substance use in the cycling environment and a trivialization of the side effects of the banned substances. Finally, younger cyclists were not concerned about the long-term health consequences of banned substances; they were more focused on the short-term performance-enhancing benefits. There is a need to implement more effective preventive programs to change athletes' attitudes toward doping and its health risks [12021].

Young athletes attitudes towards doping

There is evidence of a small but significant proportion of adolescents engaging in doping practices. Young athletes face very specific pressures to achieve results as they strive for a career at an elite level. This study used an anonymized questionnaire to survey 403 (12-21 years old) talented young athletes' attitudes toward performance-enhancing substances and supplements. Two-thirds of the sample comprised males. Athletes were generally against the use of doping substances to enhance sporting performance. Within this generally unfavorable view, males tended to express a more permissive attitude toward performance-enhancing methods than females. Those convinced of the necessity of supplementation for sporting success were also more likely to express permissive attitudes. When asked whether they would take a "magic" drug that, while undetectable, would significantly enhance performance, the overwhelming majority of athletes said "no," but many thought others would take the substance. Interestingly, there was a significant association between the projected use of the hypothetical drug by competitors and the individual respondent's willingness to take the hypothetically "magic" substance. The study offers an insight into young athletes'
attitudes toward specific forms of performance enhancement, and the strength of their beliefs in the face of a tempting hypothetical scenario [12022].

**Other athletes’ attitudes regarding doping**

Review articles suggest a small but significant proportion (between 3 and 12%) of male adolescents have used anabolic-androgenic steroids (AAS) at some point. A total of 40 talented male and female athletes (mean average age 20 years) from 13 different sports attended 12 focus groups held over the UK intended to investigate athletes' attitudes toward doping. Focus group transcriptions were analysed and coded with the use of QSR NVivo 8. Athletes in general did not report a significant national doping problem in their sport, but exhibited sporting xenophobia with regard to both doping practices and the stringency of testing procedures outside of the UK. Athletes often viewed doping as "unnatural" and considered the shame associated with doping to be a significant deterrent. Athletes perceived no external pressure to use performance enhancing drugs. In response to hypothetical questions, however, various factors were acknowledged as potential "pressure" points: most notably injury recovery and the economic pressures of elite sport. Finally, a significant minority of athletes entertained the possibility of taking a banned hypothetical performance enhancing drug under conditions of guaranteed success and undetectability. It was concluded that the athletes in this study generally embraced those values promoted in anti-doping educational programmes, although there were some notable exceptions. That the social emotion of shame was considered a significant deterrent suggests anti-doping efforts that cultivate a shared sense of responsibility to remain “clean” and emphasise the social sanctions associated with being deemed a “drugs cheat”, resonate with this atypical social group [10314].

**How athletes discuss on doping**

One study investigates the discursive management of taking prohibited substances in sport. In particular, it explores how one high-profile athlete, Australian cricketer Shane Warne, accounted for his drug-taking behavior when talking to the media. Discursive psychology (DP) is used as the theoretical and methodological framework to study drug discourses in sport. Emphasis in DP is on making explicit how psychological concepts (e.g. drug explanations) are used in everyday talk to perform certain actions. The research data is Warne's first public press statement concerning his 2003 positive test for hydrochlorothiazide and amiloride. Analysis reveals that Warne constructed his drug taking as not being related to performance enhancement and substantiated this with a history of negative test results. Warne worked up his taking as the result of ignorance rather than deliberate deception. Further, he presented this as a one-off event and not reflective of systematic drug usage. It is argued in this study that to better understand drug taking in sport, sport researchers need to understand how athletes talk about drugs. For it is through talk that most sporting activities are conducted and maintained, and it is this talk that needs to be understood and analyzed [07022].

**Attitudes against dopers**

It was investigated the social image of anabolic steroids (AS) users grounding our analysis on the achievement goal theory of Nicholls. The main goal was to explore how an athlete's acceptance of AS use would impact on the way that athlete will be perceived by others. Non-AS-using participants reacted to one of two scenarios portraying a male athlete either accepting or refusing to engage in drug use behavior. The results suggested that the acceptance of anabolic steroids yielded an unfavorable social image – perceivers inferred a predominant ego orientation to characterize the AS-user's motivation as well as weaker
sportspersonship and a stronger proclivity for reactive aggression than instrumental aggression. Moreover, the analyses did not yield significant gender or interaction effects. Finally, those findings are commented in view of methodological shortcomings and of the perspectives that they may offer for future research concerning the motivational aspects of the social perceptions of drug use in sport [13050].

**Body image and attitudes toward male roles**

The authors sought to expand on previous observations suggesting that body-image pathology is associated with illicit use of anabolic-androgenic steroids (AAS). In particular, the authors compared current versus past AAS users and short-term versus long-term users in this respect. The authors assessed 89 heterosexual men who lifted weights regularly-48 AAS users and 41 nonusers-on measures of self-esteem, attitudes toward male roles, body image, eating-related attitudes and behaviors, and muscle dysmorphia ("reverse anorexia nervosa"). AAS users as a whole showed few differences from nonusers on most measures but showed greater symptoms of muscle dysmorphia (e.g. not allowing their bodies to be seen in public, giving up pleasurable activities because of body-appearance concerns). The current and past AAS users each differed only modestly from nonusers on most measures. Short-term AAS "experimenters" were also largely indistinguishable from nonusers, but the long-term AAS users showed striking and significant differences from nonusers on many measures, including marked symptoms of muscle dysmorphia and stronger endorsement of conventional male roles. It was concluded that both body-image pathology and narrow stereotypic views of masculinity appear to be prominent among men with long-term AAS use. Although our cross-sectional observations cannot confirm that these factors help to cause or perpetuate AAS use, a causal hypothesis is certainly plausible and deserving of further testing in longitudinal studies. If these factors are indeed causal, then AAS users might respond to cognitive behavior approaches that simultaneously take aim at both types of maladaptive beliefs [06016].

Men's fitness goals are influenced by the lens through which they view their bodies, which is different from the way women view their bodies. Their increased focus on a muscular, hairless body means that they exercise to enhance their physical bulk and are more likely to engage in depilatory behaviors. In addition, the drive for muscularity may be associated with an increased risk anabolic-androgenic steroids and other nutritional supplements whose utility not clearly demonstrated. In the extreme, the drive for muscularity may manifest itself as a form of body dysmorphic disorder referred to as muscle dysmorphia. However, not all men focus on their muscularity. Gay men are more likely than heterosexual men to experience a desire to be thin and are at greater risk for eating and body image disorders [07015].

**Polypharmacy**

A review of the literature was conducted to examine the relationship between the use of anabolic androgenic steroid (AAS) use and the use of other drugs. Studies published between the years of 1995 and 2010 were included in the review. The use of AAS is positively associated with use of alcohol, illicit drugs and legal performance enhancing substances. In contrast, the relationship between AAS and the use of tobacco and cannabis is mixed. It was concluded that the results of the review indicate that the relationship between AAS use and other substance use depends on the type of substance studied [01108].

The aim of one study was to describe qualitatively and quantitatively dietary supplements
(DS) and medication use in elite athletes. Athletes (n=912; age 24 ± 6 years; 72 % male) reported medications and DSs taken within 3 days before doping control. It was analyzed data collected from 2006 to 2008, identified and classified substances. Total of 75 percent athletes reported use of at least one substance, 61 percent took DS (3 per user) and 41 percent took medications. Among users, 21 percent reported the use of six and more different products, and one took 17 different products at the same time. Majority of medication users took non-steroidal anti-inflammatory drugs (NSAID) (25 %), and 22 percent used more than one NSAID. It was found no gender differences in DS use. Individual sport athletes used more DS. The study showed widespread use of DS and drugs by elite athletes. Consumption of DS with no evident performance or health benefits, demonstrated the need for specific educational programs focused on DS use. Amount, quantity and combination of the reported products raised concern about the risk of potential side effects [11009].

Medical and nonmedical use of stimulant drugs
Smith and Farah (2011) presented a scholarly review of critical areas related to their intriguing title "Are Prescription Stimulants 'Smart Pills'?") It was contend that they accomplished the main goal of the article, to get the facts straight about possible cognitive enhancement via the nonmedical use of stimulant drugs by individuals without a diagnosis of attention-deficit/hyperactivity disorder (ADHD). At the same time, they justified their main conclusions that (a) individuals are seeking and engaging in nonmedical use of stimulant drugs with the expectations of cognitive enhancement despite uncertainty whether such expectations are valid and (b) on some tasks, there are small average benefits of nonmedical use, but the overall pattern is not clear (e.g., small beneficial effects across most individuals or large beneficial effects only in a few individuals, both of which result in small average effects). It was offeedr comments in 3 areas to amplify key topics mentioned but not emphasized by Smith and Farah: (a) characterization of the cognitive effects of medical use of stimulants to contrast with the cognitive effects of nonmedical use; (b) justification of medical use of stimulants by placement on a normally distributed dimension of behavior rather than categorical diagnosis of ADHD, which varies widely across countries; and (c) evaluation of the potential risks of nonmedical use to individuals and to society (e.g., the likelihood of addiction to stimulant drugs in a small minority of the population) rather than just the potential benefits of cognitive enhancement [11418].

Concomittant use of other substances
Reports of illicit substance use by college athletes have become commonplace in recent years, yet comparatively little effort has been put forth by the research community to understand these behaviors. Data for this study came from a large, national dataset collected by the National Collegiate Athletic Association (NCAA). The study compared substance use behaviors of male undergraduate student athletes who reported using ergogenic performance enhancing substances (e.g. anabolic steroids and peptide hormones) during college (PES users) to those who did not (PES non-users). A consistent pattern of higher substance use rates was observed among PES users compared to non-users, including heavier drinking, higher prevalence rates of cigarettes, marijuana, amphetamines, narcotics, and a variety of permissible and impermissible dietary supplements. An unexpected finding was that there were large discrepancies in reported prevalence rates between similar or overlapping survey items (e.g. past year use of "narcotics" versus "I have taken Vicodin, Oxycontin or Percocet with/without a prescription"). The findings suggest that male college athletes who use PES while in college demonstrate a general tendency to engage in alcohol and drug use behaviors, regardless of whether these behaviors improve or impede athletic performance. The results further suggest that college athletes may not fully appreciate drug categorizations that are commonly employed to gauge substance use behaviors. Changes to
drug education and prevention programs may be needed to enhance understanding of drug properties and actions [13037].

Neuropharmacy addiction

The use of psychoactive substances to neuroenhance cognitive performance is prevalent. Neuroenhancement (NE) in everyday life and doping in sport might rest on similar attitudinal representations, and both behaviors can be theoretically modeled by comparable means-to-end relations (substance-performance). A behavioral (not substance-based) definition of NE is proposed, with assumed functionality as its core component. It is empirically tested whether different NE variants (lifestyle drug, prescription drug, and illicit substance) can be regressed to school stressors. Participants were 519 students (26 years old, 73 % female). Logistic regressions indicate that a modified doping attitude scale can predict all three NE variants. Multiple NE substance abuse was frequent. Overwhelming demands in school were associated with lifestyle and prescription drug NE. Researchers should be sensitive for probable structural similarities between enhancement in everyday life and sport and systematically explore where findings from one domain can be adapted for the other. Policy makers should be aware that students might misperceive NE as an acceptable means of coping with stress in school, and help to form societal sensitivity for the topic of NE among our younger ones in general [13038].

Connexion to use of dietary supplement

Recently published case reports, coupled with a large observational study of 1017 deployed servicemen to Iraq (January 2009), has highlighted the issue and potential concerns regarding the unregulated use of dietary and exercise supplements within the British military. Consequently, an exploratory pilot study was undertaken to assess whether the findings of the previous Iraq study were applicable to current deployed British servicemen in Afghanistan. From 150 questionnaires handed out there were 87 completed questionnaires (58 % return). The mean age was 28 with 90 percent being male. From the total of 87 persons 46 percent were self-declared current smokers with 38 percent admitting to drinking >6 caffeinated drinks per day. Forty nine persons (56 %) admitted to a history of supplement use with 35 (40 % compared with 32 % in 2009 in Iraq) declaring current use. The average duration of supplement use among current users was 3 (2-9) months. The main sources of supplement supply were via local NAAFI purchase (57 %), internet purchase (40 %) and via their local chemist (3 %). The main types of supplement used were proteins/amino acids (86 %), creatine (34 %), chromium (31 %), stimulants (17 %), hydroxycut (6 %), and testosterone boosters (1 %) with no persons admitting to the use of ephedra or anabolic steroids. It was concluded that a significant proportion of the British servicemen employed on operations in Afghanistan who were sampled, admitted to current dietary and exercise supplement use whilst on deployment. The results of this small study suggest that their use on operations may be increasing. Smoking rates and caffeine consumption, on deployment, remain high in the British military. A larger detailed study with greater representation among soldiers deployed to forward operating bases would be helpful to fully appreciate the scale of supplement use [11423].

The aim of one study was to describe the prevalence, trends and associated factors of dietary supplements (DS) and anabolic-androgenic steroids (AAS) use among Finnish adolescents. The sample comprised 30 511 adolescents aged 12-18 years, of which 22 519 (74 %) answered a questionnaire. It was also studied associations between 14 socioeconomic, health and health behavioural variables and DS and AAS use by logistic regression. The proportion of respondents using DS was 45 percent during the past year and
it increased linearly by age. Vitamins (37 %) and herbal products (13 %) were the most common DSs. In 1991, 9 percent of the boys aged 16-18 years reported protein use, while the frequency in 2005 was 17 percent, which was a significant increase. AAS use was uncommon; only 53 boys (0.5 %) and 20 girls (0.2 %) reported AAS use. The strongest factors associated with DS use in multivariate model were physical exercise outside sports clubs (odds ratio 1.9; 95 % confidence interval 1.6 to 2.2), and in sports clubs (odds ratio 1.7; 95 % confidence interval 1.5 to 1.9). Recurrent drunkenness (odds ratio 5.8; 95 % confidence interval 1.5 to 21.6) and peer drug use in boys (odds ratio 2.1; 95 % confidence interval 1.2 to 3.7) were the risk factors for AAS use, whereas physical exercise outside sports clubs (odds ratio 0.3; 95 % confidence interval 0.1 to 0.5) was a protecting factor. It was concluded that although the overall use of DS remained at the same level during the study period, there was a slight trend towards increasing use of vitamin and protein supplements. Dietary supplements use is associated with frequent sports participation and poorer than average health, while anabolic-androgenic steroids use is associated with health-compromising behaviours [10015].

Nutritional supplement (NS) use is widespread in sport. This study applied an integrated social cognitive approach to examine doping attitudes, beliefs, and self-reported doping use behavior across NS users (n=96) and nonusers (n=116). Following ethical approval, 212 competitive athletes (age mean 21; 137 males) completed self-reported measures of doping-related social cognitions and behaviors, presented in an online format where completion implied consent. Significantly more NS users (23 %) reported doping compared with nonusers (6 %). NS users presented significantly more positive attitudes toward doping and expressed a significantly greater belief that doping is effective. When presented with the scenario that performance-enhancing substances are effective and increase the possibility of winning, NS users were significantly more in favor of competing in situations that allow doping. In sum, doping use is three-and-a-half times more prevalent in NS users compared with nonusers. This finding is accompanied by significant differences in doping attitudes, norms, and beliefs. Thus, this article offers support for the gateway hypothesis; athletes who engage in legal performance enhancement practices appear to embody an "at-risk" group for transition toward doping. Education should be appropriately targeted [13035].

Fitness supplements as a gateway substance for AAS use
Approximately 3 percent of young Americans have used anabolic-androgenic steroids (AAS). A traditional model of adolescent substance use, the gateway hypothesis, suggests that drug use follows a chronological, causal sequence, whereby initial use of a specific drug leads to an increased likelihood of future drug use. Therefore, the use of illicit appearance and performance enhancing drugs (APED), such as AASs, also follows an analogous progression, whereby legal APEDs, (e.g. nutritional supplements) precedes illicit APED use. It was examined the relationship between nutritional supplement use, beliefs about APEDs, and APED use in 201 male (n=100) and female (n=101) undergraduates. Participants completed measures of muscle dysmorphia (MDDI), body checking (BCQ, MBCQ), eating disorder symptoms (EDE-Q), perfectionism (FMPS), positive beliefs about the efficacy-safety of AAS use and APED use patterns. A series of covariance structure models (CSM) showed body image disturbance, compulsive exercise, illicit drug use, and perfectionism, independent of gender, were significant predictors of positive beliefs about AAS. Those who used both fat burning and muscle building supplements reported the strongest beliefs in AAS efficacy-safety, which was associated with higher likelihood of current illicit APED use. There was evidence of significant indirect relationships between supplement use and illicit APED use through contact with other AAS users and beliefs about AAS. The potential role for nutritional supplement use in the initiation of illegal APED use is discussed. Future prevention efforts may benefit from targeting legal APED users in youth. (PsycINFO Database Record) [12024].
**Complementary and alternative medicine (CAM)**

Athletes are high achievers who may seek creative or unconventional methods to improve performance. Western medicine as is practiced in many industrialised countries is generally regarded as conventional, or orthodox, and its use has a long-established history in these societies. Interest and use of complementary and alternative medicine (CAM) has, however, been growing in recent times in Western countries, as reflected by the increasing number of research papers in medical and scientific journals. The literature indicates that athletes are among the heaviest users of complementary and alternative medicine (CAM) and thus may pioneer population trends in CAM use. Surveys in many countries have suggested a high use of CAM: in the United States, about a third of adults aged 18 years or older use CAM. While nonathletes may use CAM for prevention, treatment or rehabilitation from illness/injuries, athletes may possibly also use CAM for performance enhancement. If links between sport motivation and doping exist, and athletes' sport motivation and CAM use are related, a connection between athletes' CAM use and doping may also occur. Despite this growing interest, the definition of what is complementary or alternative remains very subjective and is certainly not universally accepted. While individual organisations have their own definitions, the perceptions of the general population or the end users and even the practitioners of the various forms of medicine of what constitutes CAM vary tremendously. In addition, More physicians are also seeking training in CAM: there is an estimated 3,000 American physicians who integrate acupuncture into their practice and an estimated one-third of homeopaths who are physicians or osteopaths. Unlike non-athletes, athletes may use CAM not just for prevention, treatment or rehabilitation from illness or injuries, but also for performance enhancement. Assuming that athletes' creative use of anything unconventional is aimed at "legally" improving performance, CAM may be used because it is perceived as more "natural" and erroneously assumed as not potentially doping. This failure to recognise CAMs as pharmacological agents puts athletes at risk of inadvertent doping. The general position of the World Anti-Doping Authority (WADA) is one of strict liability, an application of the legal proposition that ignorance is no excuse and the ultimate responsibility is on the athlete to ensure at all times whatever is swallowed, injected or applied to the athlete is both safe and legal for use. This means that a violation occurs whether or not the athlete intentionally or unintentionally, knowingly or unknowingly, used a prohibited substance/method or was negligent or otherwise at fault. Athletes are therefore expected to understand not only what is prohibited, but also what might potentially cause an inadvertent doping violation. Yet, as will be discussed, athlete knowledge on doping is deficient and WADA itself sometimes changes its position on prohibited methods or substances. The situation is further confounded by the conflicting stance of anti-doping experts in the media. These highly published disagreements may further portray inconsistencies in anti-doping guidelines and suggest to athletes that what is considered doping is dependent on the dominant political zeitgeist. Taken together, athletes may believe that unless a specific and explicit ruling is made, guidelines are open to interpretation. Therefore doping risk-taking behaviours may occur because of the potential financial, social and performance gains and the optimistically biased interpretation (that trying alternatives is part of the "spirit of sport") and doping risk-taking behaviours may occur. This discussion paper seeks to situate the reader in a world where elite level sports and CAM intersects. It posits that an understanding of the underlying motivation for CAM use and doping is currently lacking and that anti-doping rules need to be repositioned in the context of the emerging phenomenon and prevalence of CAM use. Data from non-athlete patients suggests that ingested CAM substances are not viewed as medications because they are perceived as "natural". Thus athletes may use CAM in the belief that it is more natural and, erroneously, not potentially doping (either as a "method" or "substance" under the anti-doping Code). The lack of understanding of various forms of medicine by patients and athletes may result in their not informing doctors (or coaches)
about non-conventional treatment use because it is not viewed as important or relevant to their medical management. Patients thus risk complications from CAM drugs and their interaction with prescribed medications. The additional consequence from failure to recognise CAMs as pharmacological agents puts athletes at risk of inadvertent doping [12015].

**Connexion to eating habits**

Health professionals concerned about the risks of adolescent obesity and disordered eating practices need greater understanding of how families with adolescents manage food in today's fast paced environment. One paper sought to gain conceptual understanding of the food and eating routines of families with a female adolescent athlete from the perspectives of mothers and daughters. Ten white, non-Hispanic mothers and their daughters were purposively sampled from high school track and cross country teams in Upstate New York. Informants completed in-depth, qualitative interviews. Researchers used the constant comparative method to analyze transcripts for emergent themes and to build a conceptual framework that represented the many factors and processes involved in the construction of family food routines. Families varied in forms and patterns of family eating activities with mothers playing a pivotal role in these routines. Family members' individual needs and values were negotiated in constructing these routines. In this sample the daughters' involvement in sports influenced family eating routines, but mothers' employment, ethnicity, social support, income, and areas of residence also played a role. The model describes how individual participants' food choice processes interact to produce family food routines. The conceptual model can inform research and practice related to the family environments in which adolescents experience food and eating [10016].

**Eating disorders**

Eating disorders do occur in male athletes. They are less prominent than in female athletes, and therefore in danger of being missed. The high-risk sports fall into the same categories as with females: aesthetic sports, sports in which low body fat is advantageous, such as cross-country and marathon running, and sports in which there is a need to "make weight", including wrestling and horse racing. Athletic involvement may foster the development of an eating disorder. Some male athletes, in their preoccupation with body image, will abuse anabolic steroids. While sports participation may contribute to the aetiology of an eating disorder, the converse is also true. Exercise may be used as therapy for some cases of eating disorder. In order to adequately treat eating disorders in the male athlete, it is first essential to identify cases. Psychoeducation of athletes, their families, coaches and trainers is an important first step. Counselling an athlete to pursue a sport appropriate to his body type, or to leave his sport behind altogether (an unpopular recommendation from a coach's perspective) can be important to treatment. Treatment of co-morbid psychiatric conditions is essential. Treatment can be structured using a biopsychosocial approach, and all appropriate modalities of therapy, including individual, family and group, as well as psychopharmacotherapy, where appropriate, should be applied [06017].

**Eating disorders and anabolic steroids**

Dissatisfaction with the body is very common in the population, in females in all ages as well as among males. Studies on female and male body image show the role of the media in defining and perpetuating body ideals, e.g. a muscular ideal male body type, or a thin female ideal. A meta-analysis of the effects of the media on male body image concerns, yielded similar effect sizes as those found with women. As a result of internalization of cultural norms, females become dissatisfied with the lower part of their bodies from the waist down and try to lose weight while males primarily want to change the shape of upper part of their
bodies (stomach and chest) and are more likely to desire an increase in weight. Body dissatisfaction has been reported as a risk factor, and one of the strongest predictors for onset of an eating disorder (ED) and is also associated with low self-esteem and depression. Dissatisfaction with the body seems to be the common and prominent denominator, not only between the sexes, but also between males with ED and males using anabolic androgenic steroids (AAS). Both ED and the use of AAS may seriously affect physical health and the psychological and social wellbeing of those who suffer from those problems. Comparisons between males with ED, male bodybuilders and normal controls revealed that bodybuilders more closely resembled the ED group than normal controls regarding body dissatisfaction and loss of sexual desire. Other similarities found between males with ED and body-builders including AAS users were characteristics such as perfectionism, ineffectiveness and low self-esteem. An essential question is whether more similarities can be found between males with ED and AAS users or if these groups differ in some essential respects. It is for example unclear whether there is a distinction between males with ED and males using AAS regarding the occurrence of underlying interpersonal profiles like negative self-image and the severity of psychiatric symptoms. Based on earlier studies showing several similarities between these groups, we anticipated that negative self-image and psychiatric symptoms would be similar between males with eating disorders and males who recently used AAS. Few studies have however investigated if there are other similarities in respect to self-image or psychiatric symptoms between clinical samples of eating disordered males and males in treatment for negative effects of AAS use. The aim of one study was to compare two clinical samples, one of males with ED and one of males who used AAS, regarding self-image and psychiatric symptoms. The study compared males with eating disorders (n=13) and males who recently stopped AAS use (n=29) on self-image and psychiatric symptoms, using The Structural Analysis of Social Behavior self-questionnaire and a shortened version of The Symptom Check List. The eating disorder group reported significantly lower scores for Self-emancipation and Active self-love and higher scores for Self-blame and Self-hate. Both groups reported serious psychiatric symptoms. The common denominator between groups was serious psychiatric symptomatology rather than negative self-image. It was concluded that the negative self-image profile, especially self-hate, found among males with Eating Disorders may indicate that the studied groups differ in aetiology of the underlying problems. The serious psychiatric symptoms in both groups call staff to pay attention to any thoughts of suicide due to severe depressive symptoms where by specialized psychiatric treatment may be needed [13036].

Concomittant symptoms and signs

To determine whether adolescents who participate in a weight-related sport are at increased risk for unhealthful weight-control behaviors and steroid use a study was performed. Subjects were 4,746 adolescents (50 % males, 50 % females) from 31 public middle and high schools in the Minneapolis/St Paul area of Minnesota. More males (20 %) than females (16 %) reported participation in a weight-related sport. Males who reported participation in a weight-related sport had an increased risk of past-week vomiting (odds ratio 5.7), laxative use (OR 6.8), as well as past-year vomiting (OR 4.9), laxative use (OR 3.4), diuretic use (OR 6.0), and steroid use (OR 3.7), compared with those males who did not report participation. Females who reported participation in a weight-related sport had an increased risk of past week vomiting (OR 2.1), as well as past year vomiting (OR 2.0), laxative use (OR 2.6), and steroid use (OR 2.6), compared with those who did not report participation in a weight-related sport. The current study shows that participation in a sport that adolescents perceive as emphasizing weight is strongly associated with unhealthful weight-control behaviors and steroid use. Preventive efforts, targeting parents, coaches, and adolescents are needed to decrease this risk [07020].
Adolescents with learning difficulties
One study investigated the relationships among sleep problems, learning difficulties and substance use in adolescence. Previous research suggests that these variables share an association with executive functioning deficits, and are intertwined. The sample comprised 427 adolescents (median age 16 years) attending remedial schools and 276 adolescents (median age 15 years) attending a mainstream school. Participants completed anonymous self-report questionnaires. Results indicated that adolescents without learning difficulties were more likely to use tobacco, methamphetamine and cannabis, whereas those with learning difficulties engaged in more inhalant use. Adolescents who had more sleep problems were more likely to use tobacco, alcohol, methamphetamine, cannabis, inhalants, cocaine, ecstasy and any other illegal drug. Adolescents with learning difficulties had more sleep problems than those without learning difficulties. However, sleep problems remained independently associated with tobacco, cannabis and inhalant use when learning difficulties were taken into account [11422].

Female adolescents
During the 1990s, 3 different national surveys of US adolescents documented a 2-fold to 4-fold increase in the prevalence of anabolic steroid use among adolescent girls. Public awareness concerning escalating female anabolic steroid use further heightened in 2004 when the Centers for Disease Control and Prevention reported that more than 7 percent of ninth-grade girls indicated current or prior anabolic steroid use, a level exceeding that of some young male subgroups. National attention focused on steroid use in adolescent girls when it became a topic discussed during the 2005 congressional hearings on drug use in sports. Previous associations with female anabolic steroid use have been limited to older women, and most reports of mature women taking anabolic steroids have related the use to competitive athletics and to bodybuilding. Using the nationally representative 2003 Youth Risk Behavior Surveillance System data set, it was examined the characteristics of girls reporting anabolic steroid use. Because of the association between steroid use and sports participation among older women, we particularly explored that relationship among girls reporting prior or ongoing anabolic steroid use. Female students in grades 9 through 12 (n=7544) self-reported anabolic steroid use was compared with other health-related behaviors and with sports participation. Prior or ongoing anabolic steroid use was reported by 5.3 percent of female high school students. Those adolescent girls had a marked increase in other health-compromising behaviors, including past 30-day use of alcohol (odds ratio, 8.8; 95 % confidence interval 5.5 to 14.2), cigarettes (OR, 5.1), marijuana (OR, 7.9), cocaine (OR, 10.8), and diet pills (OR, 4.9). They were more likely to carry a weapon (OR, 7.5), have had sexual intercourse before age 13 years (OR, 2.9), and have had feelings of sadness or hopelessness almost every day for at least 2 consecutive weeks (OR, 4.1). They were less likely to play school-sponsored team sports (OR, 0.52). Those who reported anabolic steroid use were more likely to use extreme measures to lose weight, including greater 30-day use of vomiting and laxative use (OR 5.0) and diet pills, powders, or liquids taken for weight loss (OR, 4.9). Last, psychological problems were prominent among these adolescents. Anabolic steroid users were more likely to indicate having had feelings of sadness or hopelessness for 2 consecutive weeks or more (OR, 4) and were more likely to have attempted suicide (OR, 7.3). Overall, 52 percent of female students indicated participation on team sports. Team sports participants were less likely to be steroid users compared with team sports nonparticipants (OR, 0.52). In addition, being in sports did not seem to identify a unique subgroup of steroid users. Anabolic steroid users in team sports were more likely to use seatbelts (OR 3.1) and condoms or birth control pills (OR, 5.2), compared with anabolic steroid users who were not in team sports. Steroid users participating in sports shared the same problem behaviors as steroid users not participating in team athletics. It was concluded that self-reported anabolic steroid use is not confined to adolescent girls in competitive athletics and is an indicator of adolescent girls with a marked increase in a cluster of other...
health-harming behaviors. Adolescent female anabolic steroid users are characterized by polysubstance abuse and by a marked increase in other health-harming behaviors. Compared with nonusers, odds are higher that they became sexually active at a younger age and have had more sexual partners; they are more likely to carry weapons and to have experience with violence. Along with greater controlled and illicit drug use, they are more likely to resort to harmful weight loss practices. These adolescent girls' mental health is more likely to be impaired, with more than two thirds having had feelings of depression and with almost half having planned or attempted suicide [07021].

Performance versus recreational drugs

The relationships between projected use, self-reported behavior and attitudes to performance-enhancing (PED) and recreational (RD) drugs were investigated among 82 competitive Hungarian athletes, with 15 percent admitting using PED and 32 percent using RD. Both the observed doping estimations (even those made by non-users) and self-admitted use were considerably higher than the average rate of positive doping tests (2 % of all tests). The notable overestimation by PED users (35 % vs 17 %) was in keeping with the false consensus effect. A prediction model with attitude and projection to the likelihood of PED use suggested at least a 70 percent chance of self-involvement of athletes, with responses at or above the median scores (Performance Enhancement Attitude Scale ≥ 60 and estimation ≥ 50%) on the two independent measures. Users overestimated the prevalence of doping in their sport but not RD use, with the converse holding for RD users' views of doping. PED users also showed a significantly more lenient attitude toward doping. This domain-specific characteristic adds new information to the ongoing research effort in understanding drug-doping co-morbidity. The reasons for elevated in-group projection are discussed, along with the potential application of this phenomenon in doping epidemiology studies [11011].

Health promoting effects of sports

To study whether participation in organized sports during adolescence predicts increased smoking of tobacco, alcohol intoxication and cannabis use from late adolescence to adulthood when controlling for potential confounders by a survey of national sample of Norwegian high school students (aged 13-19 years) in 1992 (T1) followed-up in 1994 (T2), 1999 (T3) and 2006 (T4) (n=3251). Outcome measures included smoking of tobacco and 12-month prevalences of alcohol intoxication and cannabis use, respectively. Confounders included pubertal timing, friends' drug use, perceived social acceptance, grades and parental socio-economic status. Latent growth curve analyses showed that initial level of participation in organized sports predicted growth in alcohol intoxication. Those involved initially in team sports had greater growth in alcohol intoxication, but lower growth in tobacco use and cannabis use, during the adolescent and early adult years compared to those involved in technical or strength sports. Practising endurance sports, as opposed to technical or strength sports, predicted reduced growth in alcohol intoxication and tobacco use. Sports participation in adolescence, and participation in team sports in particular, may increase the growth in alcohol intoxication during late adolescent and early adult years, whereas participation in team sports and endurance sports may reduce later increase in tobacco and cannabis use [09007].

Lifestyle alteration

Lifestyle's alterations are hazardous for health. On one hand they produce a high rate of mortality and disease, on the other hand they cause a reduction of work outcomes and an
increase of occupational accidents with important consequences for both worker’s health and his/her financial status. The aim of one study was to review the scientific literature for possible relationships between mental health and lifestyle alterations of young workers. It was considered as lifestyle factors the attitudes towards: smoking, alcohol consumption, eating, use of medications and doping substances, physical activity and sleeping. From the study it clearly emerges the existence of correlation between lifestyle habits and mental health; in fact behavioral alterations can produce problems of mental health and vice versa. Furthermore, some work peculiarities can lead to psychic disturbances and/or to unhealthy habits which can themselves cause negative effects on working activity. It is very important for young workers to understand that unhealthy behaviors, which can be corrected, are hazardous in terms of health and safety for both the single worker and the collectivity and that those behaviors can enhances the other working risks. Because there is a close interaction between mental health and lifestyles, it would be necessary a careful promotion of mental health on workplaces and to take all the preventive measures, with particular regard for those related to the work organization, in order to reduce the onset, exacerbation and unmasking of mental disorders and psychological difficulties. In working environment, the occupational health physician and his relationship with the patient are of fundamental importance. During preventive and periodic medical examinations, the occupational health physician should take detailed information on young worker's habits regarding smoking, alcohol consumption, eating, physical activity, sleeping, pharmacological abuse and possible presence of mental disorders and furthermore he/she should actively take part in the information and education process of the worker [07012].

**Prediction of later life-style**

The aim of one study was to examine the associations between self-rated health (SRH), physical activity and other lifestyle habits among former athletes and referents in late adulthood. Male athletes (n=514) who represented Finland from 1920 through 1965 and referents (n=368) who were classified healthy at the age of 20 years participated in this population-based cohort study. The present analysis was based on a questionnaire study in 2001. SRH was assessed by a single question. Univariate binary and multivariate logistic regression analyses were used to examine the associations of health-related behaviours with SRH. The majority of former athletes (64 %) rated their health better than referents (48 %). A higher percentage of the athletes (54 %) compared to the referents (44 %) belonged to the most physically active groups (MET quintiles IV-V). A high percentage of the athletes (77 %) and referents (79 %) were occasional or moderate alcohol users. The proportion of never smokers among athletes was 59 percent and among referents 37 percent. Among current smokers there were no differences in nicotine dependence between athletes and referents. In the univariate analysis the odds of reporting good SRH was 2 times higher for athletes than for referents. In multivariate logistic regression analysis, former participation in team and power athletic groups had significantly higher SRH than the referents even after adjusting for age, level of physical activity, alcohol and smoking habit, and occupation. People, who participated in very active physical exercise in their youth, as indexed by participation in competitive sports by elite athletes, continue a physically active lifestyle, and maintained healthier lifestyle. They had significantly higher SRH than the referents in their senior years, which were not totally explained by their physically active and healthier lifestyles [10437].

**Risk factors for doping**

Grounded conceptually in social cognitive theory, this research examines how personal, behavioral, and environmental factors are associated with risk perceptions of anabolic-androgenic steroids. Ordinal logistic regression and logit log-linear models applied to data
gathered from high-school seniors (n=2,160) in the 2005 Monitoring the Future study showed significant explanatory effects for gender, race, exposure to drug spots, steroid availability, peer use of steroids, sensation-seeking, depression, and self-esteem. Females, African Americans, and those who had seen drug spots the most frequently estimated higher levels of risk associated with steroid use, while those who indicated ease in obtaining steroids and those with close friends who had used the drugs estimated lower risk. Also estimating lower levels of risk were sensation seekers, those who appeared depressed, and those with low levels of self-esteem. Analyses reveal how steroid risk determinants may differ from those related to methylenedioxy-methamphetamine (i.e., MDMA, ecstasy) and marijuana use [09008].

Illicit anabolic-androgenic steroid (AAS) abuse, though an important public health problem, remains inadequately studied. Almost all AAS abusers are male and lift weights, but the risk factors for AAS use among male weightlifters remain poorly understood. It was recruited 233 experienced male weightlifters, of whom 102 (44 %) reported lifetime AAS use, and assessed their childhood and adolescent attributes retrospectively, using structured clinical interviews and computerized questionnaires. This cross-sectional cohort approach—a design that we have formally presented in the recent methodological literature-utilizes a study cohort, not selected for outcomes of interest, and assesses exposures and outcomes retrospectively. It was hypothesized that conduct disorder and body-image concerns would be major risk factors for subsequent AAS use among male weightlifters. Within the study population, many attributes showed little association with AAS use, but conduct disorder and body-image concerns showed strong associations. For individuals with prior conduct disorder versus those without, the hazard ratio (95 % confidence interval) for subsequent AAS use was 2.2 (1.5 to 3.4). For individuals in the middle versus lowest tertile of scores on a retrospective adolescent muscle-dysmorphia scale, the hazard ratio was 1.5 (0.84 to 2.6); for the highest versus lowest tertile, the hazard ratio was 3.3 (2.0 to 5.3). It was concluded that conduct disorder and body-image concerns represent important risk factors for AAS use among male weightlifters. Thus, assessment of these attributes may help to identify individuals most likely to require interventions to discourage this form of substance abuse [12029].

Much of the literature investigating the relationship between sports participation and substance use has focused upon student populations, with little focus being given to athletes who participate at elite levels. Identifying why some athletes may be at a greater risk for substance use can help in the design and implementation of prevention initiatives. Data for one study was from 1684 self-complete surveys with elite Australian athletes. Eight percent (n=134) of the sample reported the use of at least one of the six illicit drugs under investigation (ecstasy, cannabis, cocaine, meth/amphetamine, ketamine and GHB) in the past year. Having been offered or having had the opportunity to use illicit drugs in the past year, knowing other athletes who use drugs and identifying as a 'full-time athlete' were significant predictors of past-year illicit drug use, while having completed secondary education or a post-school qualification was associated with a lower likelihood of past-year illicit drug use. Athletes are part of a sportsnet that includes family, coaches, support staff and other athletes, and these relationships may encourage the use, supply and demand for drugs. The current findings suggest that relationships with some of those in the sportsnet may play an important role when understanding illicit drug use among elite athletes. As education appears to be associated with a lower likelihood of illicit drug use among this group, initiatives should encourage athletes to engage in off-field pursuits which may also help prepare them for life after sport [12030].

*Psychiatric comorbidity diseases*
"Comorbidity phenomenon" defines the not univocal interrelation between medical illnesses and psychiatric disorders, each other negatively influencing morbidity and mortality. Most severe psychiatric disorders, such as schizophrenia, bipolar disorder and depression, show increased prevalence of cardiovascular disease, related to poverty, use of psychotropic medication, and higher rate of preventable risk factors such as smoking, addiction, poor diet and lack of exercise. Moreover, psychiatric and organic disorders can develop together in different conditions of toxic substance and prescription drug use or abuse, especially in the emergency setting population. Different combinations with mutual interaction of psychiatric disorders and substance use disorders are defined by the so called "dual diagnosis". The hypotheses that attempt to explain the psychiatric disorders and substance abuse relationship are examined as common risk factors, psychiatric disorders precipitated by substance use, psychiatric disorders precipitating substance use (self-medication hypothesis), and synergistic interaction [13033]

Diagnostic and therapeutic difficulty concerning the problem of dual diagnosis, and legal implications, are also discussed. Substance induced psychiatric and organic symptoms can occur both in the intoxication and withdrawal state. Since ancient history, humans selected indigene psychotropic plants for recreational, medicinal, doping or spiritual purpose. After the isolation of active principles or their chemical synthesis, higher blood concentrations reached predispose to substance use, abuse and dependence. Abuse substances have specific molecular targets and very different acute mechanisms of action, mainly involving dopaminergic and serotoninergic systems, but finally converging on the brain's reward pathways, increasing dopamine in nucleus accumbens. The most common substances producing an addiction status may be assembled in depressants (alcohol, benzodiazepines, opiates), stimulants (cocaine, amphetamines, nicotine, caffeine, modafinil), hallucinogens (mescaline, LSD, ecstasy) and other substances (cannabis, dissociatives, inhalants). Anxiety disorders can occur in intoxication by stimulants, as well as in withdrawal syndrome, both by stimulants and sedatives. Substance induced mood disorders and psychotic symptoms are as much frequent conditions in ED, and the recognition of associated organic symptoms may allow to achieve diagnosis. Finally, psychiatric and organic symptoms may be caused by prescription and doping medications, either as a direct effect or after withdrawal. Adverse drug reactions can be divided in type A, dose dependent and predictable, including psychotropic drugs and hormones; and type B, dose independent and unpredictable, usually including non psychotropic drugs, more commonly included being cardiovascular, antibiotics, anti-inflammatory and antineoplastic medications [13033].

**Sponsoring of sports**

Organised sport provides an important setting for health promotion. Peak sporting organisations have a role in assisting and overseeing sports clubs, including providing funding opportunities. As such, sponsorship of these organisations may influence the funding of community sport. One study aimed to describe the nature and scope of peak sporting organisations' sponsorship, and particularly food and beverage company sponsors. An analysis of national and state sporting organisations' websites for the nine most popular sports for children and from four Australian states and territories was conducted using a structured survey tool. Information collected included the number and type of sponsors and sponsorship policies. The nature of food and beverage sponsors was defined as more healthy or less healthy using criteria from a Delphi survey. 443 sponsors were identified across 55 websites. Overall, 9 percent of sponsors were food companies and 3 percent were alcohol manufacturers. The majority of food companies (63 %) and alcohol manufacturers (100 %) did not meet criteria as healthy sponsors. It was thus concluded that sponsorship of peak
sporting organisations is widespread and consists of a relatively high proportion of alcohol manufacturers and food companies, some of which produce products considered to be unhealthy. This sponsorship may influence community sport through sponsored sporting programs or by indicating sponsors’ acceptability [11005].

To examine the relationship between direct alcohol and non-alcohol sponsorship and drinking in Australian sportspeople 652 (51 % female) individuals completed questionnaires on alcohol and non-alcohol industry sponsorship (from bars, cafes etc.), drinking behaviour (Alcohol Use Disorders Identification Test (AUDIT)) and known confounders. Thirty-one percent reported sponsorship (30 % alcohol industry; 4 % both alcohol and non-alcohol industry and 2 % non-alcohol industry only.) Multivariate regression showed that receipt of alcohol industry sponsorship was predictive of higher AUDIT scores, but non-alcohol industry sponsorship and combinations of both were not. Governments should consider alternatives to alcohol industry sponsorship of sport. Hypothecated taxes on tobacco have been used successfully for replacing tobacco sponsorship of sport in some countries, and may show equal utility for the alcohol industry’s funding of sport [11006].

Determining children’s exposure to food and beverage company sponsorship, and the effect of this exposure, is important in establishing the extent to which there may be health and societal consequences. One paper aimed to provide preliminary evidence on the scope and potential effects on children of unhealthy food and beverage sponsorship. A review of published literature and media and marketing reports was conducted to determine the types of food and beverage sponsorship campaigns that children are exposed to, and the effect of corporate sponsorship (including tobacco and alcohol) on children and adolescents. A large range of food and beverage sponsorship activities, in Australia and internationally, were identified for both school and sport settings. In particular, food and beverage companies have attempted to develop a marketing presence at all levels of professional and community sport. No information was identified measuring the effect of food and beverage company sponsorship on children and adolescents. However, empirical evidence from consumer studies relating to tobacco and alcohol sponsorship has repeatedly demonstrated that sponsorship has an impact on children’s product recall and product-related attitudes and behavioural intentions. It was concluded that while there is no available research on the direct effect of food and beverage sponsorship, the demonstrated effects of tobacco and alcohol sponsorship on children’s product awareness, preferences and consumption are likely to be applicable to food companies [11420].

**Information to dopers**

One study was designed to investigate anabolic steroid users’ experiences of, and motivations for, use. Five men and six women users took part in in-depth interviews. Four themes emerged: Steroid Use vs Abuse; Side-effects; Trusted Information Sources; and Social Pressure. Many users believed that steroids used in moderation were safe. Serious side-effects (liver and kidney damage, hypertension) were not significant disincentives. Information from health professionals tended to be mistrusted because it was not based on first-hand experience of use. Social support, especially from within the body building community, was an important motivator. It is concluded that intervention programmes need the support of the body building community in order to be effective [06018].

**Medical practitioners’ knowledge, attitudes and beliefs**

Central to the work of many medical practitioners is the provision of pharmaceutical support for patients. Patients can include athletes who are subject to anti-doping rules and
regulations which prohibit the use of certain substances in and out of competition. One paper examined the evidence on medical practitioners’ knowledge, attitudes and beliefs towards doping in sport. A systematic search strategy was followed. Research questions and relevance criteria were developed a priori. Potentially relevant studies were located through electronic and hand searches limited to English language articles published between 1990 and 2010. Articles were assessed for relevance by two independent assessors and the results of selected studies were abstracted and synthesised. Outcomes of interest were knowledge, attitudes and beliefs in relation to doping in sport. Six studies met the inclusion criteria and were examined in detail. Samples reflected a range of medical practitioners drawn from the UK, France, Greece, Italy and Ireland. The investigations varied with respect to outcome focus and quality of evidence presented. It was concluded that whilst the extant empirical research posits a negative attitude towards illegal performance enhancement combined with a positive inclination towards doping prevention, it also exposes a limited knowledge of anti-doping rules and regulations. Insufficient education, leading to a lack of awareness and understanding, could render this professional group at risk of doping offences considering Article 2.8 of the World Anti-Doping Agency Code (WADC). Moreover, in light of the incongruence between professional medical codes and WADC Article 2.8, medical professionals may face doping dilemmas and therefore further discourse is required. At present, the current evidence-base makes it difficult to plan developmentally appropriate education to span the exposure spectrum. Addressing this situation appears warranted [11007].

One study examined General Practitioner's (GP) knowledge, practice and training requirements in relation to doping in sport in Ireland. All 2083 GPs on the Irish College of General Practitioners register received a postal questionnaire, yielding a 37 percent response rate (n=771, 63 % male, average age 46 years). Results revealed that 14 percent deemed their knowledge of doping agents to be good or very good, 12 percent had completed specific training modules in doping or sport, and 24 percent were connected with a specific sport as a team doctor or advisor. Over one in four (28 %) had been consulted for advice on doping in sport, 33 percent possessed the current list of prohibited substances, and 25 percent knew of the Irish Sports Council’s drug-testing procedures. The current initiatives to discourage doping in sport were felt to be ineffective, and although 92 percent indicated that GPs had a role to play in the prevention of doping in sport, only 9 percent felt adequately trained for such a role. There was overwhelming support for further training among GPs, although the most appropriate method of providing training is complex and requires strategic planning [09009].

**Contagiousness of doping**

Using a psychosociological approach, the purpose of one study was to identify and understand the use of doping substances by young elite cyclists in Swizerland. Semi-structured interviews were conducted with young cyclists who were hoping to find a professional team and cyclists who had recently become professional. All of the young cyclists interviewed took nutritional supplements and believed that they improved their performance, which has been shown by other scholars to be a risk factor for doping. These cyclists believed that doping at the professional level in cycling was acceptable but did not approve of it at the amateur level. They were attracted to doping; they were open to using doping substances themselves if it was the key to continuing their cycling career, but only after they became professional. Team staff, doctors, parents and friends helped to create a “clean” environment that prevented the young cyclists from doping before becoming professional. The more experienced cyclists, who doped or used to dope, transmitted the culture of doping to the young cyclists, teaching them doping methods and which substances to use. This study could help to improve prevention and help to detect doping, as it is clear

311
that doping behaviours begin at the amateur level [09010].

**Economical aspects**

In 1990, the illicit steroid market was estimated to be USD 400 million. Steroid cycles, typically lasting 6 to 14 weeks, can cost hundreds of dollars. A cycle consists of daily oral doses plus weekly or monthly intramuscular depot injections. Some users take multiple cycles per year [07031].

Illegal steroids entering the United States and distributed to athletic and at-risk populations has increased dramatically. It is now estimated to be an over 100 million US dollar black market for steroids in the US alone, with more than 80 percent manufactured in Mexico. Today performance-enhancing programs and drugs are not the exclusive province of elite athletes, but have spread to health clubs, high schools and other at-risk populations, creating an over $1.4 billion US dollar industry that is growing daily as new compounds are synthesized and marketed. Projecting these figures internationally suggests that the illegal steroid market alone approaches a billion US dollars annually [08006].

It is estimated that an eight weeks performance enhancement regime of pharmaceutical grade recombinant human growth hormone will cost about USD 2000, well out of the range of an adolescent and the majority of weekend athletes [08006].

**Importance of sports medicine as a medical specialty**

In Europe, participation in physical activity has been growing among people of all ages. Thus, there is an increasing demand for care relating to sports medicine and this has promoted the development of specialised sports physicians. Sports medicine involves a wide range of professionals with functions of taking care of active population, recreational and competitive athletes upon different aspects: curative, rehabilitative and preventive. In the light of an higher demand of expertise and sport-specific burden of knowledge, such as a further development of the phenomenon doping with all the related moral, legal and health implications, the sport physician has to deal with a complex picture. As a result, the need to provide prevention at all levels has become one of the most important objectives of sports medicine. One article aimed to give a brief overview of the state of the art of this specialty in Europe and to describe definitions, scopes and educational perspectives of the Sport Medicine Specialty Training Core Curriculum to be adopted in the EU [09013].

**Impact of national programs**

Physical activity and sports are considered as one of the determinants of health. The aim of the study was to review the rationale for the formulation of this public health issue and its integration in national action plans. The study shows that fourteen national programmes were drafted and implemented between 2001 and 2006 by seven institutions. The research methodology was based on crossing data obtained from semi-directed interviews and documents regarding the design, implementation and follow-up of these programmes. For the conditions of the success, the fourteen actions scored an average of 175 ± 67 out of 300 percent. Public health actors and professionals must be given more opportunities to involve themselves and engage in developing stronger relationships and linkages, in particular with the institutional and community settings. In general, the most invested parts of a programme are the structural and operational aspects of activities. Six significant points surfaced from the study; consideration of drug use as an addictive behaviour; recognition of the psychological stress of professional athletes; acknowledgment of youth as being at high risk
for doping behaviour; integration of the concept that physical activity and sports must take the benefit/risk perspective into account; and the necessity to promote health. Through the exchange of numerous local and regional experiences, an optimisation of their synergistic connections was made possible on a continuum extending from "health promotion through physical activity and sports" to "prevention of drug-use and doping behaviours". Professionals have been able to develop actions in the above-mentioned domains across this continuum that have, to date, remained isolated. Proposals are made to strengthen these dynamics. Other health determinants and public health priorities could be investigated with the same methodology [09014].

**Staffing protocols in high-performance sport**

A common feature of the current doping scandals appears to be that those organisations under scrutiny have allowed external "experts" or "gurus" to drift in and out of their organization and control supplementation practices in the organisation, without actually being a bona fide part of the organisation’s staffing structure. There is no point having antidoping policies and codes of conduct if individuals are allowed to drift into the organisation and influence the supplementation policy, without those individuals being legally bound to the anti-doping policy and code of conduct. No individual other than those on the supplementation panel should be able to influence or administer supplements. The issue of doping and the challenges for anti-doping organisations in staying ahead of scientific developments and remain [13027].

**Law issues**

The field of science and technology that proposes to apply enhanced understanding of the human genetic code to reshaping our individual and collective destinies has generated more interest among the general public, as well as in the athletic community, than the potential for physical enhancement of the human body and its performance. Genometric experiments have produced physically enhanced mice, and the production of similarly enhanced humans may not be far off. Although it is not the objective of most genometric research, the day will come when gene-based "treatments" will enable individuals to build muscle or increase endurance faster than is possible through conventional methods. One article described developments in the area of physical enhancement that may find application in the "gene doping" of athletes. For example, human performance-related genes may be delivered to athletes using tools developed for research in gene therapy; the protein products of these genes may be administered in recombinant form; and recently discovered small-molecule activators of the major genetic regulatory pathways of physical prowess may be taken orally, providing "exercise in a pill". The article also described the attempts to regulate and punish the use of prohibited techniques for performance enhancement among athletes. As science advances, defining and detecting "gene doping" becomes increasingly complex. Thus, the study of physical enhancement provides an ideal starting point for the interdisciplinary examination of the intersection between law and science [09015].

**Doping + violence**

Within the context of problem-behaviour theory, one study investigated the intra-relationship between attitudes and behaviours towards exercise, sport involvement, violence in sport-related events, eating fruits, smoking and hashish or ecstasy use in a sample of Greek adolescents. Participants were 5991 Greek school pupils who responded to questionnaires assessing behaviour and attitudes towards health-related behaviours. Positive associations were found between pupils’ reports of violence in sport-related events, smoking and hashish
or ecstasy use on the one hand, and eating fruits and participation in sport and exercise on
the other. In contrast, small positive association was observed between sport involvement
and violence in sport-related events. Attitudes towards health risk behaviours were inversely
related to attitudes towards health-promoting behaviours, and attitudes were positively
related to corresponding behaviours. Sport involvement and regular exercise decreased but
smoking and use of hashish or ecstasy increased with age. More males than females
participated in organized sport and violent acts in sport-related events. Males' involvement
in sport violence increased with age. Sport is a suitable context for the promotion of several
health-related behaviours apart from exercise. Nevertheless, the present sport structure
excludes most young people and is positively linked with sport violence. A less demanding
sport context should be provided for the majority of young people, particularly for females.
Sport programmes designed to promote health behaviours should be encouraged. More
concentrated actions to combat sport violence are required [10439].

Knowledge in different countries

Austria

Strategies for doping prevention are based on prior identification of opportunities for
intervention. There is no current research focusing on the potential role in doping prevention,
which might be played by the parents of junior elite athletes. The purpose of this study was to
evaluate the knowledge and attitudes toward doping among parents of Austrian junior
athletes and to analyze factors potentially influencing these beliefs. In one study, two
questionnaires were distributed to 1818 student athletes, each with instructions that these
surveys were to be completed by their parents (n\textsubscript{total} 3636). Parents filled in questionnaires at
home without observation. Responses from 883 parents were included in this analysis.
Compared to female parents, male parents demonstrated significantly better knowledge
about doping and its side effects and were more likely to be influenced by their own sporting
careers and amounts of sports activities per week. Parental sex did not demonstrate a
significant influence on responses reflecting attitudes toward doping. Additional research is
needed to compare these results with young athletes' knowledge and attitudes to determine
if and to what degree parental attitudes and beliefs influence the behavior and attitudes of
their children [13046].

Austrian juniors

An important factor while developing efficient doping prevention strategies is to identify
relevant target groups, to evaluate the state of knowledge about this topic as well as to
evaluate motivations behind using prohibited substances. Measures to prevent doping
substances abuse have to be supported in early stages of childhood. The aim of one
prospective study was to evaluate the knowledge of Tyrolean junior athletes about doping in
sport. Next to the knowledge, their attitudes in regard to doping practices have also been a
focus of this project. Within a prospective cross-sectional study, Tyrolean junior athletes
aged between 14 and 19 years (n=408) were anonymously questioned by distributing
questionnaires in three Tyrolean sport schools as well as two Tyrolean sport-training centers.
To collect the data, an anonymous questionnaire with close-ended questions was used. Next
to sociodemographic data, questions also evaluated the knowledge about prohibited
substances as well as attitudes and behaviors towards doping. The concept was set up
based on contents of comparable studies and publications. The knowledge about doping
among junior athletes was moderate. The consumer behavior of the young athletes on the
other hand has turned out to be satisfactory. Nevertheless, the overall knowledge especially
regarding potential negative side effects of doping agents is poor. Thus, to incorporate an
effective doping-prevention strategy, improved education, particularly in terms of side effects,
is clearly needed. To achieve sustainable doping-prevention effects, focus has to be generally set on education within the frame of junior competitive sport [13047].

**Australia**

One study presents a comprehensive examination of the Sport Drug Control Model via survey data of elite Australian athletes. A cross-sectional nationwide mail survey of 1237 elite Australian athletes was conducted. Structural equation modelling was employed to test the model. Morality (personal moral stance on performance-enhancing substances use), reference group opinion (perceived moral stance of reference group on performance-enhancing substances use) and legitimacy (perceptions of the drug testing and appeals processes) evidenced significant relationships with attitude towards performance-enhancing substances use, which in turn was positively associated with doping behaviour. The model accounted for 81 and 13 percent of the variance in attitude towards performance-enhancing substances use and doping behaviour, respectively. These findings validate the usefulness of the Sport Drug Control Model for understanding influences on performance-enhancing substances use. Nevertheless, there is a need to survey athletes representing a broader range of competition levels and cross-cultural research to test the model's applicability to other populations of athletes [13048].

**Italy**

It was explored use and attitudes toward drugs and dietary supplements (DS) and knowledge concerning doping in cycling retrospective cross-sectional study. Forty cyclists aged 19 to 23 years and practicing for 14 to 30 h/wk were interviewed. Previous use (last 3 months) of drugs or DS occurred in 33 of 40 (83 %) and 39 of 40 (98 %) cyclists, respectively. Almost all the subjects named at least 1 doping agent (range, 1-10). Within a fixed list of 18 substances (among which only 14 were doping agents), participants recognized 3 to 18 of them as doping agents. They recognized tramadol and sildenafil as doping agents, which are not doping agents, and failed to recognize probenecid and albumin, which actually are. Doping knowledge correlated with drug use. Participants deemed doping prevalence high among cyclists in general but not in their own team. It was concluded that the use of prescription drugs and DS was a common occurrence. Doping knowledge was poor and biased, and its relationship with drug use deserves consideration. Educational interventions are needed to improve knowledge and awareness about prescription drugs and DS use, as well as about doping [13049].

**Illicit drugs around an Olympic game**

It was aimed to describe presentations to emergency departments during the Sydney 2000 Olympic Games for conditions related to the use of illicit drugs; to discuss the implications of such presentations for surveillance and public health action at similar events in the future. Fifteen sentinel emergency departments in the greater Sydney metropolitan area for a 38-day period spanning the Sydney 2000 Olympic Games had 424 presentations to sentinel emergency departments with conditions related to illicit drug use. The mean daily number of presentations for adverse events due to illicit drug use was significantly higher (13.3 versus 8.8 presentations) in the 2-week Olympic Games period than in the lead-up to the Games, culminating in a large peak following the closing ceremony. There was also a significant increase (5.1 versus 1.7 presentations) in the mean daily number of presentations related to use of ecstasy or amphetamines, whereas no change was noted in presentations related to heroin use. Over half (52 %) of presentations occurred at two emergency departments in areas known as being hot-spots for illicit drug use. It was concluded that enhanced surveillance of adverse events following illicit drug use, possibly targeting known 'hot-spots', should be considered for future mass events. Advance preparation of preventive strategies,
such as 'party-safe' messages, will enable rapid response to unusual patterns of illicit drug-related harm during future mass events [10440].

**US position stand on androgen and human growth hormone use**

Perceived yet often misunderstood demands of a sport, overt benefits of anabolic drugs, and the inability to be offered any effective alternatives has fueled anabolic drug abuse despite any consequences. Motivational interactions with many situational demands including the desire for improved body image, sport performance, physical function, and body size influence and fuel such negative decisions. Positive countermeasures to deter the abuse of anabolic drugs are complex and yet unclear. Furthermore, anabolic drugs work and the optimized training and nutritional programs needed to cut into the magnitude of improvement mediated by drug abuse require more work, dedication, and preparation on the part of both athletes and coaches alike. Few shortcuts are available to the athlete who desires to train naturally. Historically, the NSCA has placed an emphasis on education to help athletes, coaches, and strength and conditioning professionals become more knowledgeable, highly skilled, and technically trained in their approach to exercise program design and implementation. Optimizing nutritional strategies are a vital interface to help cope with exercise and sport demands. In addition, research-based supplements will also have to be acknowledged as a strategic set of tools (e.g. protein supplements before and after resistance exercise workout) that can be used in conjunction with optimized nutrition to allow more effective adaptation and recovery from exercise. Resistance exercise is the most effective anabolic form of exercise, and over the past 20 years, the research base for resistance exercise has just started to develop to a significant volume of work to help in the decision-making process in program design. The interface with nutritional strategies has been less studied, yet may yield even greater benefits to the individual athlete in their attempt to train naturally. Nevertheless, these are the 2 domains that require the most attention when trying to optimize the physical adaptations to exercise training without drug use. Recent surveys indicate that the prevalence of androgen use among adolescents has decreased over the past 10-15 years. The decrease in androgen use among these students may be attributed to several factors related to education and viable alternatives (i.e. sport supplements) to substitute for illegal drug use. Although success has been achieved in using peer pressure to educate high school athletes on behaviors designed to reduce the intent to use androgens, it has not had the far-reaching effect desired. It would appear that using the people who have the greatest influence on adolescents (coaches and teachers) be the primary focus of the educational program. It becomes imperative that coaches provide realistic training goals for their athletes and understand the difference between normal physiological adaptation to training or that is pharmaceutically enhanced. Only through a stringent coaching certification program will academic institutions be ensured that coaches that they hire will have the minimal knowledge to provide support to their athletes in helping them make the correct choices regarding sport supplements and performance-enhancing drugs. The NSCA rejects the use of androgens and hGH or any performance-enhancing drugs on the basis of ethics, the ideals of fair play in competition, and concerns for the athlete’s health. The NSCA has based this position stand on a critical analysis of the scientific literature evaluating the effects of androgens and human growth hormone on human physiology and performance. The use of anabolic drugs to enhance athletic performance has become a major concern for professional sport organizations, sport governing bodies, and the federal government. It is the belief of the NSCA that through education and research we can mitigate the abuse of androgens and hGH by athletes. Due to the diversity of testosterone-related drugs and molecules, the term androgens is believed to be a more appropriate term for anabolic steroids [09016].
Influence of religion on use of doping

Strength of religious faith (SRF) is rarely studied as a protective factor against substance use and misuse in sports. Herein, we studied the potential buffering effect of the complex socio-educational, sports, and religiousness factors in the protection against substance use and misuse, including cigarettes, analgesics, appetite suppressants, potential doping behavior, and binge drinking. The sample of subjects included 40 high-class female athletes (22-26 years of age). Using a strictly anonymous questionnaire, we investigated different social, educational, and sports factors (including SRF measured by the Santa Clara Strength of Religious Faith Questionnaire) in relation to substance use and misuse. Following the calculation of simple correlations, multiple regression analysis revealed that in combination with low sports experience, SRF has a significant buffering effect against binge alcohol drinking and consumption of appetite suppressants. The data are discussed in comparison with previous findings and theoretical background. Future studies should study the topic while observing samples of recreational and competitive athletes of both genders [12023].

Religiousness is rarely studied as a protective factor against substance use and misuse in sport. Herein, it was studied the potential buffering effect of the complex socio-educational, sports, and religiousness factors in the protection against substance use and misuse, including cigarettes, analgesics, appetite suppressants, potential doping behavior, and binge drinking. The sample of subjects included 40 high-class female athletes (22-26 years of age). Using a strictly anonymous questionnaire, we investigated different social, educational, and sports factors (including strength of religious faith measured by the Santa Clara Strength of Religious Faith Questionnaire) in relation to substance use and misuse. Following the calculation of simple correlations, multiple regression analysis revealed that in combination with low sports experience, strength of religious faith has a significant buffering effect against binge alcohol drinking and consumption of appetite suppressants [10026].

Religiousness is rarely studied as protective factor against substance use and misuse in sport. Further, it was found no investigation where college-age athletes were sampled and studied accordingly. The aim of the present study was to identify gender-specific protective effects of the religiousness (measured by Santa Clara Questionnaire) and other social, educational, and sport variables as a potential factors of hesitation against doping behaviors in sport-science-students from Mostar, Bosnia, and Herzegovina (51 women and 111 men; age range, 18-26). The gender differences for the non-parametric variables were established by Kruskall-Wallis test, while for the parametric variables the t-test for independent samples was used. Multiple regression calculations revealed religiousness as the most significant predictor of the social, health, sport and legal factors of hesitation against doping behaviors in both genders. However, the differential influence of the social, educational, sport and religious factors in relation to negative consequences of the doping behaviors is found for men and women. Such differential influence must be emphasized in tailoring the anti-doping policy and interventions [11014].

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predictor of the social, health, sport and legal factors of hesitation against doping behaviors in both genders. However, the differential influence of the social, educational, sport and religious factors in relation to negative consequences of the doping behaviors is found for men and women. Such differential influence must be emphasized in tailoring the anti-doping policy and interventions [13067].

**The adolescent athlete**

The use of doping compounds is less likely in adolescent athletes, but the detection that much more difficult given that the baseline secretion of the endogenous hormone is shifting during pubertal development with the greatest rise in testosterone in boys occurring about the time of peak height velocity and maximal secretion of hGH and IGF-I. Can one grow more rapidly during childhood and early adolescence to a taller than genetically programmed adult height or to a more robust body composition given anabolic-androgenic steroids (AAS), human growth hormone (rhGH), insulin-like growth factor (rhIGF-I), insulin or erythropoietin [10001]? The more successful late childhood and early adolescent age athletes are often more physically mature than their age-peers. Early adolescent development seemingly permits them to use the strength and power in sport and performance that their average (or even slowly) developing age-peers have not yet attained. The exceptions might be those athletes in the more aesthetic sports in which flexibility and a lower center of gravity are more important than size and strength. These are virtually exclusively in females. Most boys’ sports require high levels of strength and power; however, at elite levels of competition technique is a strong measure of success; for the athlete must produce, control and efficiently use the energy in a fashion that maximizes athletic performance; for example, explosive power in some jumping sports or overall technical skill in the pole vault. Earlier developing children are taller and stronger than their age-peers. That may confer an advantage at younger ages; however, sport-specific skills are important. It is because of these that some of the "later blooming" adolescents catch-up in performance with their earlier developing peers and likely overtake them; for they have had the discipline to attain the requisite skills and are perhaps at lesser risk to "burn-out" and cease participation in that sport [10017].

The rationale for taking ergogenic "effectors", such as rhGH, rhIGF-I, anabolic steroids, insulin or erythropoietin, is that by becoming bigger and stronger the athlete will perform better. Some boys who are not athletes take anabolic steroids and perhaps other ergogenic aides to "look better". However, there are no definitive data for rhGH or insulin in young adults and none at all for any of these five agents in adolescents who are not deficient in them. In any experimental setting the study design is such that one studies the effects of a single agent with all other things being equal. For a drug one decides on a dose, or range of doses and the subjects are allocated randomly to receive or not receive a particular dose. The subjects should be selected from a common pool and all should have identical "requirements", for example, in studies of athletes, prescribed diet and exercise regimens. It is apparent that many athletes take a "cocktail" of drugs making it virtually impossible to denote any single agent as causing a specific outcome. The difficulty in adolescents is that increased secretion of testosterone (and growth hormone) is a natural part of human pubertal development. Thus it would be very difficult to ascribe any individual changes noted in strength and body composition to any pharmacological agent at this time. The ethics of human experimentation in adolescents precludes any serious objective study using anabolic steroids [10017].
Availability of illegitimate drugs in the society

A description of the illicit drug market in Denmark's second largest city was provided based upon the prevalence of narcotics and illicitly sold medicals during the years 2002 and 2003. The changes on the illicit drug market were described by comparing the results to a similar study conducted ten years earlier. The study comprised of 469 cases of seized material by Aarhus Police during 2002-2003. Additional information relating to the 341 persons charged is also included in the study. Heroine, cocaine and amphetamine were seized in 31 percent, 30 percent, and 28 percent of the cases, respectively, and comprise the most frequently encountered hard drugs on the market. The prevalence of cocaine in Aarhus Police District has increased more than tenfold during the past ten years. The purity of the three drugs decreased significantly during the same period, although large variations in the quality of drugs were observed. Medicals were found in 16 percent of the seizures (containing 32 different active substances). The most frequent group of medicals was benzodiazepines, which made up a total of 74 percent of the medicals in the study. Anabolic steroids, ecstasy and methamphetamine were each found in 4 percent of the seizures. Men with an average age of 29 years comprised 92 percent of the persons charged in the study. Persons with a foreign nationality comprised 15 percent of the charged, while 25 percent had a birthplace outside Denmark. This means that the prevalence of stimulants especially cocaine have increased significantly during the past ten years. Meanwhile the purity of the drugs has decreased. The benzodiazepines are still the most frequent group of medicals on the illicit market [08034].

Internet drug availability

Today's Internet provides extensive "underground" guidelines for obtaining and using illicit substances, including especially anabolic-androgenic steroids (AAS) and other appearance- and performance-enhancing drugs (APEDs). It was attempted to qualitatively characterize APED-related Internet sites. It was used relevant Internet search terms (e.g. "steroids bodybuilding" and "buy steroids online") to assess (i) the numbers of site visitors; (ii) offers of drugs for sale; and (iii) the quality of online medical information. We also chose the examples of (iv) "site-enhancing oils" and (v) "cattle implants" to illustrate the volume of available Internet information as compared with that in the medical literature. It was found thousands of sites involving AAS and other APEDs. Most sites presented an unashamedly pro-drug position, often openly questioning the qualifications and motivations of mainstream medical practitioners. Offers of AAS and other APEDs for sale, together with medical advice of varying legitimacy, were widespread across sites. Importantly, many sites provided detailed guidelines for exotic forms of APED use, some likely associated with serious health risks, which are probably unknown to most practicing clinicians. It seems important for practitioners to be aware of the extent of this "underground literature," which may strongly influence their patients' decisions about use and abuse of APEDs [13058].

Psychoactive drugs in the society

Analysis of urine samples collected across a city centre, for the detection of novel psychoactive substances (NPS) was performed with a cross-sectional study of anonymized urine samples used for the analysis of classical recreational drugs, NPS and metabolites. Pooled urine samples collected from portable stand-alone four-person urinals across a city centre were analysed using full-scan accurate-mass high-resolution liquid chromatography coupled to tandem mass spectrometry. Data were processed against compound databases
containing >1700 drug compounds and metabolites. Seven established recreational drugs (3,4-methylenedioxyamphetamine, cocaine, cannabis, ketamine, 3,4-methylenedioxy-N-methylamphetamine, methamphetamine and amphetamine) and six potential NPS [hordenine (all 12 urinals), cathine (11), methylhexaneamine (9), 4-methylmethcathinone (6), methiopropamine and metabolites (2) and methoxetamine and metabolites (1)] were detected. Methylhexaneamine, methiopropamine and hordenine are currently uncontrolled in the UK, whereas methoxetamine is currently subject to a Temporary Class Drug Order. Metabolites of the anabolic steroid nandrolone were found in two urinals and trenbolone metabolites and clenbuterol in one urinary. It was concluded that analysis of pooled urine samples collected anonymously from stand-alone urinals in a large inner city can detect the use of recreational drugs, NPS and anabolic steroids. Metabolite detection indicates actual drug use, metabolism and elimination rather than simply discarded drugs in the urinals. This technique by confirming the actual drug(s) used has the potential to be additive to currently used datasets/key indicators providing more robust information for healthcare authorities, legislative and law enforcement on the drugs actually being used [13059].

Organised crime and drugs in sport

A report was from the Australian Crime Commission and entitled “Organised Crime and Drugs in the Sport” had the main following conclusions:

- Australian professional athletes, facilitated by sports scientists, coaches and support staff are using prohibited substances including peptides and hormones
- This behaviour is occurring in a number of professional sporting codes in Australia
- The use of illicit drugs within some sporting codes is higher than previously recorded
- Organised crime is involved in the domestic distribution of peptides and hormones
- There are significant integrity concerns within professional sports in Australia
- The use of prohibited substances by athletes is leading to an association between professional athletes and criminal identities
- There is a culture in some professional sports of administering untested and experimental substances to athletes
- Some sports scientists and medical practitioners are involved in supplying peptides and hormones to athletes.

These revelations rocked the Australian sporting community and have caused significant soul-searching and reflection within sporting organisations, particularly at high-performance level. A number of organisations conducted or are conducting internal reviews of their processes and protocols around the administration of supplements and medications. What was initially perceived as a widespread issue across elite sport in Australia now appears to be a more focal issue, concentrated in small pockets of professional sport. There are assertions, yet to be validated, that in some professional clubs there were systemic injection programmes as part of the supplementation strategy. It is almost inconceivable that such practices did not cause alarm bells to ring for club officials. If the claims are proved to be correct, then one can only conclude that those organisations do not have robust internal governance processes or have drifted away from such processes [13027].
Legitimate use of drugs in sports

It was assessed and compared the prevalence of declared medication, such as corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), beta2-agonists, narcotic analgesics, anaesthetics, and antidepressant drugs, in time and between different sports among athletes tested for doping control in a 4-year period in Belgium. Data were obtained from 18,645 doping control forms gathered between 2002 and 2005 from national doping organisations in Belgium and The Netherlands, the International Cycling Union (UCI), and the Belgian Cycling Federation. All athletes were asked by doping control officers to declare the medication taken in the last three days before competition after which the doping control forms were double blinded and handed over to the laboratory. The overall declared use of medication belonging to one of the monitored categories increased from 20 percent in 2002 to 25 percent in 2005. Differences in use of medication were observed between sports with a higher prevalence of use of NSAIDs in ball sports compared to other sports and a higher use of beta-agonists and corticosteroids in cycling with percentages of declared corticosteroid use in samples from the UCI exceeding 36 percent in 2005. These results indicate that the current granting of therapeutic use exemption for corticosteroids and beta-agonists needs to be revised and that threshold levels for beta-agonists should be implemented [08035].

Enquiries made in the Drug Information Database (DID™) were retrospectively analysed to develop a better understanding of athletes’ interests and concerns regarding the prohibited status of available substances regarding anonymous enquiries recorded in the DID in 2006 and 2007. The DID recorded 223,717 enquiries with 200 of the >6000 UK Licensed Pharmaceutical products receiving over 100 enquiries each. The majority (79 %) of these enquiries were in the pharmaceutical product category, followed by recreational drugs (10 %). A variety of common medications was subject to enquiry with anti-inflammatory agents, decongestants and bronchodilators being most common; a trend in keeping with reported medication use by athletes. Of all enquiries, 42 percent were not found owing to misspelled words or enquiries about unregulated substances. The proportion of enquiries about substances not listed in the database is relatively high and has increased over the 24-month observation period. The DID is a well used information resource with some 10,000 enquiries being made each month. With some 73 percent of enquiries being made by the athletes themselves, further investigations are warranted to explore enquiry patterns in relation to specific sports. Of the unsuccessful enquiries, a large number were related to nutritional supplements which warrants further investigation. The DID database appears to be a valid mirror of athletes’ chemically assisted practices and may be successfully used to inform health professionals as well as anti-doping prevention programmes [08036].

Over-the-counter medicine

A questionnaire was administered to elite athletes from Australia, Canada, the UK, and the USA representing 10 Olympic sports in order to explore knowledge and understanding of over-the-counter (OTC) medication since the removal of many of these substances from the World Anti-Doping Agency Prohibited List, in 2004. Athletes demonstrated limited knowledge and understanding. Around half (51 %) knew the penalty incurred following a doping violation involving a banned OTC stimulant. The terms Monitoring Program and Specified Substance List were understood by 43 and 68 percent of respondents, respectively. Overall, the status of substances in relation to the Prohibited List was correctly identified in just 35 percent of cases. As a whole, athletes were of the opinion that OTC stimulants posed a risk to health, were performance enhancing and that their use was against the spirit of sport. They were undecided as to whether these drugs should be returned to the Prohibited List. Elite athletes require targeted education programmes that will enable them to make informed decisions on the potential of OTC medications for therapeutic or performance enhancing purposes.
Doping agents as medical treatment

Many performance-enhancing supplements and/or drugs are increasing in popularity among professional and amateur athletes alike. Although the uncontrolled use of these agents can pose health risks in the general population, their clearly demonstrated benefits could prove helpful to the critically ill population in whom preservation and restoration of lean body mass and neuromuscular function are crucial. Post-intensive care unit weakness not only impairs post-intensive care unit quality of life but also correlates with intensive care unit mortality. A review covered a number of the agents known to enhance athletic performance, and their possible role in preservation of muscle function and prevention/treatment of post-intensive care unit weakness in critically ill patients. These agents include testosterone analogues, growth hormone, branched chain amino acid, glutamine, arginine, creatine, and beta-hydroxy-beta-methylbutyrate. Three of the safest and most effective agents in enhancing athletic performance in this group are creatine, branched-chain amino acid, and beta-hydroxy-beta-methylbutyrate. However, these agents have received very little study in the recovering critically ill patient suffering from post-intensive care unit weakness. More placebo-controlled studies are needed in this area to determine efficacy and optimal dosing. It is very possible that, under the supervision of a physician, many of these agents may prove beneficial in the prevention and treatment of post-intensive care unit weakness [09031].

Pharmacists

When athletes consult sports outpatient or orthopedic clinics it is possible to undergo drug treatment with the medical staff having prior knowledge of that patient being an athlete. However, if athletes seek any other diagnosis and treatment as an ordinary patient, the possibility of medical staff realizing the potential for imposing a doping issue on the athlete is extremely low. As a result, if the athlete fails to provide medical staff with information regarding anti-doping regulations when receiving clinical treatment, drug treatment administered as part of medical practices could be viewed as doping, resulting in the athlete being disciplined. In order to avoid this, pharmacists should participate in training in order to be able to provide information for anti-doping purposes. There is an opinion that knowledge regarding anti-doping is something that should be shared by all pharmacists, as pharmacists are educated in the fields of pharmacology and pharmacokinetics during the pharmacy education process, and sports pharmacology is a part of this. However, in order for pharmacists to understand sports pharmacology, it is necessary to provide education not only on the benefits and adverse effects of pharmaceutical products, but also on the concept of banned substances. It can be considered one of the pharmacist's duties to protect athletes who purchase drugs at a pharmacy or consult medical institutions as patients. With this, it may be proposed considering the potential for introducing sports pharmacology to pharmaceutical education, and specialist pharmacist training in the sports spectrum [12038].

Compared to general practitioners

Information about doping awareness among medical professionals is scarce. It was evaluated the attitudes, level of knowledge and experience among general practitioners (GPs) and pharmacists (Ps) with regard to doping. In a cross-sectional national survey a 59-item self-administered questionnaire was sent to a representative random sample of 645 GPs and 330 Ps. Overall, 204/975 (133 GPs, 71 Ps) questionnaires were returned and
available for analysis. Fewer than half (39 % GPs vs 48 % Ps) of respondents were familiar with the formal definition of doping. The abbreviation WADA was correctly interpreted by 42 percent (33% vs 59 %), and 65 percent knew that the European Commission has the legislation to fight against doping. More GPs (69 % vs 31 %) agreed to have a role to play in doping prevention, similar proportions considering themselves to have sufficient knowledge of prevention initiatives (65 % vs 35 %). Overall, 12 percent of respondents (9 % GPs, 19 % Ps) reported being directly confronted with a request for prescription of doping agents in the previous 12 months (mainly stimulants, anabolic agents, hormones, corticosteroids). It was concluded that GPs and Ps are frequently exposed to questions about and requests for doping agents. They have acceptable level of general knowledge but are in need for more specific information on prohibited substances and legislature [12039].

Pharmacy students

Doping is one of the most serious problems for the sport community, and it is important that pharmacists have more interaction with athletes to ensure safer drug usage. Education is one of the most important roles of sports pharmacists, who are specialists regarding drug usage for athletes. It was investigated pharmacy students' interests and comprehension regarding drug usage, doping and supplement intake by using the form of a questionnaire, since it is important to know how they understand these subjects as part of their greater educational program. The subjects were sophomore and junior pharmacy students at three universities. It was revealed that most of the students have negative images regarding doping violation, and they answered that they are familiar with doping. However, only sixteen percent of the students had attended lectures by specialists on doping. In addition, one third of pharmacy students did not know that some over-the-counter (OTC) drugs might contain doping substances. With regard to supplement intake, approximately two thirds of the respondents had an interest in and positive image of supplement intake. However, it was revealed that only one third of them recognized supplements as food, and their information regarding supplements was obtained from uncertain media. It was suggested that it is important for pharmacy students to have more opportunities to learn about what doping is. More education and enlightenment by sports pharmacists would be effective for pharmacy students as well as athletes, and it would help us to broaden the scope of what we can do for athletes and society [13045].

A cancer risk of doping?

Anabolic steroid and peptide hormones or growth factors are utilized to increase the performance of athletes of professional or amateur sports. Despite their well-documented adverse effects, the use of some of these agents has significantly grown and has been extended also to non-athletes with the aim to improve appearance or to counteract ageing. Pre-clinical studies and epidemiological observations in patients with an excess of hormone production or in patients chronically treated with hormones/growth factors for various pathologies have warned about the potential risk of cancer development and progression which may be also associated to the use of certain doping agents. Anabolic steroids have been described to provoke liver tumors; growth hormone or high levels of its mediator insulin-like growth factor-1 (IGF-1) have been associated with colon, breast, and prostate cancers. Actually, IGF-1 promotes cell cycle progression and inhibits apoptosis either by triggering other growth factors or by interacting with pathways which have an established role in carcinogenesis and cancer promotion. More recently, the finding that erythropoietin (Epo) may promote angiogenesis and inhibit apoptosis or modulate chemosensitivity in cancer cells expressing the Epo receptor, raised the concern that the use of recombinant
Epo to increase tissue oxygenation might favor tumor survival and aggressiveness. Cancer risk associated to doping might be higher than that of patients using hormones or growth factors as replacement therapy, since enormous doses are taken by the athletes often for a long period of time. Moreover, these substances are often used in combination with other licit or illicit drugs and this renders almost unpredictable all the possible adverse effects including cancer. Anyway, athletes should be made aware that long-term treatment with doping agents might increase the risk of developing cancer [07032].

**Dependence in clinical practice**

The nonmedical use of anabolic-androgenic steroids (AAS) appeals to athletes across several sports, particularly those whose activity makes muscle size and strength advantageous, and in individuals (usually men) with body dysmorphic disorder. Patterns of nonmedical use, including supratherapeutic doses of illicitly obtained drugs, increase the risk for adverse psychiatric and other medical consequences. Although AAS users may be more likely to consult physicians for nonpsychiatric medical consequences than changes in their mental status, it is argued that the motivation for persistent use despite adverse consequences is sustained in large part by psychological variables. Therefore, all physicians who treat nonmedical AAS users will benefit from an understanding of these psychological variables, including the potential for AAS to cause dependence [09032].

**Treatment of drug addicts**

The objective of this review is to describe self-administration procedures for modeling addiction to cocaine, cannabis and heroin in the human laboratory, the benefits and pitfalls of the approach, and the methodological issues unique to each drug. In addition, the predictive validity of the model for testing treatment medications will be addressed. The results show that all three drugs of abuse are reliably and robustly self-administered by non-treatment-seeking research volunteers. In terms of pharmacotherapies, cocaine use is extraordinarily difficult to disrupt either in the laboratory or in the clinic. A range of medications has been shown to significantly decrease cocaine's subjective effects and craving without decreasing either cocaine self-administration or cocaine abuse by patients. These negative data combined with recent positive findings with modafinil suggest that self-administration procedures are an important intermediary step between pre-clinical and clinical studies. In terms of cannabis, a recent study suggests that medications that improve sleep and mood during cannabis withdrawal decrease the resumption of marijuana self-administration in abstinent volunteers. Clinical data on patients seeking treatment for their marijuana use are needed to validate these laboratory findings. Finally, in contrast to cannabis or cocaine dependence, there are three efficacious Food and Drug Administration-approved medications to treat opioid dependence, all of which decrease both heroin self-administration and subjective effects in the human laboratory. In summary, self-administration procedures provide meaningful behavioral data in a small number of individuals. These studies contribute to our understanding of the variables maintaining cocaine, marijuana and heroin intake, and are important in guiding the development of more effective drug treatment programs [08038].

**Educational programs**

Continuing educational programs developed for these at-risk populations by national olympic
organizations and athletic federations are important first steps to curb these doping behavior. Medical professionals, teachers, coaches and sports organizations must all be made aware of this continuing problem in our adolescent and at-risk populations and contribute to its solution by open, honest discussion. Most importantly, professional athletes must serve as role models and spokesmen for drug-free sport and lifestyle. This position must be actively supported by the media, owners of teams and international sports federations by providing consistent leadership and advocacy of anti-doping programs in sport, regardless of costs and consequences. Accepting the magnitude of doping in at-risk populations and developing education, prevention and treatment programs is the only way we can prevent the continuing spread of the abuse of doping in sport and its spread into the most fragile groups in our society, our youth and at-risk populations [08006].

**Social perceptions**

It was explored social perceptions related to the use of anabolic steroids in sports. More specifically, 78 women and 102 men read one of two scenarios depicting a male athlete facing either a drug use or non-drug use situation. Then, participants reported their perceptions of the scenario protagonist in terms of self-determined sport motivation, sportspersonship orientations, and athletic aggression. Results of a multivariate analysis of covariance indicated that, in comparison with a non-using protagonist, the anabolic steroid-using athlete was regarded as less self-determined in one's motivation (i.e. sport participation based on predominant feelings of pressure to obtain external rewards or avoid punishment) and as displaying weaker sportspersonship orientations (i.e. lesser concerns for opponents, the social conventions of sport, and for one's own athletic commitment). In addition, the steroid-using athlete was perceived as resorting more readily to reactive aggression than to instrumental aggression (i.e. intent to injure one's opponent vs merely hinder his performance). Finally, the analyses did not disclose significant gender or interaction effects [08040].

The aim of one study was to improve understanding of the development of multiple drug use in patients seeking treatment at an addiction clinic for steroid-related problems. It was interviewed six patients (four men and two women) with experience of AAS use who were attending an addiction clinic for what they believed were AAS-related problems. The patients were interviewed in-depth about their life stories, with special emphasis on social background, substance use, the development of total drug use and subjective experienced psychological and physical side effects. There was significant variation in the development of drug use in relation to social background, onset of drug use, relationship to AAS use and experience of steroid effects. All patients had initially experienced positive effects from AAS but, over time, the negative experiences had outweighed the positive effects. All patients were dedicated to excess training and took AAS in combination with gym training, indicating that the use of these drugs is closely related to this form of training. Use of multiple drugs was common either in parallel with AAS use or serially. The study shows the importance of understanding how AAS use can develop either with or without the concomitant use of other drugs of abuse. The use of anabolic steroids can, however, progress to the use of other drugs. The study also indicates the importance of obtaining accurate, comprehensive information about the development of AAS use in designing treatment programmes and prevention strategies in this area [08041].

**Medical risks with illegal drugs**
Unfortunately, cadaver extracts of pituitary human growth hormone may still be in circulation. It has been reported that a Russian coach was arrested and, upon searching his apartment in Moscow, over 1000 cadaver pituitary glands were found preserved in a large container [08042]. Moreover, the problem of counterfeit drugs also exists with hGH: illegal pharmaceutical manufacturers are now flooding the black market with hGH vials of unknown quality and safety [08006].

Human growth hormone is marketed on the internet in many forms: pills, drops and aerosol formulations; most are ineffective and shams. The normal route of administration of hGH is injection, posing an additional health risk of infection from non-sterile counterfeit drugs and the risk of HIV and hepatitis transmission caused by shared needles. However, also using high quality human growth hormone may lead to life-threatening health conditions, especially since some estimates report that athletes who use growth hormone to enhance performance are taking 10 times the therapeutic dosage. Some reported side effects of hGH are abnormal bone growth, hypertension, cardiovascular disease, cardiomyopathy, glucose intolerance, colonic polyps, decreased life span, and cancer [08043].

As with steroids and growth hormone, doping with erythropoietin is often injected in supernormal doses that could cause increased blood viscosity, deep vein and coronary thromboses, cerebral thromboses, pulmonary embolism, arrhythmias, stroke and death. It has been estimated that 20 European cyclists have died since 1987 due to abuse of erythropoietin, making it one of the most deadly doping agents [08006].

**Doping and the respiratory system**

Historically many different drugs have been used to enhance sporting performances. The magic elixir is still elusive and the drugs are still used despite the heavy adverse effects. The respiratory system is regularly involved in this research probably because of its central location in the body with several connections to the cardiovascular system. Moreover people are aware that O$_2$ consumption and its delivery to mitochondria firstly depend on ventilation and on the respiratory exchanges. The second step consists in the tendency to increase VO$_{2\max}$ and to prolong its availability with the aim of improving the endurance time and to relieve the fatigue. Many methods and substances had been used in order to gain an artificial success. Additional oxygen, autologous and homologous transfusion and erythropoietin, mainly the synthetic type, have been administered with the aim of increasing the amount of oxygen being delivered to the tissues. Some compounds like stimulants and caffeine are endowed of excitatory activity on the CNS and stimulate pulmonary ventilation. They did not prove to have any real activity in supporting the athletic performances. Beta-adrenergic drugs, particularly clenbuterol, when administered orally or parenterally develop a clear illicit activity on the myosin fibres and on the muscles as a whole. Salbutamol, terbutaline, salmeterol and formoterol are legally admitted when administrated by MDI in the treatment of asthma. The prevalence of asthma and bronchial hyperactivity is higher in athletes than amongst the general population. This implies that clear rules must be provided to set a correct diagnosis of asthma in the athletes and a correct therapy to align with the actual guidelines according to the same rights of the "other" asthmatic patients [07132].

The purpose of one study was to investigate the effects of resistance training and long-term anabolic androgenic steroids (AASs) administration on respiratory function. Subject groups consisted of AAS users (n=9) who were still using AAS at time of testing (SU); AAS users (n=6) who had been abstinent for > 3 months (SA), bodybuilding controls (n=8) (BC), and (n=8) sedentary male controls (SC). FEV$_1$, FVC, and PEF were measured. The results found that all subjects were within normal range, and there were no differences between groups.
Maximum inspiratory pressure (MIP), and grip strength were both significantly greater in SU compared with SC; no significant difference was found between the other groups. Their MIP and grip strength was significantly correlated. The data from this study suggest that the combination of resistance training and AAS administration produce a significant increase in MIP in a cohort of long-term AAS users [11569].

Information on doping

Many sporting organisations in Australia conduct drug information seminars for their athletes; however, it is uncertain whether these programs provide athletes with pertinent drug information in formats that are conducive to information retention. The aims of one study were to investigate self-reported confidence in knowledge of illicit drugs and information seeking behaviours among elite athletes. Data were collected from two sources: quantitative surveys with elite Australian athletes; and qualitative interviews with key experts who come into contact with elite athletes. Athletes were confident in their knowledge of the effects of illicit drugs such as cannabis and meth/amphetamine, but less confident in their knowledge of the effects of illicit drugs such as GHB and ketamine. A substantial proportion felt that athletes in their sport would benefit from more information concerning illicit drugs. Both athletes and key expert believed that information on illicit drugs should be delivered to athletes in a specific and relevant manner. There may be stigma attached to information seeking within a sports club or organisation. Accordingly, improving the accessibility to creditable information via the Internet may prove to be an effective means by which to educate athletes on the effects of illicit drugs [11022].

Internet

Identifying the use of non-approved drugs by cheating athletes has been a great challenge for doping control laboratories. This is due to the additional complexities associated with identifying relatively unknown and uncharacterized compounds and their metabolites as opposed to known and well-studied therapeutics. In 2010, the prohibited drug candidates and gene doping substances AICAR and GW1516, together with the selective androgen receptor modulator (SARM) MK-2866 were obtained by the Cologne Doping Control Laboratory from Internet suppliers and their structure, quantity, and formulation elucidated. All three compounds proved authentic as determined by liquid chromatography-high resolution/high accuracy (tandem) mass spectrometry and comparison to reference material. While AICAR was provided as a colourless powder in 100 mg aliquots, GW1516 was obtained as an orange/yellow suspension in water/glycerol (150 mg/mL), and MK-2866 (25 mg/mL) was shipped dissolved in polyethylene glycol (PEG) 300. In all cases, the quantified amounts were considerably lower than indicated on the label. The substances were delivered via courier, with packaging identifying them as containing “amino acids” and “green tea extract”, arguably to circumvent customs control. Although all of the substances were declared “for research only”, their potential misuse in illicit performance-enhancement cannot be excluded; moreover sports drug testing authorities should be aware of the facile availability of black market copies of these drug candidates [11023].

Internet websites offering androgenic anabolic steroids (AAS) were identified and available products were examined. Keywords for the website search were: "anabolic steroids," "anabolic steroids buy," "anabolic steroid purchase." The first 10 websites offering AAS in the first 10 pages of results were considered. At least two AAS-containing products per website were selected. Thirty AAS-selling websites were identified, mainly located in the United States (47 %) and Europe (30 %). Most websites sold other anabolic/ergogenic products
(clenbuterol, 77 %; GH/IGF, 60 %; thyroid hormones, 47 %; erthropoietin, 30 %; insulin, 20 %) or products for AAS-related adverse effects (mainly: estrogen antagonists, 63 %; products for erectile dysfunction, 57 %; 5alpha-reductase inhibitors, 33 %; anti-acne products, 33.3%). AAS were sold as medicines (70 %) or as dietary supplements (30 %). AAS in medicines were mainly: nandronole (20 %), methandrostenolone (18 %), and testosterone (12 %). Dietary supplements contained mainly DHEA and included several fake compounds. Manufacturers were declared for 98 percent of medicines and 67 percent of dietary supplements; however, several manufacturers were not found on the Internet. Described benefits were usually few adverse effects and no estrogenicity. Toxicity was seldom reported and presented as mild. Recommended doses were two-fourfold higher than current medical recommendations. In conclusion, misleading information and deceiving practices were common findings on AAS-selling websites, indicating their deleterious potential for public health [11024].

This study examined whether different types of media affect the use of dietary proteins and amino acid supplements, and intent to use anabolic-androgenic steroids. A random sample of 618 boys aged 11-18 years from eight schools in the Flemish part of Belgium completed standardized questionnaires as part of the Media and Adolescent Health Study. The survey measured exposure to sports media, appearance-focused media, fitness media, use of dietary supplements, and intent to use anabolic-androgenic steroids. Data were analyzed using logistic regressions and are presented as adjusted odds ratios (OR); 9 percent indicated to have used dietary proteins, 4 percent indicated to have used amino acid supplements, and 12 percent would consider using anabolic-androgenic steroids. After adjusting for fitness activity, exposure to fitness media was associated with the use of dietary proteins (OR 7.2) and amino acid supplements (5.2). Intent to use anabolic-androgenic steroids was associated with exposure to fitness media (2.4) and appearance-focused media (6.0). Sports media did not correlate with the use of dietary supplements and intent to use anabolic-androgenic steroids. Specific types of media are strong predictors of the use of supplements in adolescent boys. This provides an opportunity for intervention and prevention through the selection of fitness media as a communication channel. Health practitioners should also be aware that the contemporary body culture exerts pressure not only on girls but also on boys [13044].

Telephone hot-line

The aim of one retrospective study was to analyze the calls concerning anabolic products (AP), received at Écoute Dopage, a French anti-doping hot-line. It was reviewed all phone calls handled between 2000 and 2008, among them 214 concerned AP. Information collected include demographic data, reason for the phone call, name of AP, characteristic of consumption, adverse reactions. Fifteen different AP (mainly testosterone) were reported. Calls concerned information about side-effects (42 % of calls), risk for doping (28 %), and risk for health (10 %), psychological assistance (10 %), and legislation (2 %). Most calls came from fitness practitioners or bodybuilders (85 %). The reason for use was documented in 137 subjects: to increase muscular strength (76 %), improve social life ability (15 %), improve sporting ability (6 %), and losing weight (3 %). Eighty subjects (37 %) reported at least one side-effect mainly uro-genital (40 cases) or psychic disorders (25 cases), both 15 cases. Among these 80 patients, 17 patients (21.25%) presented signs of AP dependence. The abuse of AP in sport is a public health problem well known, but data on the dependence on AAS are sparser. Information and education should be emphasise to fight against doping. [13055].

Media medicine
One paper uses UK media coverage of the sleep drug modafinil to investigate the medicalisation of sleep at a conceptual level. Using metaphorical frame analysis it was investigated the conceptual links created in media discourse between sleep and health, and the body and technology in the UK. Using this novel analytical tool it was explored under what circumstances modafinil is constructed as a necessary medical treatment or a (il)legitimate performance enhancement and, how in this process, various images of the body are constructed. It was found that media discourse on modafinil was structured through four types of sleep discourse: patient, sports, recreational, and occupational. Each discourse was built up around the specific deployment of three central metaphorical frames “war”, “commodity” and “competition” that acted to construct the biological body in a particular way. How the body was framed in each discourse impacted upon how modafinil use was portrayed in terms of therapy or enhancement and the level of engagement with a medical rhetoric. This had distinct normative implications strongly influencing the legitimacy afforded to modafinil use in each domain. It was argued that medical authority acts to legitimise modafinil use for repair, restoration and relief of suffering, whilst being deployed to pass judgment on its use in bodies already perceived as functioning normally. This led the authors to conclude that conceptually, the acceptability of “enhancement” is strongly tied to context of use and intricately related to medical social control [08044].

An integrated approach

Doping use is an important issue in both competitive and non-competitive sports, and poses potentially irreversible health consequences to users. Scholars increasingly call for theory-driven studies on the psychosocial processes underlying doping use that will inform subsequent policy-making and prevention interventions. The aim of one study was to implement an integrative theoretical model to assess the direct and indirect effects of motivational variables, moral orientations, and social cognitions on doping intentions. A randomly selected and representative sample of 750 elite athletes anonymously completed a battery of questionnaires on motivational and moral constructs, and social cognitions related to doping. Hierarchical linear regression analysis and multiple mediation modeling were used. The effects of achievement goals and moral orientations were significantly mediated by attitudinal, normative, and self-efficacy beliefs, in both lifetime ever and never doping users. Moral orientations indirectly predicted the doping intentions of never users, but did not predict ever users' doping intentions. Achievement goals and sportspersonship orientations influence doping intentions indirectly, through the effects of attitudes and self-efficacy beliefs. Sportspersonship (moral) orientations were relevant to doping intentions among athletes with no prior experiences with doping, while achievement goals and situational temptation were relevant to both lifetime never and ever dopers [13043].
INADVERTENT DOPING

In the past years, an increasing number of dietary supplements containing undeclared doping substances has been identified. The consumption of these supplements can lead to inadvertent doping cases. Although warnings about the risk of inadvertent doping have been communicated, recent studies show that athletes' knowledge of the problem is inadequate. Furthermore, it seems that the risk has been growing due to the increased availability of pharmaceutical substances via the internet, which are admixed by criminal manufacturers to their, arguably, non-effective supplement products. The main candidates from the dietary supplement market for inadvertent doping with stimulants are products containing ephedrine and analogues, sibutramine and methylhexaneamine. Such products are mainly advertised as fat burners or mood enhancers, and their use may lead to positive doping results in competition. The risk of inadvertent doping with such supplements is based on different reasons. In the case of supplements containing ephedrines, the natural sources of ephedrine such as Ma Huang or ephedra sinica are frequently mentioned on the label rather than the names of the active ingredients (ephedrine, pseudoephedrine, methylephedrine, etc). Despite extensive education of athletes regarding unclear labelling, or the variety of names by which banned substances may be referred to, many athletes still fall into this doping trap.

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In case of supplements enriched with sibutramine, the ingredient is not declared on the label and the consumer is only provided with the information that the product contains 'pure herbal ingredients' that are advertised to have considerable weight loss capabilities. Sibutramine can be found in therapeutically or even supratherapeutically doses in slimming capsules, powders and even slimming teas. Sibutramine is a synthetic anorectic drug, only approved as a pharmaceutical preparation and available only on prescription. Because of its enormous side effects (stroke and heart attack risk for patients with a history of cardiovascular disease), the European Medicines Agency recommended in January 2010 that this drug be withdrawn from the market. Sibutramine has been on the list of prohibited substances from the World Anti-Doping Agency (WADA) since 2006. Since 2006–2009, there has been a high risk for inadvertent doping with the stimulant methylhexaneamine, which was added to the WADA prohibited list in 2009. The issue of inadvertent doping arises from the fact that methylhexaneamine can be found on the labels in numerous different names such as dimethylamylamine, dimethylpentylamine, pentylamine, geranamine, forthane and 2-amino-4-methylhexane. On WADA's 2011 prohibited list, only the names methylhexaneamine and dimethylpentylamine are mentioned in the group of stimulants, which complicates the identification of the substance as a prohibited compound. In some supplements, geranium
root extract or geranium oil is mentioned as an alleged natural source of methylhexaneamine. However, recent investigations have shown that methylhexaneamine is not a natural ingredient of geranium oil, which means that synthesised methylhexaneamine must have been added. Despite warnings by different national antidoping agencies in 2009 and 2010, numerous elite athletes in competition have been found to have a positive test for methylhexaneamine [11425].

It is clear that there is a real risk that athletes who use dietary supplements may unknowingly ingest a banned substance that will cause them to record a positive doping outcome. There are cases in which a doping infringement can be traced back to supplement use and for which the athlete has undertaken some strategies to reduce this risk. For example, the athlete has received written advice from a supplement manufacturer that their produce does not contain banned substances, but following a positive doping test, a sealed container of the dietary supplement has been examined and found to contain the banned ingredient. Unfortunately, strict liability applies to these situations and even if athletes have been successful in having the terms of their ban from sport reduced, a doping infringement will still be recorded against their name. The loss of a career, livelihood and reputation are stakes that an athlete must take into account when using dietary supplements [11425].

Until now only two cases have been detected in which dietary supplements contained therapeutic (30 µg per tablet) and supratherapeutic (2 mg/capsule) doses of the beta2-agonist clenbuterol. In the supratherapeutically dosed product, clenbuterol was not declared on the label. Both supplements were advertised as weight loss products. Because of the extremely high concentration of clenbuterol in the second product (100-fold more than the therapeutic dose), severe side effects could be expected; in addition, a high risk of cross-contaminations of other products with clenbuterol is likely. Because WADA has classified clenbuterol as an anabolic agent, its detection in doping control may lead to severe sanctions. In 2009 and 2010, dietary supplements containing the prohibited growth hormone-releasing peptide-2 (GHRP-2) were detected. The products were advertised to produce anabolic, fat-reducing and anticasabolic effects and to improve regeneration. One product, in an ampoule of a drinking solution, contained an orally active concentration of GHRP-2. Such a product may lead to inadvertent doping cases because the name GHPR-2 is not specifically listed on the WADA prohibited list and is unknown to the majority of the sports community. However, GHRP-2 belongs as a releasing factor to the prohibited substance group S2 on the WADA list [11425].

**Sibutramine**

In case of supplements enriched with sibutramine, the ingredient is not declared on the label and the consumer is only provided with the information that the product contains “pure herbal ingredients” that are advertised to have considerable weight loss capabilities. Sibutramine can be found in therapeutic or even supratherapeutic doses in slimming capsules, powders and even slimming teas. Sibutramine is a synthetic anorectic drug, only approved as a pharmaceutical preparation and available only on prescription. Because of its enormous side effects (stroke and heart attack risk for patients with a history of cardiovascular disease), the European Medicines Agency recommended in January 2010 that this drug be withdrawn from the market. Sibutramine has been on the list of prohibited substances from the World Anti-Doping Agency (WADA) since 2006 [11010].

**Methylhexaneamine**
Since 2008-2009, there has been a high risk for inadvertent doping with the stimulant methylhexaneamine, which was added to the WADA prohibited list in 2009. The issue of inadvertent doping arises from the fact that methylhexaneamine can be found on the labels in numerous different names such as dimethylamylamine, dimethylpentylamine, pentyramine, geranamine, forthane and 2-amino-4-methylhexane. On WADA's 2011 prohibited list, only the names methylhexaneamine and dimethylpentylamine are mentioned in the group of stimulants, which complicates the identification of the substance as a prohibited compound. In some supplements, geranium root extract or geranium oil is mentioned as an alleged natural source of methylhexaneamine. However, recent investigations have shown that methylhexaneamine is not a natural ingredient of geranium oil, which means that synthesised methylhexaneamine must have been added. Despite warnings by different national antidoping agencies in 2009 and 2010, numerous elite athletes in competition have been found to have a positive test for methylhexaneamine [11010]

**Illicit blue tablets containing anabolic androgen steroids**

The necessity of specific, confirmatory tests in the identification of seized illicit products was highlighted by the analysis of eighteen heart shaped, blue tablets confiscated by Police at a street control in the North East of Italy. The tablets responded as amphetamines to a preliminary color test (Marquis); a subsequent, confirmatory assay by gas chromatography-mass spectrometry revealed the presence of two anabolic androgen steroids (AAS), methandienone and methyltestosterone, in concentration of 1.7 and 1.5 mg respectively per tablet; no trace of amphetamine-like or nitrogen containing compounds was found. The observed orange coloration was due to the reaction of concentrated sulphuric acid, contained in the Marquis reagent, with keto group of steroids. The two AAS, banned under the world antidoping code, are not considered as psychoactive drugs of abuse in most countries, although their trafficking may entangle severe public health concerns [13076].

**Dietary supplements containing prohibited anabolic agents**

The extent of the contamination of dietary supplements with anabolic agents was evaluated in 2001 and 2002. A well-publicised study showed that about 15 percent of non-hormonal supplements such as vitamins, minerals, proteins and creatine contained anabolic androgenic steroids (mainly prohormones) that were not declared on the label. The reason for the contamination was most probably the fact that manufacturers of prohormones also manufactured other supplements on the production line without sufficient cleaning. Another source of cross-contamination could have been the unclean transport containers from raw material suppliers of prohormones. The amount of detected prohormones, especially prohormones of nandrolone, could produce positive doping cases. Since 2002, dietary supplements have appeared on the market, which are probably intentionally spiked with high amounts (more than 1 mg/g) of “classic” anabolic steroids, not declared or declared with non-approved or fancy names on the label. Among these, steroids including stanozolol, metandienone, dehydrochloromethyltestosterone and oxandrolone have been identified. All these steroids are orally effective drugs based on their 17-alkyl group. These dietary supplements are advertised as leading to enormous enhancement of strength and lean body mass. The concentrations of the anabolic androgenic steroids are in the therapeutic or supratherapeutic range per serving leading to positive doping cases detectable for several days and weeks, respectively, depending on the type of steroid administered. Because the manufacturers of these faked products also prepare other nutritional supplements on the
same production line, the risk of cross-contaminations with such "classic" anabolic androgenic steroids is very high. Such contaminations have been found in fizzy tablets of vitamin C, magnesium and multivitamins produced for Spanish and German supermarkets containing, for example, small amounts of stanozolol and metandienone with the potential to produce a positive doping response [11425].

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Nutritional supplements can be a source of positive doping cases as some supplements contain prohibited substances without showing this on their label. This problem has existed for some time and has been extensively studied in the past 8 years. The sport of tennis has played a particular role in this problem because of some peculiar doping cases within its community. For more than a decade, it has been known that nutritional supplements can be "contaminated" with doping substances, which means that the contents of the supplements are not identical to the list of ingredients on the label. Tennis has played a particular role in this debate because of the complexity of the cases of Bohdan Ulìhrach and Greg Rusedski, who tested positive for nandrolone or nandrolone prohormones in 2002 and 2003, respectively. In June 2007, Guillermo Coria sued an American nutritional company for the financial damages he suffered during his 2 year suspension after also testing positive for nandrolone in 2001. This problem is of major concern to elite athletes, who can test positive in a doping test without knowingly taking banned substances. This so-called "inadvertent doping use" has resulted in an unknown number of positive cases because doping tests often rely on the presence of metabolites of banned substances in urine, and cannot discern between intentional and inadvertent use [07025].
**Dietary supplements contaminated with prohormones**

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**Dietary supplements with clenbuterol**

Until summer 2011 only two cases have been detected in which dietary supplements contained therapeutic (30 microg per tablet) and supratherapeutic (2 mg/capsule) doses of the beta2-agonist clenbuterol. In the supratherapeutically dosed product, clenbuterol was not declared on the label. Both supplements were advertised as weight loss products. Because of the extremely high concentration of clenbuterol in the second product (100-fold more than the therapeutic dose), severe side effects could be expected; in addition, a high risk of cross-contaminations of other products with clenbuterol is likely. Because WADA has classified clenbuterol as an anabolic agent, its detection in doping control may lead to severe sanctions [11010].

**Dietary supplements containing prohibited peptide hormones**

In 2009 and 2010, dietary supplements containing the prohibited growth hormone–releasing peptide-2 (GHRP-2) were detected. The products were advertised to produce anabolic, fat-reducing and anticatabolic effects and to improve regeneration. One product, in an ampoule of a drinking solution, contained an orally active concentration of GHRP-2. Such a product may lead to inadvertent doping cases because the name GHRP-2 is not specifically listed on the WADA prohibited list and is unknown to the majority of the sports community. However, GHRP-2 belongs as a releasing factor to the prohibited substance group S2 on the WADA list [11010].

**Designer drugs**

Since 2002, the so-called designer steroids can also be found on the dietary supplement market. These steroids are neither listed as ingredients in any currently available medication, nor do their names appear in the WADA list of prohibited substances. Most of these designer steroids have been synthesised in the 1960s and were tested only in animal studies for their anabolic and androgenic effects. Nowadays, these steroidal agents are produced exclusively for the nutritional supplement market and are advertised for their anabolic- or aromatase-inhibiting capacities. With regard to the effects and side effects of these steroids for humans, there is limited or no knowledge. In most cases, the labelling of these products contains non-approved or fancy names of the steroids. More than 40 such designer steroids have been detected. The detection of metabolites of such a steroid in an athlete’s urine sample is likely to lead to a positive doping case [11425].
Emerging drugs

In 2009 and 2010, the first prohibited selective androgen receptor modulators (SARMs) and the gene doping substances AICAR and GW1516 were detected on the black market. All these substances are still in clinical trials and have not yet been approved as medications. From the experience, it can be expected that these substances will appear very soon on the dietary supplement market, with advertising that the SARM products will achieve anabolic effects whereas the gene doping substances will enhance endurance. If these substances are added to other supplement products without being declared on the label, new sources of risk for inadvertent doping will be created [11010].
PREVENTION OF DOPING

The potential for adverse effects and the prevailing attitudes towards “unethical” performance-enhancing substances have prompted various efforts to prevent anabolic steroids use in adolescent athletes over the past 25 years. Principal among these has been a punitive approach consisting of in- and out-of-competition doping controls and suspension or expulsion for refusing testing or failed tests. Although these actions may have some impact on elite-level adolescents athletes, they are of questionable utility for those at lower levels where testing is not conducted and of no use for young AAS users not involved in organised sport. Prevention programs founded on cognitive-behavioural or social environment paradigms that do not focus on a fear-based philosophy may be more effective. Based on their findings that a significant proportion of steroid users “have access to models that have used or use anabolic steroids.” It has also been recommended interventions that focus on “environments and groups where the general level of drug use is high.” Furthermore, as these adolescents know users, the claims by authorities of the significant adverse effects of steroid use “may be perceived as unsubstantiated.” Therefore, a different model than “fear based” is needed to deter use among children and adolescents, whether involved in organised sports or not. This view is supported by early prevention efforts when it was compared a “balanced education program (potential risks and benefits)” to a “risks-only (negative or scare tactics) program” of AAS use and found that while the balanced approach resulted in significant improvements in the athletes’ attitudes to adverse AAS effects, there was no change in the fear-based group [09023].

Anti-doping activities in sport have shifted from secondary prevention (intervening after athletes have used) to educational strategies focused on primary prevention through promoting abstinence. There is no empirical evidence to guide targeting of anti-doping education initiatives. In one paper, a heuristic to guide education initiatives was derived by re-analysing a series of interviews (n=20) with athletes, coaches, sports managers, physiotherapists and sports nutritionists. The findings indicate primary prevention of doping may be enhanced by timing it around periods of career instability where athlete vulnerability to doping may increase as a function of winning or losing sponsorship. Sponsorship is broadly defined as financial (e.g. salary stipend) and non-financial support (e.g. training facilities). This provides a basis for targeting education interventions to promote abstinence. Two options are offered to mitigate the need to time prevention activity around career instability by lessening the effect of sponsorship on athlete doping. The first is liberalising access to legitimate performance enhancing technologies (e.g. training techniques or nutritional supplements). The second is to delay access to financial sponsorship (beyond living expenses) until retirement, with monetary gains (e.g. prize money) deposited into an account where penalties are debited if the athlete is caught doping [10304].

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access to legitimate performance enhancing technologies (e.g. training techniques or nutritional supplements). The second is to delay access to financial sponsorship (beyond living expenses) until retirement, with monetary gains (e.g. prize money) deposited into an account where penalties are debited if the athlete is caught doping [11025].

One study examined the extent to which the trajectory of participation in sports, athletics or exercising (PSAE) covaried with substance use in early adulthood controlling for team sports participation using parallel process latent growth curve modeling. Analysis of data collected from a series of panel studies using a cohort-sequential design. Specifically, the analyses used longitudinal data from 11 741 individuals from the graduating classes of 1986-2001, first surveyed as seniors in American high schools. Up to four additional follow-up surveys were administered to age 26 years. Data were collected using in-school and mailed self-administered questionnaires. Level of PSAE, past-30-day alcohol, cigarette and marijuana use frequency and any past-30-day use of illicit drugs other than marijuana (IOTM) were the main processes of interest. Self-reported race/ethnicity, college status at age 19/20 years, parental education, gender and team sports participation during high school were included as covariates. Results indicate that higher initial levels of PSAE related to lower initial substance use prevalence rates other than alcohol, and lower initial prevalence rates of substance use then corresponded with lower substance use rates throughout early adulthood. Further, as individuals increased PSAE levels throughout early adulthood, the frequency of their use of cigarettes, marijuana and IOTM correspondingly decreased. It was concluded that increased participation in sports, athletics or exercising (PSAE) is related to significantly lower substance use frequency at modal age 18 and through significantly and negatively correlated growth trajectories through early adulthood. Encouraging PSAE among adolescents and early adults may relate to lower substance use levels throughout early adulthood [11026].

The US National Institute on Drug Abuse has called for increased research into the use of physical activity in substance abuse prevention, specifically research into physical activity type and context. One paper examines the relationships between secondary school student substance use and exercise in general and school athletic team participation, and examines such relationships over time. Nationally representative cross-sectional samples of 8th-, 10th-, and 12th-grade students were surveyed each year from 1991 to 2009. Substance use measures included past 2-week binge drinking and past 30-day alcohol, cigarette, smokeless tobacco, marijuana, and steroid use. Analyses were conducted during 2009-2010. Across grades, higher levels of exercise were associated with lower levels of alcohol, cigarette, and marijuana use. Higher levels of athletic team participation were associated with higher levels of smokeless tobacco use and lower levels of cigarette and marijuana use across grades and to higher levels of high school alcohol and steroid use. Exercise helped suppress the undesired relationship between team participation and alcohol use; exercise and athletic team participation worked synergistically in lowering cigarette and marijuana use. Observed relationships were generally stable across time. In conclusion, there appear to be substantive differences between exercise and team sport participation in relation to adolescent substance use. These findings from cross-sectional data suggest that interventions to improve levels of general physical activity should be evaluated to determine if they help delay or reduce substance use among youth in general as well as among student athletes [11027].

**Athletes Targeting Healthy Exercise and Nutrition Alternatives (ATHENA)**

To explain, through mediation analyses, the mechanisms by which ATHENA (Athletes Targeting Healthy Exercise and Nutrition Alternatives), a primary prevention and health promotion intervention was designed to deter unhealthy body shaping behaviors among
female high school athletes, produced immediate changes in intentions for unhealthy weight loss and steroid/creatine use, and to examine the link to long-term follow-up intentions and behaviors. In a randomized trial of 1668 athletes, intervention participants completed coach-led peer-facilitated sessions during their sport season. Participants provided pre-test, immediate post-test, and 9-month follow-up assessments. ATHENA decreased intentions for steroid/creatine use and intentions for unhealthy weight loss behaviors at post-test. These effects were most strongly mediated by social norms and self-efficacy for healthy eating. Low post-test intentions were maintained 9 months later and predicted subsequent behavior. It was concluded that ATHENA successfully modified mediators that in turn related to athletic-enhancing substance use and unhealthy weight loss practices. Mediation analyses aid in the understanding of health promotion interventions and inform program development [09030].

Almost one half of male and female students participate in high school-sponsored athletics, and high school also is a time when classroom health promotion curricula are less effective. The Athletes Training and Learning to Avoid Steroids is a sport team-centered drug-use prevention program for male high school athletes, which has been shown to reduce alcohol and illicit drug use. Just as anabolic steroid use is associated with male athletes, female sport participants may be at a greater risk for disordered eating and body-shaping drug use. Extending sport team-centered programs to young women athletes required defining and ranking factors related to developing those harmful behaviors. Survey results from a cross-sectional cohort of female middle and high school student athletes were used to identify and prioritize potential curriculum components, including mood and self-esteem, norms of behavior, perceptions of healthy body weight, effects of media depictions of women, and societal pressures to be thin. The derived sport team-centered program was prospectively assessed among a second group of female student athletes from 18 high schools, randomized to receive the intervention or the usual care control condition. The Athletes Targeting Healthy Exercise and Nutrition Alternatives (ATHENA) intervention is a scripted, coach-facilitated, peer-led 8-session program, which was incorporated into a team’s usual training activities. The ATHENA program significantly altered the targeted risk factors and reduced ongoing and new use of diet pills and body-shaping substances (amphetamines, anabolic steroids, and sport supplements). These findings illustrate the utility of a structured process to define curriculum content, and the program’s positive results also confirm the sport team’s potential as a vehicle to effectively deter health-harming behaviors [06030].

**Telephone counseling**

Drug abuse, most notably anabolic-androgenic steroid (AAS) use, in athletes is widespread. As a result, athletes and exercise enthusiasts who abuse these drugs are troubled by the side effects of these illicit drugs, especially AAS. In an attempt to improve this situation, since 1993, it was counseled athletes who abuse drugs and others with questions about AAS via telephone and tabulated the results. Counseling sessions took place by telephone every Monday between 19:00-23:00 h. The number of cases was tabulated each year and the specific items discussed during each consultation were categorized by key words. Cases consisted of both drug abusers and athletes who did not abuse drugs and were concerned about the side effects or other various problems surrounding the use of AAS. From 1993 to 1996, there were about 50 cases yearly; thereafter, the number of consultations dropped to about 30 to 40 cases each year. In 2002, consultations with drug abusers accounted for 52 percent of all consultations, compared with 46 percent of all consultations from 1993 to 2002. It was found that abusers of hormones exist in Japan, as well as elsewhere. It was hoped these results will demonstrate the necessity of employing public institutional counseling systems for athletes who are drug abusers in Japan, similar to the successful system instituted by the Swedish National Service [07029].
National policy against doping

Sweden

In 2003, the Swedish Parliament adopted a cross-sectorial national public health policy based on the social determinants of health, with an overarching aim – to create societal conditions that will ensure good health, on equal terms, for the entire population – and eleven objective domains. At that time the policy was globally unique, and serves as guidance for public health practice at the national, regional and local levels. The development of the public health policy and the determinants of health are presented regularly in various reports by the Swedish National Institute of Public Health. In order to provide a holistic approach to analysing implemented measures and providing new recommendations within the eleven objective domains of the Swedish national public health policy, we have divided these in three strategic areas. These are: Good Living Conditions, Health-Promoting Living Environments and Living Habits, and Alcohol, Illicit Drugs, Doping, Tobacco and Gambling, each described in the respective introductions for Chapters 3-5. The production of the report was supported by a common analytical model that clarified the societal prerequisites for health in the eleven objective domains. These are factors that can be influenced by political actions in order to create a change. Economic analyses have also been developed to provide a priority basis for political decisions. Analyses of the development of public health determinants were based on data from the National Public Health Survey and data delivered from about 15 various national agencies. Measures that have been implemented between 2004 and 2009 are analysed in details, as the basis for new recommendations for future measures [13060].

Brazil

A retrospective study reports data obtained from the National Institute of Criminalistics of the Brazilian Federal Police Department (DPF) on 3676 anabolic products seized between 2006 and 2011. Anabolic androgenic steroids (AAS) were declared on the labels of 96 percent of the products. About one third of the products declared to be from Paraguay, and 14 percent from Brazil. Stanozolol, testosterone and nandrolone were the substances most declared on the labels. Package and qualitative chemical analyses (performed on 2818 products) found that 32 percent of the seized products were counterfeit, with an increase in the counterfeit detection rate during the period. Almost half of the fake products did not contain the declared substances, and 28 percent had only non-declared substances. Testosterone and its esters were responsible for 45 percent of the 582 cases of non-declared drug detection. Package analysis alone was responsible for the identification of 5 percent of all counterfeit products. These results indicate the need for a continuous effort by the government aimed at decreasing the availability of these products in the country [13061].
OVERVIEWS OF GENERAL LABORATORY TECHNIQUES

Unification of the screening protocols for a wide range of doping agents has become an important issue for doping control laboratories. This study presents the development and validation of a generic liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) screening method of 241 small molecule analytes from various categories of prohibited substances (stimulants, narcotics, diuretics, beta-2-agonists, beta-blockers, hormone antagonists and modulators, glucocorticosteroids and anabolic agents). It is based on a single-step liquid-liquid extraction of hydrolyzed urine and the use of a rapid-resolution liquid chromatography/high-resolution time-of-flight mass spectrometric system acquiring continuous full scan data. Electrospray ionization in the positive mode was used. Validation parameters consisted of identification capability, limit of detection, specificity, ion suppression, extraction recovery, repeatability and mass accuracy. Detection criteria were established on the basis of retention time reproducibility and mass accuracy. The suitability of the methodology for doping control was demonstrated with positive urine samples. The preventive role of the method was proved by the case where full scan acquisition with accurate mass measurement allowed the retrospective reprocessing of acquired data from past doping control samples for the detection of a designer drug, the stimulant 4-methyl-2-hexanamine, which resulted in re-reporting a number of stored samples as positives for this particular substance, when, initially, they had been reported as negatives [10027].

The PubMed and Google Scholar search engines were used to identify publications addressing various forms of doping, methods employed in their detection, and adverse effects associated with their use. The list of drugs prohibited by the World Anti-Doping Agency (WADA) has grown in the last decade. The newer entries into this list include gonadotropins, estrogen antagonists, aromatase inhibitors, androgen precursors, and selective androgen receptor modulators. The use of mass spectrometry has revolutionized the detection of various compounds; however, challenges remain in identifying newer designer androgens because their chemical signature is unknown. Development of high throughput bioassays may be an answer to this problem. It appears that the use of anabolic steroids continues to be associated with premature mortality (especially cardiovascular) in addition to suppressed spermatogenesis, gynecomastia, and virilization. The attention that androgen abuse has received lately should be used as an opportunity to educate both athletes and the general population regarding their adverse effects. The development of sensitive detection techniques may help discourage (at least to some extent) the abuse of these compounds [10028].

The increasing number of samples and target substances in doping control requires continuously improved screening methods, combining high-throughput analysis, simplified sample preparation, robustness and reliability. The issue of doping in sport is multifaceted. New drugs not only with anabolic properties such as selective androgen receptor modulators, synthetic insulins, blood doping with erythropoietins or homologous and autologous blood transfusions but also with sample manipulation have necessitated sensitive, comprehensive and specific detection assays allowing for the identification of cheats. New methods based on mass spectrometry, flow cytometry and immunological techniques have been introduced and improved in the past years to support and enhance the antidoping fight [08051].

The analysis of sports samples for prohibited substances began in the 1960s and has developed since then using modern technologies close to the latest scientific discoveries. For small molecules, apart from the routine use of GC-MS, the newer techniques include the use of isotope ratio MS to detect testosterone and nandrolone administration and LC-MS/MS
(liquid chromatography-tandem MS) to detect diuretics. For large molecules, several applications of LC-MS/MS are described as well as immunoprocesses for erythropoietin and human growth hormone [08052].

The ability to measure isotope distribution at natural abundance with high accuracy and precision has increased the application of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) to doping control in recent years. GC-C-IRMS is capable of measuring the carbon isotope ratio (delta$^{13}$C) of urinary steroids and confirm their synthetic origin based on the abnormal $^{13}$C content. One tutorial describes some of the complexities encountered by obtaining valid delta$^{13}$C measurements from GC-C-IRMS and the need for careful interpretation of all relevant information concerning an individual's metabolism in order to make an informed decision with respect to a doping violation [08053].

Technical advances are being made in many areas of biotechnology and genetics that are facilitating the detection of doping in sport. These improvements have been catalyzed by the need to counter the ever-increasing sophistication of the community of athletes and their retinues who are intent on the illicit use of physical, pharmacological and genetic tools and methods to enhance athletic performance, in contravention of established international ethical and legal standards and of international treaty. The methods described in one article present a partial and general picture of only some of these advances [12047].

**Olympic laboratories**

Summer and Winter Olympic anti-doping laboratories, accredited by the International Olympic Committee in the past and the World Anti-Doping Agency in the present times, acquire worldwide interest to apply all new analytical advancements in the fight against doping in sports, hoping that this major human event will not become dirty by association with this negative phenomenon. One article summarized the new analytical progresses, technologies and knowledge used by the Olympic laboratories, which for the vast majority of them are, eventually, incorporated into routine anti-doping analysis [12048].

2005 London was awarded the Games of the XXX Olympiad. Following the IOC motto of “Citius, Altius, Fortius”, it was decided that this would be an appropriate strategy to ensure faster analysis with higher sensitivity, and stronger proof in cases of doping. A number of new analytical methods were developed. Faster analysis was obtained with excellent sensitivity using a modified gas chromatography–tandem mass spectrometry (GC-MS/MS) screening method. After extensive development and optimisation, the run-times were reduced from 40 min to 14 min using short capillary GC columns. This GC-MS/MS screen was complemented with an ultra high pressure chromatography (UHPLC) with high resolution mass spectrometry (HRMS) screen. For the first time, and after much research, it was concluded that UHPLC with HRMS could reliably screen over 200 analytes. This needed both positive and negative ionisation modes together with collision induced dissociation with all three MS experiments occurring rapidly so as not to compromise chromatographic performance. Working in full scan mode, the volume of data acquired was enormous and review of the vast amount of data was a challenge. After extensive collaboration with the manufacturer there was a software that will identify the targeted analytes in 2 min after a 10 min acquisition time. Any new designer substance can now be readily searched for since we are working in full scan mode. Furthermore, now that multiple samples are collected from a single athlete over time (the athlete biological passport) it can review such data to search for any change in biomarkers. Indeed, as the electronic files, like the samples, are stored for 8
years, it will now be possible to review the data at a later date to confirm that no prohibited substance had been taken at the time of sample collection. Although the qualitative identification of a foreign substance is all that is required to provide such evidence, for some substances, WADA rules establish a quantitative threshold. This requires the laboratory to quantify the substance with less than a documented uncertainty and establish that the concentration exceeds the reporting threshold. Proving the administration of a pseudo-endogenous substance, i.e. one that is virtually identical to the endogenous substance, is an even more difficult task. Combustion isotope ratio mass spectrometry is used to evidence exogenous administration by identification of a foreign substance [12049].

Practical testing in Brazil

It was summarized the results obtained from the doping control analysis during the period of the 2007 Pan American Games held in Rio de Janeiro, Brazil. Approximately 5600 athletes from 42 different countries competed in the games. Testing was performed in accordance to World Anti-Doping Agency technical note for prohibited substances. One 8 mL urine sample was used for the analysis of five steroid metabolites with two separate analyses by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). Urine samples were submitted to GC/C/IRMS for confirmation analysis to determine the $^{13}$C/$^{12}$C ratio of selected steroids. Fifty-seven urine samples were analyzed by GC/C/IRMS and the delta $^{13}$C values (per thousand) of androsterone, etiocholanolone, 5beta-androstane-3alpha, 17beta-diol (5beta-diol), 5alpha-androstane-3alpha, 17beta-diol (5alpha-diol) and 5beta-pregnane-3alpha, 20alpha-diol (5beta-pdiol), and the endogenous reference compound were presented. One urine sample with a testosterone/epitestosterone (T/E) ratio of 4.7 was confirmed to be positive of doping by GC/C/IRMS analysis. The delta values of 5beta-diol and 5alpha-diol were 3.8 and 10.8, respectively, compared to the endogenous reference compound 5beta-pdiol, which exceeded the WADA limit of 3 per thousand [09046].

The worldwide network of World Anti-Doping Agency (WADA)-accredited anti-doping laboratories plays a fundamental role in supporting the global fight against doping in sport. This role is dependent on the ability to provide accurate, reliable and comparable data in identifying and measuring the presence of prohibited substances and methods. The accredited laboratories participate in WADA's External Quality Assessment Scheme (EQAS) program, which provides the structure to continuously assess and improve laboratory performance in compliance to the requirements of the International Standard for Laboratories and related Technical Documents. The WADA EQAS is comprised of various programs, including a blind EQAS, a double-blind EQAS and an educational EQAS, each with specific goals with regard to monitoring and improving laboratory competence. In this article, the anti-doping rules and processes that govern granting and maintenance of WADA laboratory accreditation, aimed at ensuring a high-quality of laboratory operations within the framework of the global fight against doping in sport, are reviewed [12050].

Transportation

The transportation of urine samples, collected for doping control analysis, does not always meet ideal conditions of storage and prompt delivery to the World Anti-Doping Agency (WADA) accredited laboratories. Because sample collection is not conducted under sterile conditions, microbial activity may cause changes to the endogenous steroid profiles of samples. In one work, funded by WADA, a chemical mixture consisting of antibiotics, antimycotic substances and protease inhibitors was applied in urine aliquots fortified with
conjugated and deuterated steroids and inoculated with nine representative microorganisms. Aliquots with and without the chemical mixture were incubated at 37 degrees C for 7 days to simulate the transportation period, whereas another series of aliquots was stored at -20 degrees C as reference. Microbial growth was assessed immediately after inoculation and at the end of the incubation period. Variations in pH and specific gravity values were recorded. Gas chromatography-mass spectrometry (GC-MS) analysis was performed for the detection of steroids in the free, glucuronide, and sulfate fractions. The addition of the chemical stabilization mixture to urine samples inhibited microorganism growth and prevented steroid degradation at 37 degrees C. On the other hand, four of the nine microorganisms induced alterations in the steroid profile of the unstabilized samples incubated at 37 degrees C [09034].

Quality of doping testing

Large amount of efforts are wasted on anti-doping testing and courts, while the current anti-doping practices are not deem to be infallible or thoughtfully foolproof according to one author. In one article published in the Journal of Applied Physiology, eight human subjects were studied for 7 weeks and treated with recombinant human erythropoietin (rHuEpo) for 4 weeks and a post period of 3 weeks. Urine samples were obtained during all periods and sent to two WADA-accredited laboratories. Whereas one of the two laboratories determined rHuEpo misuse in all subjects during the boosting period, the second laboratory found no misuse, with one sample to be negative, and the remaining seven to be suspicious. More interestingly, while one laboratory found only two of 24 samples to be positive and three to be suspicious during maintenance and post period, the second laboratory found no positive or suspicious samples. As in other areas of healthcare, errors might also occur in laboratory diagnostics. Given the high amount of tests performed every day in clinical laboratories, even a low prevalence still reflects meaningful numbers, harbouring important public health and patient safety implications. For a variety of reasons, false-positive and false-negative results can occur in any area of laboratory diagnostics, including anti-doping testing. One may support the hypothesis that the International anti-doping system might fail or, incidentally, do fail. As such, it might produce contradictory outcomes, because false-negative results may reassure the athletes that some forms of doping are hardly detectable, whereas falsepositive results might lead to adverse clinical, ethical and economical consequences for those found guilty. It is also to mention that the high number of athletes testing positive during anti-doping controls clearly attests that the current strategy is probably ineffective to prevent athletes to dope and modify this otherwise upsetting trend. A more suitable and less expensive strategy might be planned, where identification of abnormal deviations from reference individual values, regardless of pathological or artificial (doping) sources, would allow to follow and target the athlete by an armamentarium of conventional and relatively inexpensive laboratory tests, which are affordable to Governments and healthcare systems, and are also available to vast majority of clinical laboratories [09035].

Blood sampling and blood samples handling

Although not (yet) a frequent doping control specimen, blood samples are advantageous over urine specimens in a doping control context in at least two ways

- they commonly contain the intact drug rather than metabolites, which represents a work-around when new or entirely unknown (designer) compounds are misused and metabolism studies are not (or not publicly) available
they provide information on drug concentrations at the time of sampling, which is of uttermost importance concerning those drugs prohibited in-competition only.

As a consequence, the option to expand doping controls from urine and (less frequently) plasma or serum to whole blood shortly before or after competition was evaluated and assays for the analysis of minimal-invasively collected dried blood spots (DBS) were reported in 2011 and 2012. DBS, created from a volume of 25 microl, were excised from blood collection cards and consecutively extracted into methanol/tert.-butyl-methyl ether and acetone. The combined extracts were concentrated, reconstituted and analyzed on a C-18 UHPLC column (2.1 × 50 mm, 1.9 microm particle size) with 0.2 percent formic acid (solvent A) and acetonitrile (solvent B) connected via ESI to a quadrupole-orbitrap hybrid mass spectrometer. Here, various MS modes were successively used comprising scan-to-scan polarity switching combined with accurate mass full scan MS and target analyte inclusion list (for online single-event product ion scan experiments) as well as all-ion fragmentation. Hence, the combined targeted qualitative and quantitative analysis was possible and data for non-target substances for retrospective evaluation or homology searches based on conserved and common molecular structures were recorded. The model assay included a total of 24 substances covering the prohibited classes S1, S3-S6, S8, S9, and P2, and LODs ranged from 0.05-0.5 ng/mL. Moreover, LOQs were determined for four model substances (tetrahydrocannabinol, cocaine, clenbuterol, and salbutamol) and were found between 0.25 and 2 ng/mL, meeting the required sensitivity to measure physiologically relevant concentrations of these drugs [13012].

Handling of urine

Stability of doping substances in urine

For a correct interpretation of analytical results in doping control, knowledge on the stability of prohibited substances in the urinary matrix is a prerequisite. So far, limited data is available on the stability of prohibited substances in unaltered urine because most of the studies investigating the stability of drugs have used stabilized, sterilized, or filtered urine. In this work, the long-term stability of ephedrine, methylephedrine, cathine, 19-norandrosterone glucuronide, and a wide range of diuretics was determined over a period of 9 months at -20 degrees C, 4 degrees C, 22 degrees C, and 37 degrees C. Short-term stability, including the influence of 6 freeze-thaw cycles and 15 h storage at 60 degrees C, was also investigated. Often, a tolerance limit of 15 percent, similar to what is commonly used in the evaluation of precision data during method validation, is used to evaluate stability. One paper described an alternative approach, using measurement uncertainty data to evaluate long-term stability with a probability of 95 percent, and proposes a simple alternative for investigating the stability for non-threshold substances. The results indicate that all the investigated substances are stable when stored at -20 degrees C and 4 degrees C, but that at higher temperatures significant degradation effects can occur. The study also shows that degradation can be dependent on the urinary matrix and that the results from stability studies using stabilized, filtered, or sterilized urine can underestimate degradation effects [07041].

Transportation of doping control urine samples from the collection sites to the World Anti-doping Agency (WADA) Accredited Laboratories is conducted under ambient temperatures. When sample delivery is not immediate, microbial contamination of urine, especially in summer, is a common phenomenon that may affect sample integrity and may result in misinterpretation of analytical data. Furthermore, the possibility of intentional contamination of sports samples during collection with proteolytic enzymes, masking the abuse of
prohibited proteins such as erythropoietin (EPO) and peptide hormones, is a practice that has already been reported. Consequently, stabilization of urine samples with a suitable method in a way that protects samples’ integrity is important. Currently, no stabilization method is applied in the sample collection equipment system in order to prevent degradation of urine compounds. An overview of a study, funded by WADA, on degradation and stabilization aspects of sports urine samples against the above threats of degradation. Was presented. Extensive method development resulted in the creation of a mixture of chemical agents for the stabilization of urine. Evaluation of results demonstrated that the stabilization mixture could stabilize endogenous steroids, recombinant EPO, and human chorionic gonadotropin in almost the entire range of the experimental conditions tested [11033].

**Diluted urine**

Excessive fluid intake can substantially dilute urinary drug concentrations and result in false-negative reports for drug users. Methods for correction (“normalization”) of drug/metabolite concentrations in urine have been utilized by anti-doping laboratories, pain monitoring programs, and in environmental monitoring programs to compensate for excessive hydration, but such procedures have not been used routinely in workplace, legal, and treatment settings. It was evaluated two drug normalization procedures based on specific gravity and creatinine. These corrections were applied to urine specimens collected from three distinct groups (pain patients, heroin users, and marijuana/cocaine users). Each group was unique in characteristics, study design, and dosing conditions. The results of the two normalization procedures were highly correlated. Increases in percent positives by specific gravity and creatinine normalization were small for heroin users (normally hydrated subjects), modest for pain patients (unknown hydration state), and substantial (2- to 38-fold increases) for marijuana/cocaine users (excessively hydrated subjects). Despite some limitations, these normalization procedures provide alternative means of dealing with highly dilute, dilute, and concentrated urine specimens. Drug/metabolite concentration normalization by these procedures is recommended for urine testing programs, especially as a means of coping with dilute specimens [09036].

**Urinary screening**

The general strategy to perform anti-doping analyses of urine samples starts with the screening for a wide range of compounds. This step should be fast, generic and able to detect any sample that may contain a prohibited substance while avoiding false negatives and reducing false positive results. The experiments presented in one work were based on ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry. Thanks to the high sensitivity of the method, urine samples could be diluted 2-fold prior to injection. One hundred and three forbidden substances from various classes (such as stimulants, diuretics, narcotics, anti-estrogens) were analysed on a C(18) reversed-phase column in two gradients of 9 min (including two 3 min equilibration periods) for positive and negative electrospray ionisation and detected in the MS full scan mode. The automatic identification of analytes was based on retention time and mass accuracy, with an automated tool for peak picking. The method was validated according to the International Standard for Laboratories described in the World Anti-Doping Code and was selective enough to comply with the World Anti-Doping Agency recommendations. In addition, the matrix effect on MS response was measured on all investigated analytes spiked in urine samples. The limits of detection ranged from 1 to 500 ng/mL, allowing the identification of all tested compounds in urine. When a sample was reported positive during the screening, a fast additional pre-confirmatory step was performed to reduce the number of confirmatory analyses [09037].

Lots of the steroids are extensively metabolised in the human body. Thus, knowledge of
urinary excretion is extremely important for the sensitive detection of steroid misuse in doping control. The methods routinely used in steroid screening mainly focus on substances, that are excreted unconjugated or as glucuronides. Common procedures include deconjugation using a beta-glucuronidase enzyme. Following extraction and concentration the analytes are submitted to LC-MS/(MS) analysis and/or GC-MS/(MS) analyses. Besides the classical steroids, more and more products appear on the market for "dietary supplements" containing steroids that have never been marketed as approved drugs, mostly without proper labelling of the contents. To cover the whole range of potential products comprehensive screening tools have to be utilised in addition to the classical methods. Endogenous steroids, e.g. testosterone, represent a special group of compounds. As classical chemical methodology is incapable of discriminating synthetic hormones from the biosynthesised congeners, the method of steroid profiling is used for screening purpose. Additionally, based on isotope signatures a discrimination of synthetic and natural hormones can be achieved [09038].

Direct injection of urine

Direct injection of urine has gained interest in the field of analytical toxicology, including doping control analysis. However, implementation of a direct urinalysis method for the LC-MS/MS detection of 34 diuretics and 9 other doping agents yielded several analytical problems, which were not observed using a traditional liquid-liquid extraction. Therefore a comparative study was made between liquid-liquid extraction and direct injection. Comparison of validation results showed that the liquid-liquid extraction at pH 7 allows to analyze samples without major drawbacks regarding matrix effects. Hence, good sensitivity was observed and detection limits ranged between 1 and 250 ng/mL for all compounds. In the direct injection approach shifted retention times were observed for several acidic and basic compounds due to unwanted matrix effects. This shift was reduced by a 25-fold dilution of the urine samples. Besides the improved retention time stability the diluted samples also exhibited lower ion suppression than the undiluted ones. After 25-fold dilution, detection limits ranged between 10 and 250 ng/mL for all compounds. Since these detection limits are at or below the minimum required performance level, imposed by the World Anti-Doping Agency, the method could be applied to routine anti-doping analysis. Samples, previously declared positive, were reanalysed using both the liquid-liquid extraction and direct injection. With both techniques all 26 samples were found to be positive, showing the applicability of direct injection for the analysis of diuretics [09039].

Proteases in doping control analysis.

Urine manipulation in sports drug testing has become a serious problem for doping control laboratories, and recent scandals in elite endurance sports have revealed the problem of urine manipulation presumably using proteases, which will impede the detection of drugs such as erythropoietin (EPO) or other peptide hormones. Using commonly accepted analytical strategies, a protocol was developed enabling the determination of elevated protease activities in doping control specimens followed by the visualization of protein degradation and identification of proteases such as chymotrypsin, trypsin and papain. Therefore, protease detection kits based on fluorescein isothiocyanate-labeled casein were employed, and protease concentrations greater than 15 microg/mL of urine entailed subsequent 1-dimensional gel electrophoretic visualization of urinary proteins. The presence of 20 microg of proteases per mL of urine caused a complete degradation of proteins usually observed in urinary matrices ("trace of burning"), while respective proteases were still detected in spiked urine samples after 10 days of storage at + 4 and - 20 degrees C. Identification of target proteases at respective molecular weights was accomplished using bottom-up sequencing approaches based on in-gel digestion of separated enzymes followed
Forensic toxicology

Forensic toxicology has developed as a forensic science in recent years and is now widely used to assist in death investigations, in civil and criminal matters involving drug use, in drugs of abuse testing in correctional settings and custodial medicine, in road and workplace safety, in matters involving environmental pollution, as well as in sports doping. Drugs most commonly targeted include amphetamines, benzodiazepines, cannabis, cocaine and the opiates, but can be any other illicit substance or almost any over-the-counter or prescribed drug, as well as poisons available to the community. The discipline requires high level skills in analytical techniques with a solid knowledge of pharmacology and pharmacokinetics. Modern techniques rely heavily on immunoassay screening analyses and mass spectrometry (MS) for confirmatory analyses using either high-performance liquid chromatography or gas chromatography as the separation technique. Tandem MS has become more and more popular compared to single-stage MS. It is essential that analytical systems are fully validated and fit for the purpose and the assay batches are monitored with quality controls. External proficiency programs monitor both the assay and the personnel performing the work. For a laboratory to perform optimally, it is vital that the circumstances and context of the case are known and the laboratory understands the limitations of the analytical systems used, including drug stability. Drugs and poisons can change concentration postmortem due to poor or unequal quality of blood and other specimens, anaerobic metabolism and redistribution. The latter provides the largest handicap in the interpretation of postmortem results [10029].

Testing in famous cases

Katrin Krabbe et al

Manipulation of urine sampling in sports drug testing is considered a violation of anti-doping rules and is consequently sanctioned by regulatory authorities. In 2003, three identical urine specimens were provided by three different athletes, and the identity of all urine samples was detected and substantiated using numerous analytical strategies including gas chromatography-mass spectrometry with steroid and metabolite profiling, gas chromatography-nitrogen/phosphorus detector analysis, high-performance liquid chromatography-UV fingerprinting, and DNA-STR (short tandem repeat) analysis. None of the respective athletes was the donor of the urine provided for doping analysis, which proved to be a urine sample collected from other unidentified individual(s). Samples were considered suspicious based on identical steroid profiles, one of the most important parameters for specimen individualization in sports drug testing. A database containing 14,224 urinary steroid profiles of athletes was screened for specific values of 4 characteristic parameters (ratios of testosterone/epitestosterone, androsterone/etiocholanolone, androsterone/testosterone, and 5alpha-androstane-3alpha,17beta-diol/5beta-androstane-3alpha,17beta-diol) and only the three suspicious samples matched all criteria. Further metabolite profiling regarding indicated medications and high-performance liquid chromatography-UV fingerprinting substantiated the assumption of manipulation. DNA-STR analyses unequivocally confirmed that the 3 urine samples were from the same individual and not from the athletes who provided DNA from either buccal cell material or blood specimens. This supportive evidence led to punishment of all three athletes according to the rules of the World Anti-Doping Agency. Application of a new multidisciplinary strategy employing
common and new doping control assays enables the detection of urine substitution in sports drug testing [07043].

**Lance Armstrong**

One article examined the metabolic performance of an elite cyclist, Lance Armstrong, before and after his diagnosis with testicular cancer. Although a champion cyclist in 1-day events prior to his diagnosis of testicular cancer at age 25, he was not a contender in multi-day endurance cycle races such as the 3-week Tour de France. His genetic makeup and physiology (high VO$_{2\text{max}}$, long femur, strong heavy build) coupled with his ambition and motivation enabled him at an early age to become one of the best 1-day cyclists in the world. Following his cancer diagnosis, he underwent a unilateral orchiectomy, brain surgery and four cycles of chemotherapy. After recovering, he returned to cycling and surprisingly excelled in the Tour de France, winning this hardest of endurance events 7 years running. This dramatic transformation from a 1-day to a 3-week endurance champion has led many to query how this is possible, and under the current climate, has led to suggestions of doping as to the answer to this metamorphosis. Physiological tests following his recovery indicated that physiological parameters such as VO$_{2\text{max}}$ were not affected by the unilateral orchiectomy and chemotherapy. It was proposed that his dramatic improvement in recovery between stages, the most important factor in winning multi-day stage races, is due to his unilateral orchiectomy, a procedure that results in permanent changes in serum hormones. These hormonal changes, specifically an increase in gonadotropins (and prolactin) required to maintain serum testosterone levels, alter fuel metabolism; increasing hormone sensitive lipase expression and activity, promoting increased free fatty acid (FFA) mobilization to, and utilization by, muscles, thereby decreasing the requirement to expend limiting glycogen stores before, during and after exercise. Such hormonal changes also have been associated with ketone body production, improvements in muscle repair and haematocrit levels and may facilitate the loss of body weight, thereby increasing power to weight ratio. Taken together, these hormonal changes act to limit glycogen utilization, delay fatigue and enhance recovery thereby allowing for optimal performances on a day-to-day basis. These insights provide the foundation for future studies on the endocrinology of exercise metabolism, and suggest that Lance Armstrong's athletic advantage was not due to drug use [07044].

**Parallel investigations of saliva and urine**

Stimulants are banned by the World Anti-Doping Agency (WADA) if used “in competition”. Being the analysis of stimulants presently carried out on urine samples only, it might be useful, for a better interpretation of analytical data, to discriminate between an early intake of the substance and an administration specifically aimed to improve the sport performance. The purpose of one study was to investigate the differences, in terms of excretion/disappearance of drugs, between urine and oral fluid, a sample that can reflect plasmatic concentrations. Oral fluid and urine samples were collected following oral administration of the following stimulants: modafinil (100 mg), selegiline (10 mg), crotetamide/cropropamide (50 mg each), pentetrazol (100 mg), ephedrine (12 mg), sibutramine (10 mg), mate de coca (a dose containing about 3mg of cocaine); analysis of drugs/metabolites was carried out by gas chromatography/mass spectrometry (GC/MS) in both body fluids. The results show that both the absolute concentrations and their variation as a function of time, in urine and in oral fluid, are generally markedly different, being the drugs eliminated from urine much more slowly than from oral fluid. The results also suggest that the analysis of oral fluid could be used to successfully complement the data obtained from urine for “in competition” anti-doping tests; in all those cases in which the metabolite(s)
concentration of a substance in urine is very low and the parent compound is not detected, it is indeed impossible, relying on urinary data only, to discriminate between recent administrations of small doses and remote administrations of higher doses [07045].

**Effects of sample storage condition on salivary hormones**

Measurement of steroid hormones in saliva is increasingly common in elite sport settings. However, this environment may enforce handling and storage practices that introduce error in measurement of hormone concentrations. It was assessed the influence of storage temperature and duration on reproducibility of salivary steroid levels. Nine healthy adults provided morning and afternoon saliva samples on two separate occasions. Each sample was divided into identical saliva aliquots which were stored long-term (i.e. 28 and 84 days) at -80°C or -20°C (testing day 1), and short-term (i.e. 1, 3, 7 and 14 days) at 4°C or 20°C (testing day 2). Samples were analyzed for cortisol, testosterone and estradiol using ELISA. In non-freezer conditions, there was a decrease from baseline to 7 days in testosterone (-26 ± 15 %) and estradiol (-58 ± 17 %) but not cortisol concentrations. This decrease was larger in samples stored at room temperature than in the refrigerator. There were small but significant changes in measured concentrations of all hormones after 28 and/or 84 days of storage in freezer conditions, but these were generally within 12 percent of baseline concentrations, and may be partly explained by inter-assay variability. Whole saliva samples to be analyzed for cortisol, testosterone and estradiol should be frozen at -20°C or below within 24 h of collection, and analyzed within 28 days. Storage of samples for measurement of testosterone and estradiol at temperatures above -20°C can introduce large error variance to measured concentrations [13113].

**Non-approved substances**

Since 2011, this category (S0) of banned substances has been a part of WADA’s prohibited list and encompasses a virtually infinite number of compounds currently not covered by any of the other sections (e.g. anabolic agents, peptide hormones, growth factors and related substances). New representatives of this class of compounds are low molecular weight luteinizing hormone (LMWLH) receptor agonists, the characterization and identification of which was presented. Focusing on two series of drug candidates based on either pyrazole or thienopyrimidine core structures, two model substances were synthesized and used to establish a targeted/non-targeted screening method employing both diagnostic precursor-product ion pair detection and precursor ion scanning. In the absence of metabolism study data, the presence of the intact drug or at least a conserved nucleus must be present to allow the detection using the proposed strategy [13012].

**Screening methods**

A comprehensive screening method for the detection of prohibited substances in doping control is described and validated. This method is capable of detecting over 150 components mentioned on the list of the World Anti-Doping Agency including anabolic androgenic steroids, stimulants and all narcotic agents that are currently analysed using different analytical methods. The analytes are extracted from urine by a combined extraction procedure using freshly distilled diethyl ether and tert-butyl methyl ether as extraction solvents at pH 9.5 and 14 respectively. Prior to GC-MS analysis the residues are combined and derivatised using a mixture of N-methyl-N-trimethylsilyl trifluoroacetamide, NH₄I and ethanethiol. The mass spectrometer is simultaneously operated in the full scan mode (mass
range varies along with GC-oven temperature program) and in the selected ion monitoring mode. Besides narcotics, stimulants and anabolic androgenic agents, this method is also capable of detecting several agents with anti-estrogenic activity and some beta-agonists. This comprehensive screening method reduces the amount of urine needed and increases the sample throughput without a loss in sensitivity and selectivity [08054].

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A simple and rapid multicomponent screening method of 130 substances for direct injections of urine samples has been developed. The fully automated method based on ultra-performance liquid chromatography (UPLC) and tandem mass spectrometry (MS/MS) is used for three different classes of doping agents: diuretics, central nervous system stimulants and opiates. The samples are diluted with buffer containing internal standards (IS) by a pipetting robot system into 96-well plates. Samples are injected on a reversed phase sub 2-microm particle column connected to a fast polarity switching and rapid scanning tandem mass spectrometer with an electrospray interface. The software used to evaluate the results produced reports containing a small-sized window for each component and a data table list with flags to indicate any adverse analytical findings in the sample. The report can also be processed automatically using an application software, which interpret the data and indicate if there is a suspicious sample. One 96-well plate can be analyzed within 16 hours [08056].

A new doping control screening method has been developed, for the analysis of doping agents in human urine, using HPLC/orbitrap with in-source collision-induced dissociation and atmospheric pressure chemical ionization. The developed method allows the detection of 29 compounds, including agents with antiestrogenic activity, beta₂ agonists, exogenous anabolic steroids, and other anabolic agents. The mass accuracy of this method is better at 2 ppm using an external reference. The detection limit for all compounds tested was better than 100 pg/ml. The recoveries of most analytes were above 70 percent. The measured median repeatability values for doping agents included in the method at concentrations of 1 and 10 ng/ml were 21 and 17 percent, respectively. The relative standard deviation (RSD) of the intraday precision (n=6) ranged from RSD 16-22 percent, whereas the interday precision (n=18), ranged from RSD 17-26 percent, depending on the solute concentration investigated [08057].

One paper presented a general screening method, based on liquid chromatography/mass spectrometry (LC/MS), for the simultaneous detection in human urine of 72 xenobiotics (21 diuretics, 16 synthetic glucocorticoids, 17 beta-adrenergic drugs, 10 stimulants, 5 anti-oestrogens and 3 anabolic steroids), excreted free or as glucuro-conjugates in urine.
Although the method has been specifically designed and evaluated in view of its potential application to anti-doping analyses, it can also be effective in other areas of analytical toxicology. Sample preparation was based on two liquid/liquid separation steps (performed at alkaline and at acid pH, respectively) of hydrolyzed human urine, and then an assay by LC/MS-MS in positive and negative ionization mode using an electrospray ionization source (ESI) and multiple reaction monitoring as the acquisition mode. The overall time needed for an LC run was less than 15 minutes. All compounds showed good reproducibility in terms of both the retention times (CV %<1) and the relative abundances of the diagnostic transitions (CV %<10). The limits of detection were in the range of 1-50 ng/mL for glucocorticoids, anti-oestrogens and steroids, and 50-500 ng/mL for diuretics, beta-adrenergic drugs and stimulants, thus satisfying the minimum required performance limits (MRPL) set by the World Anti-Doping Agency for the accredited anti-doping laboratories [08058].

**Multi-analyte testing**

Traditionally, doping control analytical assays have been drug-class dedicated and tailored to address requirements concerning sample preparation and chromatography/mass spectrometry resulting from specific physicochemical properties of target compounds. Improved analytical instrumentation (particularly based on liquid chromatography-(tandem) mass spectrometry, LC-MS/(MS)), have enabled the development of numerous cost-effective and rapid alternatives, allowing for multi-class/multi-analyte test methods. The trend towards comprehensive and preferably combined targeted/non-targeted screening procedures has been motivated in part in the requirement for analytical approaches to meet the minimum required performance levels (MRPLs) stipulated by WADA. Within 2012, several LC-MS/(MS)-based approaches were published representing options to complement or expand the currently employed methodologies of doping control laboratories. Employing targeted multiple-reaction monitoring (MRM), the detection of a total of 61 analytes (plus two internal standards) from urine covering seven classes of prohibited substances (S1–S7) and one agent categorized under M1 was reported. The apparatus employed consisted of a conventional LC equipped with a C-18 reversed-phase (RP) analytical column (2 × 50 mm, 3 μm particle size) interfaced to a triple-quadrupole MS (QqQ) via electrospray ionization (ESI), operated with scan-to-scan polarity switching. Urine samples were prepared for analysis by the addition of two internal standards. An aliquot of 5 μl was injected into the LC-MS/MS system. Gradient elution was conducted with 5 mM ammonium acetate (pH 3.5, adjusted with acetic acid, solvent A) and acetonitrile (solvent B), completing a single run within 10.75 min. For all target compounds, limits of detection (LODs) were far below the aforementioned MRPLs. The unique feature of this assay compared to other multi-analyte screening methods is the capability to detect polysaccharide-derived plasma volume expanders (e.g. hydroxyethyl starch and dextran) by combined in-source dissociation and subsequent MRM of diagnostic breakdown products was described. Another approach covering 62 analytes (plus two internal standards) and five classes of prohibited substances (S1, S3, S4, S9, and P2) was reported. Urine samples are enzymatically hydrolyzed and the liberated phase-I-metabolites (or intact drugs) are extracted into a mixture of pentane and diethylether. After evaporation to dryness and reconstitution, LC-MS/MS is conducted on two different systems, both of which use 5 mM ammonium acetate (solvent A) and acetonitrile (solvent B). Assay 1 is dedicated to the analytes of the categories S1, S3, S7, and S9 and utilizes gradient elution on a C-12 HPLC column (2 × 50 mm, 4 microm particle size) with a short overall run time of 4 min. Assay 2 aims at the detection of substances of the category S4 and employs isocratic chromatography on a C-8 HPLC column (2 × 150 mm, 5 microm particle size) at 70 percent solvent A, requiring a total run time of approximately 6 min. Mass spectrometry is conducted in both cases with QqQ instruments operated with positive ESI and MRM; unfortunately, no information on LODs is provided. In one study by, the value of hydrophilic interaction liquid chromatography (HLIC) tandem mass spectrometry compared
to the commonly used, reversed-phase chromatography (and mass spectrometry) was investigated and various options concerning column temperature, solvent composition, and stationary phase material were evaluated. Eventually, the use of a 2.1 × 150 mm HILIC column (5 µm particle size) operated at 35°C with 5 mM ammonium acetate (pH 4.5, eluent A) and acetonitrile (eluent B) was considered optimal to analyze 6, 17, 4, and 17 drugs belonging to the categories S3, S6, S7, and P2, respectively. Urine samples were prepared for analysis by liquid-liquid extraction (LLE), the extract was concentrated and analyzed by gradient elution on the above mentioned HILIC system followed by ESI in positive mode and subsequent MRM detection in a single run of 14 min. The estimated LODs sufficiently met WADA's MRPLs and the method's fitness-for-purpose was demonstrated with the required validation process; it remains to be clarified however if omitting any hydrolysis compromises the detection capability concerning agents largely excreted as conjugates. An assay enabling the determination of 23 diuretics (S5) and 23 stimulants (S6) from a single urine extract was described employing solid-phase extraction (SPE) of 1 ml of urine. The LC used in this study was equipped with a C-18 HPLC column (2.1 × 50 mm, 3 microm particle size) and employed 0.2% formic acid (eluent A) and methanol (containing 0.2% formic acid, eluent B) for gradient elution. In contrast to earlier methods, two separate injections for positive and negative ESI-MS/MS (in MRM mode) were required at run times of 17 and 16 min, respectively, necessitating the non-competitive overall measurement time per sample of 33 min. Moreover, only one internal standard that is preferably ionized in positive mode was apparently used, which is questionable when two separate analyses are conducted. The procedure was validated according to applicable guidelines and LODs were accomplished satisfying WADA's requirements. Consequently, the methodology might be fit-for-purpose if the sample/instrument ratio and thus required analysis and reporting turn-around times are met. While these assays are all designed to specifically measure a multitude of target compounds with dedicated precursor-/product-ion pairs and thus gate out all other information (for the advantage of sensitivity and speed), a trend towards combined targeted/non-targeted analytical methods has been recognized over the last few years. Here, particularly LC-MS(ESI/MS) approaches with high resolution/high accuracy mass analyzers such as time-of-flight (TOF) and orbitrap as well as hybrids consisting of quadrupole or ion trap mass selective devices and TOF or orbitraps have been used for a variety of reasons comprehensively summarized and reviewed in recent articles. The benefit of analytical information being recorded in utmost extent (limited essentially only by sample preparation and/or ionization capability) has been especially recognized and appreciated. Ionization capability was subject to investigation in the development of a complementary LC-high resolution/high accuracy MS (LC-HRMS) method in 2012. Using the so-called wrong-way-round ionization, a total of 137 analytes belonging to the prohibited substance classes S1, S3, S4-S7, S9, and P2 were measured in a single run (17 min) with positive ESI and HRMS. The LC consisted of a conventional C-18 RP ultrahigh performance liquid chromatography column (UHPLC, 2.1 × 50 mm, 1.7 µm particle size) operated under alkaline conditions with 3 mM ammonium hydroxide (pH = 10.3, solvent A) and 90% methanol (containing 3 mM ammonium hydroxide, solvent B). Despite the use of positive ESI, the alkaline milieu supported the generation and sensitive detection of protonated molecular species, adduct or product ions (hence "wrong-way-round" ionization) on an LTQ-Orbitrap mass spectrometer in full scan mode (m/z 100-650, 60,000 resolution@400 Da). Prior to analysis, urine samples underwent enzymatic hydrolysis and LLE and all target compounds were detected in this initial test method below respective MRPLs. Although not explicitly discussed, the presented assay should allow retrospective data evaluation concerning compounds that possess similar physicochemical properties as the ones tested. Covering 120 target analytes (34 diuretics, 83 stimulants, and 3 other analytes), the utility of a benchtop orbitrap mass analyzer for the combined targeted/non-targeted analysis of drugs relevant for doping controls was also presented. Following a ten-fold urine dilution (with addition of two internal standards), chromatography was conducted by means of a C-8 UHPLC column (2.1 × 50 mm, 1.8 microm.
particle size) and 1 mM ammonium acetate/0.001 percent acetic acid (solvent A) and 1 mM ammonium acetate/0.001 percent acetic acid in methanol (solvent B). Gradient elution was used yielding an overall run time of 10 min and the effluent was directed via ESI with scan-to-scan polarity switching to the orbitrap analyzer. The detector was operated in full scan mode (m/z 100–2000, 50.000 resolution@200 Da), and with the exception of glycerol, all analytes were detected at LODs between 5 and 500 ng/ml, thus fulfilling the MRPLs stipulated by WADA. Although included in the study, no further information on the capability to determine glycerol at (or below) the suggested threshold of 200 microg/ml was given. Also here it is worth mentioning that the generated and recorded data enable retrospective data mining, facilitating follow-up or prevalence studies concerning newly observed or potential future prohibited substances [13012].

Automation of sample preparation procedures in a doping control laboratory is of great interest due to the large number of samples that have to be analyzed, especially in large events where a high throughput protocol is required to process samples over 24 h. The automation of such protocols requires specific equipment capable of carrying out the diverse mechanical tasks required for accomplishing these analytical methodologies, which include pipetting, shaking, heating, or crimping. An automated sample preparation procedure for the determination of doping-related substances by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis, including enzymatic hydrolysis, liquid-phase extraction and derivatization steps, was developed by using an automated liquid handling system. One paper presents a description of the equipment, together with the validation data for 72 doping-related compounds including extraction efficiency, evaluation of carry-over, interferences, and robustness. Validation was approached as a comparison between the results obtained using the manual protocol and the transferred automated one. The described methodology can be applied for sample preparation in routine anti-doping analysis with high sample throughput and suitable performance [13085].

During the past five years, various multi-class and multi-analyte test methods have been developed for sports drug testing purposes, fuelled by the increasing demand of incorporating more and more substances (and respective metabolites) into routine doping controls without sacrificing the sample volume, faster turnaround times (particularly in case of great sport events), as well as the capability of modern mass spectrometers to provide the required scan speed and/or resolving power to cover hundreds of analytes per analytical run. Its fitness-for-purpose was impressively demonstrated by its use during the 2012 Olympic Games in London, where over 5000 samples were analyzed within a period of one month. Moreover, the generated data allow for the nowadays frequently requested re-evaluation concerning additional compounds and metabolites that were not screened for at the time of the Games [13009].

Aiming at faster and/or simpler analytical approaches for drug testing in general, the potential utility of evolving ambient mass spectrometry techniques has continued to be discussed. In proof-of-concept studies, the analyses of drugs of abuse and occasionally doping agents by means of, for example, desorption electrospray ionization (DESI), extractive electrospray ionization (EESI), or electric discharge-based methodologies such as direct analysis in real time (DART) were presented. Despite their ease-of-use and rapid generation of results, the lack in comprehensiveness and capability to allow for the often required separation of isomeric compounds, which is often required in sports drug testing, has been a major obstacle in introducing these techniques in routine doping controls. Similarly, the undisputed swiftness of matrix-assisted laser desorption ionization (MALDI)-MS/(MS) in drug detection in biological matrices has so far not been considered a viable means for sports drug testing.
mainly due to the limitations resulting from omitting chromatographic separation of analytes and, in selected cases, insufficient detection limits [13009].

"Alternative" specimens

The use of alternative specimens in the field of toxicology was first described in 1979, when hair analysis was used to document chronic drug exposure. Since then, the use of this "alternative" samples has gained tremendous importance in forensic toxicology, as well as in clinic toxicology, doping control and workplace drug testing. It is not surprising, therefore, that a large number of papers dealing with the determination of several classes of drugs in saliva, sweat, meconium and hair have been published ever since, owing to the fact that chromatographic equipment is becoming more and more sensitive, mass spectrometry (and tandem mass spectrometry) being the most widely used analytical tool, combined with gas or liquid chromatography. "Alternative" specimens present a number of advantages over the traditional samples normally used in toxicology (e.g. blood, urine and tissues), namely the fact that their collection is not invasive, their adulteration is difficult, and they may allow increased windows of detection for certain drugs. The main disadvantage of this kind of samples is that drugs are present in very low concentrations, and therefore high-sensitivity techniques are required to accomplish the analysis [08059].

Drug identification

Except for proteins, such as EPO, most prohibited drugs are identified by GC-MS, the workhorse of doping-control laboratories. LC-MS is used increasingly for diuretics, some anabolic steroids, and corticosteroids. Doping-control scientists identify a substance, in the laboratory and in court, by matching chromatographic retention time and mass spectra between unknown and standard. They need an authentic reference standard – a sample of the substance, certified to be correct. The standard may be a white powder or an excretion urine from a volunteer who took the drug. Chromatography coupled with MS makes it possible to identify not just drug classes, but specific chemicals, with absolute certainty. Pharmaceuticals include some synthetic compounds that do not occur naturally (e.g. the anabolic steroid stanozolol) and some that do (e.g. testosterone). Unfortunately, GC-MS and LC-MS cannot distinguish natural, endogenous testosterone from pharmaceutical, exogenous testosterone; however, normal human urine samples contain a testosterone isomer with no known function, epitestosterone. The urinary ratio of testosterone to epitestosterone (T/E ratio) is roughly 1:1 in most normal men, and it increases upon testosterone administration. Since the 1984 Olympics, the T/E ratio has been used to screen for testosterone use. Adverse analytical findings are defined by a T/E cut-off, which currently is 4. The two problems with any cut-off are that rare, drug-free individuals might have a naturally elevated T/E and that T/E may never exceed the cut-off in some users, either because their T/E is not responsive to administration or because they use small doses and titrate themselves. To distinguish users from nonusers, longitudinal profiling consists of plotting T/E and other urinary androgen parameters over time, expecting stability for nonusers and a spike for users. In the 1990s a new approach was introduced: isotope ratio MS (IRMS) [07046].

Blood tests

Serum can be tested by LC-MS-MS to detect hemoglobin-based oxygen carriers and by immunoassay to detect recombinant human growth hormone (GH). Natural GH is a family of isoforms, including a major one of 22 kd (22,000 atomic mass units) and some non–22-kd
isoforms, whereas recombinant GH is 100 percent 22-kd isoforms. Administration of recombinant GH suppresses endogenous GH production. The current approach to recombinant GH detection in serum is based on estimating the ratio of the 22-kd isoform to non–22-kd isoforms by immunoassay; it can detect administration for 3 hours after the last dose [07046].

Laboratory report interpretation

The laboratory urine drug test can determine what substance is present in the urine sample, not the brand, formulation, route of administration, dose, or how long before urine collection the drug was taken. Reasons why a urine drug test is negative include the drug is not prohibited by this program; the drug was never used; the drug was used long enough ago to have been eliminated completely; the drug is present below the cut-off; the drug is present below the limit of detection of the test; the drug is a prohibited (designer) drug that the laboratory does not look for; the sample was manipulated; and the sample was not real urine. The latter can be revealed by steroid screen data devoid of natural steroids in cases that would be missed by commercial adulteration tests and dipsticks. Many factors determine test retrospectivity, or how long after the end of administration the test can detect the drug in urine: among them is the dose, body burden, elimination pharmacokinetics, and test sensitivity. Anabolic steroids can be detected for as little as only a few days or as long as many months after the user stops taking them, depending on the type used (e.g. short-acting pill or long-acting oily injection), how much was used, and for how long. In addition, some steroids are easier to detect than others because of chemical differences. Individuals who have been in a drug-testing program for some time are less likely to use long-acting, easy-to-detect steroids.

<table>
<thead>
<tr>
<th>Prohibited drug</th>
<th>Period of detectability after the last dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulants</td>
<td>A few hours to a few days</td>
</tr>
<tr>
<td>Anabolic steroid</td>
<td>A few days (short acting, water soluble, small doses) to many months (long-acting oily injections, large dose)</td>
</tr>
<tr>
<td>Diuretics</td>
<td>A few hours to a few days</td>
</tr>
<tr>
<td>Marijuana</td>
<td>Some weeks</td>
</tr>
<tr>
<td>rEPO</td>
<td>A few days</td>
</tr>
</tbody>
</table>

The test results on a follow-up sample collected some times after an initial, positive sample needs to be interpreted in light of the above. If the follow-up test is positive for the same drug, it may be because the drug was not completely eliminated yet or because the athlete used the drug again in the meantime. Comparing the laboratory data from both tests may or may not provide an indication of which is the case. The follow-up test is expected to be negative if the drug was eliminated completely. This is why a negative follow-up test is not relevant to determining the accuracy or inaccuracy of a positive result on a sample collected previously. Conversely, a negative follow-up test is a valid check that the athlete has stopped using the drug. Drug users who expect to be tested at events try to time their discontinuation to pass the test; this is why no-notice, out-of-competition testing was implemented in the 1980s [07046].

Accuracy of testing

It is said that the test is blind to designer steroids, because the test is targeted and finds only
what it looks for. Typically, WADA-accredited laboratories screen for most anabolic steroids by GC-MS in the more sensitive SIM mode, monitoring only a few ions per target compound (e.g. an ion of 415 atomic mass units). A designer steroid could differ from a known one by only two extra hydrogens, give an ion of 417 atomic mass units upon fragmentation, and escape detection because the test monitors 415, not 417. Or the designer steroid could fragment to ions that happen to be monitored, in which case data readers would see suspicious signals and investigate further. The first reported designer steroid (norbolethone) was a pharmaceutical abandoned decades before, during clinical trials. It resurfaced upon further investigation of an athlete’s urine sample devoid of normal endogenous androgens, a telltale sign of endocrine suppression, which is expected after androgen administration because of negative feedback. The second designer steroid (THG) was discovered because a coach turned in a used syringe. THG simply is not detected in the standard steroid screen, probably because its chemical properties are such that it disintegrates along the way. Different modifications of the screen now allow its detection [07046].

Are the tests accurate? What are the risks of “false positive” or “false negative”? Both phrases can have widely different meanings in common language compared with antidoping jargon. In common language, a “false positive” might be any adverse analytical finding that does not result in a sanction, perhaps because the athlete had a therapeutic use exemption, because a courier’s signature was missing on a shipping document, or because the prohibited drug was a supplement contaminant. A case in which on appeal, an arbitrators’ panel had purely legal reasons to exonerate the athlete, might casually be called a “false positive.” But for the laboratory, a false positive is only the case in which the laboratory reports the presence of a drug and it is later proven scientifically that the drug was not present. The same misinterpretation or mis-semantic is for a “false negative,” in common language that might be a case where the athlete used a drug but passed the test. This could be because the metabolite was accurately detected just below the cut-off – a perfectly accurate negative result [07046].

**Chromatography**

Chromatography is an analytical chemistry technique used to separate (resolve) the chemical compounds in a mixture. Gas chromatography (GC) is done in the gas phase. A gas chromatograph has three parts: a sample introduction system (injector), an oven containing a chromatography column to achieve separation, and a detector. Typically, a microliter of liquid urine extract is automatically injected into the injector, a chamber at a high temperature. The sample is vaporized and swept along a hair-thin glass tube (capillary column, many meters long, flexible enough to be rolled up in a coil) by a carrier gas (mobile phase), such as helium. Different compounds travel at different speeds because of the differences in boiling point, polarity, and relative solubility in the carrier gas versus the coating of the inner wall of the column (stationary phase). The compounds emerge from the column outlet at different times after injection (the chromatographic retention time), separated from each other. Under identical operating conditions, the retention time is characteristic of each chemical compound. If two compounds have the same retention time, they may be identical (e.g. testosterone). If two compounds have different retention times, they certainly are different (e.g. testosterone and methyltestosterone). Matching retention times between an unknown and a reference standard is one element of identification. A graph of the amount of substance as a function of the retention time is a chromatogram. Two common GC detectors in antidoping laboratories are the nitrogen-phosphorus detector (NPD) and the mass spectrometer. The NPD detector is ideal for detecting nitrogen-containing compounds, such as stimulants [07046].
Mass spectrometry

One review introduced fundamental aspects of mass spectrometry (MS) based proteomics and illustrates how MS is an effective tool for the analysis of glycoprotein hormones. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and electrospray ionization (ESI) MS are complementary approaches that have been applied for the analysis of gonadotropins, e.g. to characterize differences in the oligosaccharide distribution of commercial human chorionic gonadotropin preparations, for isolated nicked beta-subunit, and identification of a metabolite of placental transforming growth factor in pharmaceutical hCG preparations. Immunoaffinity trapping and concentration of digested sample extract prior to MS analysis confers analytical sensitivity akin to immunoassay. A desirable objective would be to develop for clinical purposes a rapid procedure for MS detection and characterization of gonadotropins. Refinement of on-target immobilization and digestion for subsequent ionization by MALDI may eventually help to provide this capability. The advent of hybrid mass spectrometers will further advance the characterization of these complex molecules [06036].

Mass spectrometry (MS) is an analytical chemistry technique used for structure elucidation of unknowns or identification of known compounds. A mass spectrometer has three parts: an ion source where the compound is ionized to form a molecular ion and fragmented into smaller ions; a mass filter that separates ions by mass-to-charge ratio (m/z); and a detector. The graph of ion abundance as a function of m/z is a mass spectrum. The fragmentation pattern is determined by weak bonds and other physicochemical characteristics; therefore, fragmentation is reproducible and characteristic of the molecular structure, and the mass spectrum is like a fingerprint of the compound. Matching mass spectra between an unknown and a reference standard is another element of identification. Significant ions are so characteristic that matching only three ions (e.g. 143, 345, 360) and their percent abundance relative to the most intense of the three (e.g 143) has long been widely accepted as proof of chemical identification [07046].

The identification power of mass spectrometry has enabled the determination of hundreds of prohibited drugs in doping-control analysis. A few years ago, its utility was extended to peptide hormones such as erythropoietins, synthetic insulins and corticotrophins detectable in blood or urine. New assays have been established to improve the fight against doping, employing highly selective and sensitive detection methods based on chromatographic and tandem mass spectrometric techniques [07047].

Owing to the sensitive, selective, and unambiguous nature of mass spectrometric analyses, chromatographic techniques interfaced to various kinds of mass spectrometers have become the most frequently employed strategy in the fight against doping. To obtain utmost confidence in analytical assays, mass spectrometric characterization of target analytes and typical dissociation pathways have been utilized as basis for the development of reliable and robust screening as well as confirmation procedures. Methods for qualitative and/or quantitative determinations of prohibited low and high molecular weight drugs have been established in doping control laboratories preferably employing gas or liquid chromatography combined with electron, chemical, or atmospheric pressure ionization followed by analyses using quadrupole, ion trap, linear ion trap, or hyphenated techniques. The versatility of modern mass spectrometers enable specific as well as comprehensive measurements allowing sports drug testing laboratories to determine the misuse of therapeutics such as anabolic-androgenic steroids, stimulants, masking agents or so-called designer drugs in
athletes’ blood or urine specimens, and a selection of recent developments was summarized in one review [07048].

Fast and unequivocal drug detection is of considerable importance in numerous fields of analytical chemistry, and today mass spectrometry-based approaches are often the method of choice due to their sensitive and specific nature. Mass spectrometry is in constant flux with innovations and thus supports the development of new, complementary assays for rapid determination of drugs and toxins as well as their metabolic products in clinical, forensic, and doping control laboratories. Examples of such innovations that have greatly aided the worldwide bioanalytical efforts are the modern improvements in ion trap, for example, Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometer – Orbitrap, and time-of-flight (TOF) mass analyzers when coupled with sensitive ionization techniques such as electrospray ionization. In this perspective, the utility of state-of-the-art mass spectrometers and recent instrumental developments such as new and/or improved hybrid analyzers were discussed and selected applications presented [12066].

Small molecules

Recent developments in MS for the detection of small molecules in the context of doping control analysis are reviewed. Doping control analysis is evolving together with MS, which is the technique of choice in order to accomplish the analytical requirements in this field. Since these analytical requirements for the detection of a doping agent depend on the substance, in the first section we review the different scenarios. The commonly established approaches, together with their achievements and drawbacks are described. New developments in hyphenated MS techniques (both GC-MS/MS and LC-MS/MS) concerning interfaces and analyzers are mentioned. The use (or potential use) of these developments in order to minimize the limitations of the commonly established approaches in the doping control field is discussed. Finally, a brief discussion about trends and remaining limitations is presented [12067].

Small peptides in the urine

Although significant progress has been achieved during the past few years with the introduction of new assays and analytical methodologies, the detection and quantification of protein analytes, in particular of peptide hormones, continues to pose analytical challenges for the World Anti-Doping Agency-accredited anti-doping laboratories. In one article, the latest achievements in the application of MS-based methodologies and specific biochemical and immunological assays to detect some of the prohibited substances listed in section S2 of the World Anti-Doping Agency List of Prohibited Substances and Methods are reviewed. In addition, it was looked towards the future by focusing on some of the most promising analytical approaches under development for the detection of so-called biomarkers of doping [068].

In one study, a screening assay was developed comprising 11 prohibited peptides (<1.5 kDa) that are sufficiently purified from urine using weak cation exchange with subsequent determination of all substances by means of nanoUHPLC separation coupled to high resolution tandem mass spectrometry. These peptides included Gonadorelin (LH-RH), Desmopressin and 9 growth hormone releasing peptides (GHRP-1, -2, -4, -5, -6, Hexarelin, Alexamorelin, Ipamorelin and a GHRP-2 metabolite); however, the procedure is expandable to further target analytes or metabolites. The method was validated with a main focus on qualitative result interpretation considering the parameters specificity, linearity (0-500 pg/mL), recovery (45-95 %), precision (<20 % at 100 pg/mL), limits of detection (2-10 pg/mL), robustnesss and ion suppression. The proof-of-principle was shown by analysing excretion
study urine samples for LHRH, desmopressin and GHRP-2 [12065].

For most peptide hormones prohibited in elite sports the concentrations in plasma or urine are very low (pg/mL). Accordingly, hyphenated purification and enrichment steps prior to mass spectrometric detection are required to obtain sufficient doping control assays. Immunoaffinity purification in combination with nano-scale liquid chromatography coupled to high resolution/high accuracy mass spectrometry was found to have the potential of providing the necessary sensitivity and unambiguous specificity to produce reliable results. With the presented methodology 12 prohibited peptides (porcine insulin, Novolog, Apidra, Lantus DesB30-32 metabolite, Humalog and human insulin, Synacthen (synthetic ACTH analogue), luteinizing hormone-releasing hormone (LH-RH), growth hormone releasing hormone (GH-RH(1-29)) and CJC-1295 (GH-RH analogue), LongR(3)-IGF-1 and IFG-1) were simultaneously purified from plasma/serum or urine. With limits of detection for each target compound ranging in the low pg/mL level (urine), the method enables the determination of urinary peptides at physiologically relevant concentrations. For each class of peptides an appropriate antibody and a respective internal standard was implemented ensuring robust analysis conditions. Due to the fast and simple sample preparation procedure (about 25 samples per day) and the fact that all materials are commercial available, the implementation of the methodology to laboratories from other analytical fields (forensics, pharmacokinetic sciences, etc.) is enabled [12070].

Detection of misuse of peptides and proteins as growth promoters is a major issue for sport and food regulatory agencies. The limitations of current analytical detection strategies for this class of compounds, in combination with their efficacy in growth-promoting effects, make peptide and protein drugs highly susceptible to abuse by either athletes or farmers who seek for products to illicitly enhance muscle growth. Mass spectrometry (MS) for qualitative analysis of peptides and proteins is well-established, particularly due to tremendous efforts in the proteomics community. Similarly, due to advancements in targeted proteomic strategies and the rapid growth of protein-based biopharmaceuticals, MS for quantitative analysis of peptides and proteins is becoming more widely accepted. These continuous advances in MS instrumentation and MS-based methodologies offer enormous opportunities for detection and confirmation of peptides and proteins. Therefore, MS seems to be the method of choice to improve the qualitative and quantitative analysis of peptide and proteins with growth-promoting properties. This review aims to address the opportunities of MS for peptide and protein analysis in veterinary control and sports-doping control with a particular focus on detection of illicit growth promotion. An overview of potential peptide and protein targets, including their amino acid sequence characteristics and current MS-based detection strategies is, therefore, provided. Furthermore, improvements of current and new detection strategies with state-of-the-art MS instrumentation are discussed for qualitative and quantitative approaches [13091].

*Isotope ratio mass spectrometry or carbon isotope ratio*

It so happens that there is a measurable difference in carbon-13 content between endogenous and pharmaceutical testosterone. Most carbon atoms in nature are carbon-12, with a nucleus containing six protons and six neutrons. Radiocarbon dating relies on the rare carbon-14, an unstable, radioactive isotope, with a nucleus containing six protons and eight neutrons, which decays over time. Between the two is carbon-13, a stable isotope with six protons and seven neutrons. Roughly 1.1 percent of carbon in nature is carbon-13. Pharmaceutical testosterone contains a few parts per thousand less carbon-13 than does natural testosterone. This is because they arise from biosynthetic pathways that are sufficiently different. Humans make endogenous testosterone from cholesterol, itself made...
from acetate or coming from the diet. Pharmaceutical companies make testosterone by semisynthesis from plant sterols. All carbon in living beings is ultimately derived from atmospheric carbon dioxide (CO\textsubscript{2}), fixed in plants by photosynthesis. Different plants make the first multicarbon intermediates and downstream biosynthetic compounds differently. Animals eat plants, humans eat plants and animals, and “we are what we eat”. At every biosynthetic step, carbon-13 is left behind. This is because of the isotopic effect: chemical reactions go faster with lighter compounds; the molecule with a carbon-12 reacts sooner than the molecule with a carbon-13 instead. Because the pathways from atmospheric CO\textsubscript{2} to endogenous or pharmaceutical testosterone are different enough, carbon-13 is depleted to different extents; the difference happens to be measurable \[07011\].

**Full-capillary sample injection combined with sweeping CE stacking**

One study described an on-line stacking CE approach by sweeping with whole capillary sample filling for analyzing five anabolic androgenic steroids in urine samples. The five anabolic steroids for detection were androstenedione, testosterone, epitestosterone, boldenone, and clostebol. Anabolic androgenic steroids are abused in sport doping because they can promote muscle growth. Therefore, a sensitive detection method is imperatively required for monitoring the urine samples of athletes. In this research, an interesting and reliable stacking capillary electrophoresis method was established for analysis of anabolic steroids in urine. After liquid-liquid extraction by n-hexane, the supernatant was dried and reconstituted with 30 mM phosphate buffer (pH 5.00) and loaded into the capillary by hydrodynamic injection (10 psi, 99.9 s). The stacking and separation were simultaneously accomplished at -20 kV in phosphate buffer (30 mM, pH 5.0) containing 100 mM sodium dodecyl sulfate and 40 percent methanol. During the method validation, calibration curves were linear over a range of 50-1,000 ng/mL for the five analytes. In the evaluation of precision and accuracy for this method, the absolute values of the RSD and the RE in the intra-day (n=3) and inter-day (n=5) analyses were all less than 6.6 percent. The limit of detection for the five analytes was 30 ng/mL (S/N = 5, sampling 99.9 s at 10 psi). Compared with simple MECK, this stacking method possessed a 108- to 175-fold increase in sensitivity. This simple and sensitive stacking method could be used as a powerful tool for monitoring the illegal use of doping \[12064\].

**Benchtop quadrupole-orbitrap hybrid mass spectrometry**

Sensitive and unequivocal determination of analytes/contaminants in complex matrices is a challenge in the field of food safety control. In this study, various acquisition modes (Full MS/AIF, Full MS+tMS/MS, Full MS/dd MS/MS and tSIM/ddMS/MS) and parameters of a quadrupole-orbitrap hybrid mass spectrometer (Q Exactive) were studied in detail. One of the main conclusions has been that, reducing the scan range for Full MS (using the quadrupole) and targeted modes give higher signal-to-noise (S/N) ratios and thereby better detection limits for analytes in matrix. The use of Q Exactive in a complex case, for the confirmatory analysis of hormones in animal urine is presented. A targeted SIM data dependent MS/MS (tSIM/ddMS/MS) acquisition method for determination of eight synthetic hormones (trenbolone, 17\textalpha ethinylestradiol, zeranol, stanozolol, dienestrol, diethylstilbestrol, hexestrol, taleanol) and a naturally occurring hormone (zearalenone) in animal urine were optimized to have sensitive precursors from targeted SIM mode and trigger MS/MS scans over the entire chromatograph peak. The method was validated according to EC/657/2002. CC\textalpha (decision limit) for the analytes ranged between 0.11 microg/L and 0.69 microg/L and CC\textbeta (detection capability) ranged between 0.29 microg7L and 0.90 microg/L \[13094\].

**Chromatographic-mass spectrometry**
Urine samples have been the predominant matrix for doping controls for several decades. However, owing to the complementary information provided by blood (as well as serum or plasma and dried blood spots (DBS)), the benefits of its analysis have resulted in continuously increasing appreciation by anti-doping authorities. On the one hand, blood samples allow for the detection of various different methods of blood doping and the abuse of erythropoiesis-stimulating agents (ESAs) via the Athlete Biological Passport; on the other hand, targeted and non-targeted drug detection by means of chromatographic-mass spectrometric methods represents an important tool to increase doping control frequencies out-of-competition and to determine drug concentrations particularly in in-competition scenarios. Moreover, blood analysis seldom requires in-depth knowledge of drug metabolism, and the intact substance rather than potentially unknown or assumed metabolic products can be targeted. In this review, the recent developments in human sports drug testing concerning mass spectrometry-based techniques for qualitative and quantitative analyses of therapeutics and emerging drug candidates are summarized and reviewed. The analytical methods include both low and high molecular mass compounds (e.g., anabolic agents, stimulants, metabolic modulators, peptide hormones, and small interfering RNA (siRNA)) determined from serum, plasma, and DBS using state-of-the-art instrumentation such as liquid chromatography (LC)-high resolution/high accuracy (tandem) mass spectrometry (LC-HRMS), LC-low resolution tandem mass spectrometry (LC-MS/MS), and gas chromatography-mass spectrometry (GC-MS) [13096].

Liquid chromatography

A unification of doping-control screening procedures of prohibited small molecule substances may conceptually be achieved by the use of a combination of one gas chromatography-time-of-flight mass spectrometry method and one liquid chromatography-time-of-flight mass spectrometry method. In one work a quantitative screening method using high-resolution liquid chromatography in combination with accurate-mass time-of-flight mass spectrometry was developed and validated for determination of glucocorticosteroids, beta2-agonists, thiazide diuretics, and narcotics and stimulants in urine. To enable the simultaneous isolation of all the compounds of interest and the necessary purification of the resulting extracts, a generic extraction and hydrolysis procedure was combined with a solid-phase isolation modified for these groups of compounds. All 56 compounds are determined using positive electrospray ionisation with the exception of the thiazide diuretics for which the best sensitivity was obtained by using negative electrospray ionisation. The results show that, with the exception of clenhexyl, procaterol, and reprotoerol, all compounds can be detected below the respective minimum required performance level and the results for linearity, repeatability, and accuracy show that the method can be used for quantitative screening. If qualitative screening is sufficient the instrumental analysis may be limited to positive ionisation, because all analytes including the thiazides can be detected at the respective minimum required levels in the positive mode. The results show that the application of accurate-mass time-of-flight mass spectrometry in combination with generic extraction and purification procedures is suitable for unification and expansion of the window of screening methods of doping laboratories. Moreover, the full-scan accurate-mass data sets obtained still allow retrospective examination for emerging doping agents, without re-analyzing the samples [10034].

Whereas GC is done in the gas phase, liquid chromatography (LC) is done in the liquid phase. This is a crucial difference because it works for thermolabile compounds (destroyed by GC) and polar compounds (cannot be vaporized). The separation principles are the same.
A typical high-pressure or high-performance LC (HPLC) column is a steel tube the size of a fat marker pen, packed with microbeads on the surface of which is the stationary phase. The mobile phase is a liquid solvent, often a mixture whose composition is programmed to change during the run (gradient elution). Two common HPLC detectors are the diode-array detector (DAD) and the mass spectrometer. The DAD monitors UV absorption over a range of wavelengths or at selected wavelengths; it detects only those compounds that absorb UV light. When the HPLC is connected to an MS, the instrument is called LC-MS. The most advanced type of LC-MS can do tandem MS by one of several choices of conceptual and hardware approaches. It is called LC-MS-MS or LC-tandem MS. For a given class of drugs, such as diuretics, the LC-tandem MS screen is far superior to the GC-MS screen. Sample preparation time can be well less than 1 hour, down from a full day's work, because unlike GC, LC does not require the removal of water or salts, deconjugation or derivatization. Typically, the instrumental analysis run-time is two to three times shorter, well under 10 minutes per sample, because LC-MS-MS is blind to interferences; therefore, chromatographic resolution is not required, and LC run times can be shortened [07046].

The technique of liquid chromatography used in concert with (tandem) mass spectrometry (LC-MS/MS) has complemented sports drug testing strategies ever since soft ionization interfaces such as electrospray or atmospheric pressure chemical ionization (ESI or APCI, respectively) became commercially available. Numerous applications have been developed that allow the determination of prohibited therapeutics that are barely detectable or undetectable with conventional gas chromatographic-mass spectrometric techniques (GC-MS). Due to the progressive nature of doping controls, the continuously changing demands originating from the dynamic pharmaceutical market, new illegal approaches that presumably increase athletic performance, and modifications to the lists of prohibited compounds of regulative authorities such as the World Anti-Doping Agency (WADA), numerous new applications and drug-testing strategies based on LC-MS/(MS) are frequently developed in order to improve the portfolios of drug-testing laboratories. Liquid chromatography-(tandem) mass spectrometry (LC-MS/MS) has therefore revolutionized the detection assays used in doping control analysis. New methods have enabled the determination of drugs that were formerly difficult to detect or undetectable at preceding sample concentrations, and complex and/or time-consuming procedures based on alternative chromatographic-mass spectrometric or immunochemical principles have been replaced by faster, more comprehensive and robust assays. Analytical assay sensitivity and throughput are key factors for the LC-MS/(MS) approaches used in sports drug testing. Considerable improvements in liquid chromatography (such as using monolithic or UPLC columns) have resulted in shorter analysis times and significantly narrower peaks, which in turn lead to enhanced signal-to-noise ratios, better detection limits, and increased productivity. However, these fast analytical runs require very short mass spectrometric cycle times in order to obtain sufficient data points per compound and corresponding peak. In order to incorporate newly developed methods into sports drug testing systems, identification criteria are required, which have only been established for low molecular weight drugs. Several aspects are applicable and transferable to peptide and protein analysis, but specific issues such as molecular weight determination from multiply charged species or bottom-up sequencing [07050].

Doping control analytical laboratories for human sports predominantly employ nowadays chromatographic-mass spectrometric test methods for routine, high throughput screening and confirmation assays concerning low and high molecular mass analytes. Liquid chromatography-(tandem) mass spectrometry (LC-MS/(MS)) and particularly ultrahigh pressure liquid chromatography (UHPLC)-MS/MS instruments have become devices of choice due to their indispensable capabilities that compensate for limitations inherent to other commonly used strategies such as immunological and gas chromatography-(tandem) mass
spectrometry (GC-MS(/MS)) based detection methods. UHPLC-MS/MS-based assays at low mass spectrometric resolution have been established allowing for fast and sensitive targeted analyses focusing on pre-selected target analytes with diagnostic precursor-product ion pairs. Combining UHPLC to high resolution/high accuracy MS(/MS) further expanded the targeted approach (i.e., plotting extracted ion chromatograms of protonated or deprotonated molecules as well as product ions measured with accurate masses) toward non-targeted analyses enabling also retrospective data mining. In one review, recent applications of UHPLC-MS/MS in sports drug testing procedures published between 2008 and 2012 were presented and advantages as well as limitations in a short- and long-term perspective are discussed [12057].

Drug testing for sports doping control programs is extensive and includes numerous classes of banned compounds including anabolic androgenic steroids, beta2-agonists, hormone antagonists and modulators, diuretics, various peptide hormones, and growth factors. During competition, additional compounds may also be prohibited such as stimulants, narcotics, cannabinoids, glucocorticosteroids, and beta-blockers depending both on the sport and level of competition. Each of these classes of compounds can contain many prohibited substances that must be identified during the testing procedure. Various methods that have been designed to detect a large number of compounds in different drug classes are highly desirable as initial screening tools. Liquid chromatography/tandem mass spectrometry (LC-MS/MS) is widely used by anti-doping testing laboratories for this purpose and several rapid methods have been described to simultaneously detect different classes of compounds. Here, we describe a simple urine sample cleanup procedure that can be used to detect numerous anabolic androgenic steroids, beta2-agonists, hormone antagonists and modulators, glucocorticosteroids, and beta-blockers by LC-MS/MS [12058].

**Hydrophilic interaction liquid chromatography**

The chromatographic behaviour of 44 polar compounds (23 beta-adrenergic agents, 11 stimulants, 4 narcotics and 6 phenolalkylamines) included in the list of prohibited substances and methods of the World Anti-Doping Agency, has been investigated under hydrophilic interaction liquid chromatography conditions by application of different mobile phase compositions (percentage of the organic solvent, type and amount of mobile phase additive and ionic strength) and column temperatures. Detection of analytes was performed by a triple quadrupole mass spectrometer in positive ionization mode and selected reaction monitoring acquisition mode after liquid/liquid extraction. Data collected using as stationary phase type-B silica materials from different producers, showed that the best chromatographic conditions in terms of peak shape, selectivity and chromatographic retention were obtained using an initial percentage of acetonitrile of 90 percent, a column temperature of 35 °C, a mobile phase pH of 4.5 and ammonium acetate (5 mM) and acetic acid (0.1 %) as mobile phase additives. The selected chromatographic conditions were used to develop screening and confirmation analytical procedures to detect polar compounds in human urine for antidoping purpose. The developed methods were validated in terms of specificity, matrix effect, linearity, precision, accuracy, sensitivity, robustness and repeatability of retention times and relative ion abundances. Such methods offer attractive alternatives and considerable advantages over traditional approaches especially for the analysis of the phenolalkylamines [11566].

**Ultra-high-performance liquid chromatography**

Doping control analyses are generally performed in urine, a matrix that provides a prolonged detection time window, and less often in blood, serum, plasma, hair, saliva, and nails. To
identify the chemical structures of anabolic steroids the use of mass spectrometry detection is very advantageous. Gas chromatography–mass spectrometry (GC-MS) techniques allowed for the development of comprehensive screening methods. GC-MS methods are sensitive and robust but present the disadvantages of time-consuming sample pretreatment, that is often based on hydrolysis and derivatisation reactions. Liquid chromatography–mass spectrometry (LC-MS) methods have been successfully used to identify and determinate steroids in different matrices, as well as to study their metabolisms. Nowadays, automatic rapid ultra high performance liquid chromatography (UHPLC) tandem mass spectrometry has become the technique of choice for steroid analysis. Due to its generally higher speed, sensitivity, reproducibility and specificity with respect to HPLC, it can be used to simultaneously separate and determinate multi component steroid mixtures. The technique is of huge interest to separate conjugates anabolic androgenic steroids, as it allows efficiency enhancement due to the small particle (sub-2μm) column packing, which provides high peak capacity within analysis times even 5-10 fold shorter than conventional HPLC methods. Modern multiplex instruments can analyze thousands of samples per month so that, notwithstanding the generally high instrumental costs, the cost of the individual assay is affordable. In addition, the improved specificity and resolution offered by time-of-flight or quadrupole time-of-flight mass spectrometry allow their application in doping control analysis or in steroid profiling for accurate and sensitive full mass range acquisition. Aim of one review was to consider, compare and discuss the applications of the UHPLC/MS methods present in literature for the identification and determination of forbidden steroids and their metabolites in human biological matrices [12001].

Liquid chromatography/mass spectrometry (LC/MS) has been successfully applied to the detection of anabolic steroids in biological samples. However, the sensitive detection of saturated hydroxysteroids, such as androstanediols, by electrospray ionisation (ESI) is difficult because of their poor ability to ionise. In view of this, chemical derivatisation has been used to enhance the detection sensitivity of hydroxysteroids by LC/MS. One paper described the development of a sensitive ultra-high-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) method for the screening of anabolic steroids in horse urine by incorporating a chemical derivatisation step, using picolinic acid as the derivatisation reagent. The method involved solid-phase extraction (SPE) of both free and conjugated anabolic steroids in horse urine using a polymer-based SPE cartridge. The conjugated steroids in the eluate were hydrolyzed by methanolysis and the resulting extract was further cleaned up by liquid-liquid extraction. The resulting free steroids in the extract were derivatised with picolinic acid to form the corresponding picolinoyl esters and analysed by UHPLC/MS/MS in the positive ESI mode with selected-reaction-monitoring. Separation of the targeted steroids was performed on a C18 UHPLC column. The instrument turnaround time was 10.5 min inclusive of post-run equilibration. A total of thirty-three anabolic steroids (including 17β-estradiol, 5(10)-estrene-3β,17α-diol, 5α-estrane-3β,17α-diol, 17α-ethyl-5α-estrane-3α,17β-diol, 17α-methyl-5α-androstan-3,17β-diols, androstanediols, nandrolone and testosterone) spiked in negative horse urine at the QC levels (ranging from 0.75 to 30 ng/mL) could be consistently detected. The intra-day and inter-day precisions (% RSD) for the peak area ratios were around 7-51 percent and around 1-72 percent, respectively. The intra-day and inter-day precisions (% RSD) for the relative retention times were both less than 1 percent for all analytes, except the inter-day precision for boldione at 1.2 percent. The extraction recoveries for all targets were not less than 48 percent. With exceptional separation achieved by the UHPLC system, matrix interferences were minimal at the expected retention times of the selected transitions. As detection was performed with an UHPLC system coupled to a fast-scanning triple quadrupole mass spectrometer, the method could easily be expanded to accommodate additional steroid targets. This method has been validated for recovery and precision, and could be used regularly for doping control testing of anabolic steroids in horse urine samples [12060].

364
It was described a sensitive, comprehensive and fast screening method based on liquid chromatography-high resolution mass spectrometry for the detection of a large number of analytes in sports samples. UHPLC coupled to high resolution mass spectrometry with polarity switching capability is applied for the rapid screening of a large number of analytes in human urine samples. Full scan data are acquired alternating both positive and negative ionisation. Collision-induced dissociation with positive ionisation is also performed to produce fragment ions to improve selectivity for some analytes. Data are reviewed as extracted ion chromatograms based on narrow mass/charge windows (± 5 ppm). A simple sample preparation method was developed, using direct enzymatic hydrolysis of glucuronide conjugates, followed by solid phase extraction with mixed mode ion-exchange cartridges. Within a 10 min run time (including re-equilibration) the method presented allows for the analysis of a large number of analytes from most of the classes in the World Anti-Doping Agency (WADA) Prohibited List, including anabolic agents, beta2-agonists, hormone antagonists and modulators, diuretics, stimulants, narcotics, glucocorticoids and beta-blockers, and does so while meeting the WADA sensitivity requirements. The high throughput of the method and the fast sample pre-treatment reduces analysis cost and increases productivity. The method presented has been used for the analysis of over 5000 samples in about one month and proved to be reliable [13092].

Fast liquid chromatographic/mass spectrometric screening

A fast liquid chromatographic/mass spectrometric (LC/MS/MS) screening method for the detection, in urine, of synthetic glucocorticoids, stimulants (formoterol, modafinil and mesocarb), anti-oestrogens (finasteride, exemestane, anastrozole, letrozole and formestane) and synthetic anabolic steroids (stanozolol, gestrinone and tetrahydrogestrinone) is described. All these drugs (and/or their urinary metabolites) can be simultaneously extracted by a single liquid/liquid extraction step, at alkaline pH, after enzymatic hydrolysis with beta-glucuronidase, and assayed in 7 min by LC/MS/MS using electrospray ionization in positive ion mode and multiple reaction monitoring as the acquisition mode. All compounds show good reproducibility of both the retention times (CV% <2%) and the relative abundances (CV% <10 %). The limits of detection for the anti-oestrogens, glucocorticoids and steroids are in the range of 1-30 ng/mL, and for the stimulants are in the range of 100-200 ng/mL, thus satisfying the minimum required performance limits of the World Anti-Doping Agency [06037].

Orbitrap

A new doping control screening method for the analysis of diuretics and stimulants using ultra high pressure liquid chromatography-high resolution Orbitrap mass spectrometry has been developed. The screening was performed in full scan MS with scan-to-scan polarity switching which allowed to detect more than 120 target analytes. Sample preparation was limited to 10-fold dilution of the urine into the internal standard solution followed by injection. Total run time per sample was 10 min. Validation of the method yielded detection limits for diuretics between 25 and 250 ng/mL and for stimulants between 5 and 500 ng/mL. The screening method has been implemented in routine doping control [12071].

Liquid chromatography-tandem mass spectrometry

A screening method for the urinary detection of 34 exogenous anabolic steroids has been developed. The method involves an enzymatic hydrolysis, liquid-liquid extraction and detection by liquid chromatography-tandem mass spectrometry. The use of some adducts
such as (M+NH₄⁺, M+CH₃COO⁻ and M+H+MeOH⁺) was necessary in order to detect some analytes at the required level (lower than 10 ng/ml). Two transitions were selected for each analyte. Different concentration factors have been studied in order to increase the sensitivity. A concentration factor of 50 was selected for the screening method although the high ion suppression observed under these conditions can hamper its application as a quantitative method. The method was validated and limits of detection were obtained by spiking ten different blank urine samples at five different concentration levels. Up to 29 analytes were detected in all spiked urines at the required level. Limits of detection between 1 and 10 ng/ml were obtained for most analytes which fulfil current requirements. The applicability of the method was shown by analysing positive samples [07051].

A simple and accurate liquid chromatography/tandem mass spectrometry (LC/MS/MS) method has been developed and validated for the quantitative determination of ephedrine, pseudoephedrine, methylephedrine, cathine, salbutamol, morphine and epitestosterone in human urine. Urine samples were spiked with internal standard and diluted with acetonitrile. After centrifugation, the supernatants were directly analyzed by LC/MS/MS using the selected reaction monitoring (SRM) mode. The linearity, intra- and inter-day precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) were evaluated and the method was found to be accurate and reproducible for the quantitation of threshold substances. When the method was applied to the analysis of blind urine samples for the proficiency test, the results were close to the nominal concentrations, within 88-107 percent of nominal values, suggesting that the developed methods can be successfully applied to routine doping analyses [11429].

Liquid chromatography-(tandem) mass spectrometry (LC-MS/MS) has become an integral part of modern sports drug testing as it offers unique capabilities complementing immunological and gas chromatography-(tandem) mass spectrometry (GC-MS/MS)-based detection methods for prohibited compounds. The improved options of fast and sensitive targeted analysis as well as untargeted screening procedures utilizing high resolution/high accuracy mass spectrometry have considerably expanded the tools available to anti-doping laboratories for initial testing and confirmation methods. One approach is to focus on pre-selected target analytes that are measured with utmost specificity and sensitivity using diagnostic precursor-product ion pairs in low resolution tandem mass spectrometers. The other scenario is to measure and plot extracted ion chromatograms of protonated or deprotonated molecules as well as product ions as recorded in the full scan mode with high resolution/high accuracy mass spectrometry. Examples of recent applications of sports drug testing procedures published between 2007 and 2010 were presented and discussed, outlining the particular advantages of the selected approaches as well as their limitations in a short- and long-term perspective [11036].

**Liquid chromatography-mass spectrometry (LC-MS)**

Matrix effects (ion suppression/enhancement) are a well-observed phenomenon in analyses of biological matrices by high-performance liquid chromatography-mass spectrometry (LC-MS). However, few simple solutions for detecting and minimizing these adverse effects have been described so far in multianalyte analysis, especially in the field of doping control. This study describes an exhaustive characterization of matrix effects in one hundred urine samples fortified with 41 analytes (glucocorticoids and diuretics). It introduces a novel marker to identify samples in which the reliability of the results is compromised because of acute ion suppression. This new strategy strengthens the rigor of the analysis for screening purposes. Once the matrix effect is identified, a selective sample preparation is introduced to minimize the adverse ion suppression effect. That selective extraction together with the use of a deuterated internal standard permits enhancing the ruggedness of the estimation of
glucocorticoid concentration in urine [12059].

**Ultrahigh pressure liquid chromatography-(tandem) mass spectrometry**

Doping control analytical laboratories for human sports predominantly employ nowadays chromatographic-mass spectrometric test methods for routine, high throughput screening and confirmation assays concerning low and high molecular mass analytes. Liquid chromatography-(tandem) mass spectrometry [(LC-MS/(MS))] and particularly ultrahigh pressure liquid chromatography (UHPLC)-MS/MS instruments have become devices of choice due to their indispensable capabilities that compensate for limitations inherent to other commonly used strategies such as immunological and gas chromatography-(tandem) mass spectrometry [(GC-MS/(MS))] based detection methods. UHPLC-MS/MS-based assays at low mass spectrometric resolution have been established allowing for fast and sensitive targeted analyses focusing on pre-selected target analytes with diagnostic precursor-product ion pairs. Combining UHPLC to high resolution/high accuracy MS/(MS) further expanded the targeted approach (i.e., plotting extracted ion chromatograms of protonated or deprotonated molecules as well as product ions measured with accurate masses) toward non-targeted analyses enabling also retrospective data mining. In one review, applications of UHPLC-MS/MS in sports drug testing procedures published between 2008 and 2012 are presented and advantages as well as limitations in a short- and long-term perspective were discussed [13092].

**Metabolite-based liquid chromatography-mass spectrometry**

Today, immunoassays and several chromatographic methods are in use for drug screening in clinical and forensic toxicology and in doping control. For further proof of the authors' new metabolite-based liquid chromatography-mass spectrometry (LC-MS$_n$) screening concept, the detectability of drugs of abuse and their metabolites using this screening approach was studied. As previously reported, the corresponding reference library was built up with MS$_2$ and MS$_3$ wideband spectra using a LXQ linear ion trap with electrospray ionization in the positive mode and full scan information-dependent acquisition. In addition to the parent drug spectra recorded in methanolic solution, metabolite spectra were identified after protein precipitation of urine from rats after administration of the corresponding drugs and added to the library. This consists now of data of over 900 parent compounds, including 87 drugs of abuse, and of over 2,300 metabolites and artifacts, among them 436 of drugs of abuse. Recovery, process efficiency, matrix effects, and limits of detection for selected drugs of abuse were determined using spiked human urine, and the resulting data have been acceptable. Using two automatic data evaluation tools (ToxID and SmileMS), the intake of 54 of the studied drugs of abuse could be confirmed in urine samples of drug users after protein precipitation and LC separation. The following drugs classes were covered: stimulants, designer drugs, hallucinogens, (synthetic) cannabinoids, opioids, and selected benzodiazepines. The presented LC-MS$_n$ method complements the well-established gas chromatography-mass spectroscopy procedure in the authors' laboratory [11035].

**High-resolution/accurate-mass LC-MS**

Detection of androgenic-anabolic steroid abuse in equine sports requires knowledge of the drug's metabolism in order to target appropriate metabolites, especially where urine is the matrix of choice. Studying “designer” steroid metabolism is problematic since it is difficult to obtain ethical approval for in vivo metabolism studies due to a lack of toxicological data. In this study, the equine in vitro metabolism of eight steroids available for purchase on the Internet is reported; including androst-1,4,6-triene-3,17-dione, 4-chloro,17alpha-methyl-androsta-1,4-diene-3,17beta-diol, estra-4,9-diene-3,17-dione, 4-hydroxyandrostenedione,
20-hydroxyecdysone, 11-keto-androstenedione, 17a-methyldrostanolone, and tetrahydrogestrinone. In order to allow for retrospective analysis of sample testing data, the use of a high-resolution (HR) accurate-mass Thermo LTQ-Orbitrap liquid chromatography-mass spectrometry (LC-MS) instrument was employed for metabolite identification of underivatized sample extracts. The full scan LC-HRMS Orbitrap data were complimented by LC-HRMS/MS and gas-chromatography-mass spectrometry (GC-MS) experiments in order to provide fragmentation information and to ascertain whether GC-MS was capable of detecting any metabolite not detected by LC-HRMS. With the exception of 20-hydroxyecdysone, all compounds were found to be metabolized by equine liver S9 and/or microsomes. With the exception of 17alpha-methyldrostanolone, which produced metabolites that could only be detected by GC-MS, the metabolites of all other compounds could be identified using LC-HRMS, thus allowing retrospective analysis of previously acquired full-scan data resulting from routine equine drug testing screens. In summary, while in vitro techniques do not serve as a replacement for more definitive in vivo studies in all situations, their use does offer an alternative in situations where it would not be ethical to administer untested drugs to animals [11044].

With information dependent acquisition

Diuretics are a class of compounds largely used for either therapeutic or illegal (doping) purposes. Probably owing to the substantial variety of their chemical structures, which makes them hardly extractable from a biological matrix in a single procedure, a quite short list of screening methods can be retrieved in the literature. One work presented a screening procedure for 24 diuretics based on the direct injection of urine (after 50 folds dilution) in a LC-ESI-MS/MS system (Applied Biosystems 4000 QTrap). Two information dependent acquisitions (IDA), one in positive, one in negative ionization, allowed the acquisition of one selected reaction monitoring transition for each compound, which, when a significant peak was found, triggered the acquisition of the enhanced product ion (EPI) spectrum. EPI spectra were stored in a library and the procedure was able to recognize by library matching various diuretics in real positive samples. The limits of detection were comprised between 0.002 and 0.25 ml/L and ion suppression was not found to significantly influence the analysis [07052].

Liquid chromatography/time-of-flight mass spectrometry

The development of comprehensive methods able to tackle with the systematic identification of drug metabolites in an automated fashion is of great interest. In this article, a strategy based on the combined use of two complementary data mining tools is proposed for the screening and systematic detection and identification of urinary drug metabolites by liquid chromatography full-scan high resolution mass spectrometry. The proposed methodology is based on the use of accurate mass extraction of diagnostic ions (compound-dependent information) from in-source CID fragmentation without precursor ion isolation along with the use of automated mass extraction of accurate-mass shifts corresponding to typical biotransformations (non compound-dependent information) that xenobiotics usually undergo when metabolized. The combined strategy was evaluated using LC-TOFMS with a suite of nine sport drugs representative from different classes (propranolol, bumetanide, clenbuterol, ephedrine, finasteride, methoxypenamine, mephedrine, salbutamol and terbutaline), after single doses administered to rats. The metabolite identification coverage rate obtained with the systematic method (compared to existing literature) was satisfactory, and provided the identification of several non-previous reported metabolites. In addition, the combined information obtained helps to minimize the number of false positives. As an example, the systematic identification of urinary metabolites of propranolol enabled the identification of up
to 24 metabolites, 15 of them non previously described in literature, which is a valuable indicator of the usefulness of the proposed systematic procedure [13098].

A general screening method based on solid phase extraction (SPE) and liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) was developed and investigated with 124 different doping agents, including stimulants, beta-blockers, narcotics, beta2-adrenergic agonists, agents with anti-estrogenic activity, diuretics and cannabinoids. Mixed mode cation exchange/C8 cartridges were applied to SPE, and chromatography was based on gradient elution on a C18 column. Ionization of the analytes was achieved with electrospray ionization in the positive mode. Identification by LC/TOFMS was based on retention time, accurate mass and isotopic pattern. Validation of the method consisted of analysis of specificity, analytical recovery, limit of detection and repeatability. The minimum required performance limit (MRPL), established by World Anti-Doping Agency (WADA), was attained to 97 doping agents. The extraction recoveries varied between 33 and 98 percent and the median was 58 percent. Mass accuracy was always better than 5 ppm, corresponding to a maximum mass error of 0.7 mDa. The repeatability of the method for spiked urine samples, expressed as median of relative standard deviations (RSD%) at concentrations of MRPL and 10 times MRPL, were 14 and 9 percent, respectively. The suitability of the LC/TOFMS method for doping control was demonstrated with authentic urine samples [06038].

The development of comprehensive methods able to tackle with the systematic identification of drug metabolites in an automated fashion is of great interest. In this article, a strategy based on the combined use of two complementary data mining tools is proposed for the screening and systematic detection and identification of urinary drug metabolites by liquid chromatography full-scan high resolution mass spectrometry. The proposed methodology is based on the use of accurate mass extraction of diagnostic ions (compound-dependent information) from in-source CID fragmentation without precursor ion isolation along with the use of automated mass extraction of accurate-mass shifts corresponding to typical biotransformations (non compound-dependent information) that xenobiotics usually undergo when metabolized. The combined strategy was evaluated using LC-TOFMS with a suite of nine sport drugs representative from different classes (propranolol, bumetanide, clenbuterol, ephedrine, finasteride, methoxyphenamine, methylephedrine, salbutamol and terbutaline), after single doses administered to rats. The metabolite identification coverage rate obtained with the systematic method (compared to existing literature) was satisfactory, and provided the identification of several non-previously reported metabolites. In addition, the combined information obtained helps to minimize the number of false positives. As an example, the systematic identification of urinary metabolites of propranolol enabled the identification of up to 24 metabolites, 15 of them non previously described in literature, which is a valuable indicator of the usefulness of the proposed systematic procedure [12062].

High performance liquid chromatography retention time of small molecules

Quantitative structure-retention relationship (QSRR) is a technique capable of improving the identification of analytes by predicting their retention time on a liquid chromatography column (LC) and/or their properties. This approach is particularly useful when LC is coupled with a high-resolution mass spectrometry (HRMS) platform. The main aim of one study was to develop and describe appropriate QSRR models that provide usable predictive capability, allowing false positive identification to be removed during the interpretation of metabolomics data, while additionally increasing confidence of experimental results in doping control area. For this purpose, a dataset consisting of 146 drugs, metabolites and banned compounds from World Anti-Doping Agency (WADA) lists, was used. A QSRR study was carried out
separately on high quality retention data determined by reversed-phase (RP-LC-HRMS) and hydrophilic interaction chromatography (HILIC-LC-HRMS) systems, employing a single protocol for each system. Multiple linear regression (MLR) was applied to construct the linear QSRR models based on a variety of theoretical molecular descriptors. The regression equations included a set of three descriptors for each model: ALOGP, BEl6, R2p and ALOGP(2), FDI, BLTA96, were used in the analysis of reversed-phase and HILIC column models, respectively. Statistically significant QSRR models indicate a strong correlation between retention time and the molecular descriptors. An evaluation of the best correlation models, performed by validation of each model using three tests (leave-one-out, leave-many-out, external tests), demonstrated the reliability of the models [13088].

**Nano-liquid chromatography/benchtop quadrupole orbitrap tandem-mass spectrometry**

In one study, a screening assay was developed comprising 11 prohibited peptides (<1.5 kDa) that are sufficiently purified from urine using weak cation exchange with subsequent determination of all substances by means of nanoUHPLC separation coupled to high resolution tandem mass spectrometry. These peptides included Gonadorelin (LH-RH), Desmopressin and 9 growth hormone releasing peptides (GHRP-1, -2, -4, -5,-6, Hexarelin, Alexamorelin, Ipamorelin and a GHRP-2 metabolite); however, the procedure is expandable to further target analytes or metabolites. The method was validated with a main focus on qualitative result interpretation considering the parameters specificity, linearity (0-500 pg/mL), recovery (45-95 %), precision (<20 % at 100 pg/mL), limits of detection (2-10 pg/mL), robustness and ion suppression. The proof-of-principle was shown by analysing excretion study urine samples for LHRH, desmopressin and GHRP-2 [12065].

**Liquid and gas chromatography time-of-flight mass spectrometry**

A new combined doping control screening method for the analysis of anabolic steroids in human urine using liquid chromatography/electrospray ionization orthogonal acceleration time-of-flight mass spectrometry (LCoaTOFMS) and gas chromatography/electron ionization orthogonal acceleration time-of-flight mass spectrometry (GCoaTOFMS) was developed in order to acquire accurate full scan MS data to be used to detect designer steroids. The developed method allowed the detection of representative prohibited substances, in addition to steroids, at concentrations of 10 ng/mL for anabolic agents and metabolites, 30 ng/mL for corticosteroids, 500 ng/mL for stimulants and beta-blockers, 250 ng/mL for diuretics, and 200 ng/mL for narcotics. Sample preparation was based on liquid-liquid extraction of hydrolyzed human urine, and the final extract was analyzed as trimethylsilylated derivatives in GCoaTOFMS and underivatized in LCoaTOFMS in positive ion mode. The sensitivity, mass accuracy, advantages and limitations of the developed method were presented [07053].

**Gas chromatography**

The application of comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GCxGC-TOFMS) for the analysis of six anabolic agents (AAs) in doping control is investigated in this work. A non-polar-polar column configuration with 0.2 microm film thickness second dimension column was employed, offering much better spread of the components on second dimension when compared to the alternative 0.1 microm column. The proposed method was tested on the “key” AAs that the World Anti-Doping Agency (WADA) had listed at the low ngm/L levels (clenbuterol, 19-norandrosterone,
epimethandiol, 17alpha-methyl-5alpha-androstane-3alpha,17beta-diol, 17alpha-methyl-5beta-androstane-3alpha,17beta-diol and 3'-OH-stanozolol). The compounds were spiked in a blank urine extract obtained by solid-phase extraction, hydrolysis and liquid-liquid extraction; prior to analysis they were converted to the corresponding trimethylsilyl (TMS) derivatives. The limit of detection (LOD) was below or equal to the minimum required performance limit (MRPL) of 2ngmL(-1) defined by WADA, and the correlation coefficient was in the range from 0.995 to 0.999. The method allows choosing an ion from the full mass spectra which shows the least interference from the matrix and/or the best sensitivity; this can only be done if full scan mass spectral data are available. The advantage of GCxGC over classical one-dimensional GC, in terms of separation efficiency and sensitivity, is demonstrated on a positive urine control sample at a concentration of 5ngm/L. The obtained similarity to the in-house created TOFMS spectra library at this level of concentration was in the range from 822 to 932 (on the scale from 0 to 999). Since full mass spectral information are recorded, the method allows the retro-search of non-target compounds or new "designer steroids", which cannot be detected with established GC-MS methods that use selected ion monitoring mode [10035].

Urine samples have been the predominant matrix for doping controls for several decades. However, owing to the complementary information provided by blood (as well as serum or plasma and dried blood spots (DBS)), the benefits of its analysis have resulted in continuously increasing appreciation by anti-doping authorities. On the one hand, blood samples allow for the detection of various different methods of blood doping and the abuse of erythropoiesis-stimulating agents (ESAs) via the Athlete Biological Passport; on the other hand, targeted and non-targeted drug detection by means of chromatographic-mass spectrometric methods represents an important tool to increase doping control frequencies out-of-competition and to determine drug concentrations particularly in in-competition scenarios. Moreover, blood analysis seldom requires in-depth knowledge of drug metabolism, and the intact substance rather than potentially unknown or assumed metabolic products can be targeted. In this review, the recent developments in human sports drug testing concerning mass spectrometry-based techniques for qualitative and quantitative analyses of therapeutics and emerging drug candidates are summarized and reviewed. The analytical methods include both low and high molecular mass compounds (e.g., anabolic agents, stimulants, metabolic modulators, peptide hormones, and small interfering RNA (siRNA)) determined from serum, plasma, and DBS using state-of-the-art instrumentation such as liquid chromatography (LC)-high resolution/high accuracy (tandem) mass spectrometry (LC-HRMS), LC-low resolution tandem mass spectrometry (LC-MS/MS), and gas chromatography-mass spectrometry (GC-MS) [13086].

Employing gas chromatography – triple quadrupole (QqQ) tandem mass spectrometry (GC-MS/MS), a complementary methodology was presented in 2012, allowing for the qualitative and partly quantitative identification of 173 analytes including representatives of anabolic agents, beta2-agonists, hormone and metabolic modulators, masking agents, stimulants, narcotics, beta-blockers, and cannabinoids. By means of targeted multiple reaction monitoring (MRM) and 11 ISTDs (10 of which were isotopically labelled), a robust and sensitive assay was established enabling the routine screening for over 150 xenobiotic compounds plus the provision of the individual's steroid profile from a total of 1 mL of urine. In agreement with earlier approaches, the urine was subjected to enzymatic hydrolysis, liquid-liquid extraction (LLE) and finally trimethylsilylation prior to gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis [13019].
Gas chromatography-microchip atmospheric pressure photoionization-mass spectrometry

A gas chromatography-microchip atmospheric pressure photoionization-tandem mass spectrometry (GC-microAPPI-MS/MS) method was developed for the analysis of anabolic androgenic steroids in urine as their trimethylsilyl derivatives. The method utilizes a heated nebulizer microchip in atmospheric pressure photoionization mode (microAPPI) with chlorobenzene as dopant, which provides high ionization efficiency by producing abundant radical cations with minimal fragmentation. The performance of GC-microAPPI-MS/MS was evaluated with respect to repeatability, linearity, linear range, and limit of detection (LOD). The results confirmed the potential of the method for doping control analysis of anabolic steroids. Repeatability, linearity, and sensitivity (LODs 0.05-0.1 ng/mL) were acceptable. Quantitative performance of the method was tested and compared with that of conventional GC-electron ionization-MS, and the results were in good agreement [13087].

Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) is the technique that is used most widely in anti-doping labs. The GC effluent enters the mass spectrometer continuously, and the mass spectrometer continuously records roughly one mass spectrum (scan) per second. There are two main modes of MS operation: the full-scan mode and the selected ion monitoring (SIM) mod. In the full-scan mode, the mass spectrometer records the whole mass spectrum (from m/z 70 to 400), monitoring hundreds of ions. In the SIM mode, only selected ions are monitored (e.g. 143, 345, 360); therefore, a longer time is spent recording each ion. In physics, signal strength (signal-to-noise ratio) increases with the time spent collecting data. Therefore, on the same instrument SIM is more sensitive than full scan; it can detect smaller amounts of drug. Other types of MS that are more sensitive include high-resolution MS, tandem MS, and ion traps. High-resolution MS is designed to measure m/z not only to the nearest unit or decimal, but out to several more decimals. This makes it possible to mathematically deduce the molecular formula (how many carbon, hydrogen, oxygen, and other atoms it contains); the more decimals, the fewer combinations of atoms fit, the narrower the possibilities. High-resolution MS instruments happen to be inherently more sensitive. Tandem MS instruments have two mass spectrometers back to back. The first one can be used to select only one ion, the precursor ion, which can be the molecular ion. The second mass spectrometer monitors only one (or at most a few) characteristic fragmentations (transitions to product ions). This is called the multiple reaction monitoring or selected reaction monitoring mode. (Alternatively, the first mass spectrometer can be used to select only the molecular ion and the second mass spectrometer can be used to record a full scan.) Tandem MS is more sensitive because it is blind to interferences. Unlike all of the above MS types, which let all ions formed continually escape from the ion source, ion traps trap all ions until they are released, one m/z at a time, to determine their abundance [07046].

One paper described a fast gas chromatographic/mass spectrometric (GC/MS) screening method for the detection, in urine, of 36 xenobiotics (30 synthetic anabolic steroids, four narcotics, one diuretic and one stimulant) excreted free or as glucuro-conjugates in urine and detectable as trimethylsilyl (TMS) derivatives. These drugs (and/or their urinary metabolites) can be simultaneously extracted by a single liquid/liquid separation step, at alkaline pH, after enzymatic hydrolysis with beta-glucuronidase and then assayed as TMS derivatives by GC/MS using electron ionisation (EI) and single ion monitoring (SIM) acquisition mode. The total time needed for the GC run is less than 8 min. Good reproducibility of the retention times (CV % <1) and the relative abundances of the diagnostic fragment ions (CV % <10)
was observed for all target analytes. The sensitivity of the method is sufficient to match the requirements of the World Anti-Doping Agency (WADA) for the accredited laboratories, with limits of detection (LODs) that are lower than the corresponding WADA minimum required performance limits (MRPLs) for all target compounds [07049].

Gas chromatography-combustion-IRMS (GC-C-IRMS).

Before application to doping control, it had long been used to detect the fraudulent substitution of synthetic compounds in place of natural compounds in the food, flavor, and fragrance industries. The anabolic steroids are extracted from urine and separated by GC. The separated testosterone enters the pencil-size combustion oven where it is pyrolyzed. Every carbon atom in the molecule is converted to CO₂, and every hydrogen atom is converted to water (H₂O). The water is scrubbed out and only the CO₂ enters the IRMS. This type of MS measures only three m/z: 44 for 12C16O₂, and 45 and 46 for variants containing carbon-13, oxygen-17, or oxygen-18. From the relative abundances, the instrument software calculates the delta¹³C (delta) value. It reflects the ¹³C/¹²C ratio, but it actually is the difference between the ¹³C/¹²C ratio of the sample and that of an international standard. The units are ‰ (per mil). By definition, the delta value of the international standard is 0‰. Examples of values are -24 ‰ for natural testosterone and -29 ‰ for pharmaceutical testosterone. The values are negative because both compounds contain less carbon-13 than the international standard: 29 fewer parts per thousand for the pharmaceutical testosterone. After exogenous testosterone administration, the delta values of urinary testosterone metabolites become more negative. In contrast, the delta values of testosterone precursors, or of endogenous steroids not involved in testosterone metabolism, remain unchanged; therefore, they can be used as endogenous reference compounds. A gap in delta value between testosterone or its metabolites and an endogenous reference compound indicates the use of testosterone or of any steroid in its metabolism. If the difference between the delta values of one metabolite and the endogenous reference compound is three delta units or more, the WADA requirement for reporting an adverse analytical finding has been met. The power of this approach is that it can detect the use of not only testosterone itself, but also of any one of many testosterone precursors and metabolites. The second advantage is that it is not affected by factors that might influence baseline delta values. For example, diet influences the carbon-13 content of endogenous steroids – all of them to a similar extent. Although interpreting vastly different delta values from one individual to the next might be difficult, a difference in delta values between a testosterone metabolite and an endogenous reference compound clearly reveals drug use. In short, the approach compensates for individual variability. The third advantage is that it does not require identifying or even knowing what exact compound was taken. RMS testing has been applied to various testosterone precursors, testosterone metabolites, and endogenous reference compounds. It is currently done for samples with T/E greater than 4 or on request by the sports authority [07011].

An alternative calibration procedure for use when performing carbon isotope ratio measurements by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) has been developed. This calibration procedure does not rely on the corrections in-built in the instrument software, as the carbon isotope ratios of a sample are calculated from the measured raw peak areas. The method was developed for the certification of a urine reference material for sports drug testing, as the estimation of measurement uncertainty is greatly simplified. To ensure that the method is free from bias arising from the choice of calibration material and instrument, the carbon isotope ratios of steroids in urine extracts were measured using two different instruments in different laboratories, and three different reference materials (CU/USADA steroid standards from Brenna Laboratory, Cornell University; NIST RM8539 mineral oil; methane calibrated against
NIST RM8560 natural gas). The measurements were performed at LGC and the Australian National Measurement Institute (NMI). It was found that there was no significant difference in measurement results when different instruments and reference materials were used to measure the carbon isotope ratio of the major testosterone metabolites androsterone and etiocholanolone, or the endogenous reference compounds pregnanediol, 11-ketoetiocholanolone and 11beta-hydroxyandrosterone. The measurement results of this comparison were used to estimate a measurement uncertainty of delta$^{13}$C for the certification of the urine reference material being performed on a single instrument using a single reference material at NMI [11037].

The confirmation by GC/C/IRMS of the exogenous origin of pseudo-endogenous steroids from human urine samples requires extracts of adequate purity. A strategy based on HPLC sample purification prior to the GC/C/IRMS analysis of human urinary endogenous androgens (i.e. testosterone, androsterone and/or androstenediols), is presented. A method without any additional derivatization step is proposed, allowing to simplify the urine pretreatment procedure, leading to extracts free of interferences permitting precise and accurate IRMS analysis, without the need of correcting the measured delta values for the contribution of the derivatizing agent. The HPLC extracts were adequately combined to both reduce the number of GC/C/IRMS runs and to have appropriate endogenous reference compounds (ERC; i.e. pregnanediol, 11-keto-etiocholanolone) on each GC-IRMS run. The purity of the extracts was assessed by their parallel analysis by gas chromatography coupled to mass spectrometry, with GC conditions identical to those of the GC/C/IRMS assay. The method has been validated according to ISO17025 requirements (within assay precision below 0.3‰ $^{13}$C delta units and between assay precision below 0.6‰ $^{13}$C delta units for most of the compounds investigated) fulfilling the World Anti-Doping Agency requirements [12051].

In gas chromatographic-combustion-isotope ratio mass spectrometry (GC-C-IRMS) doping control analysis, endogenous androgenic anabolic steroids and their metabolites are commonly acetylated using acetic anhydride reagent, thus incorporating exogenous carbon that contributes to the measured isotope ratio. Comparison of the endogenous delta$^{13}$C of free, mono-, and di-acetylated steroids requires application of corrections, typically through straightforward use of the mass balance equation. Variability in kinetic isotope effects (KIE) due to steroid structures could cause fractionation of endogenous steroid carbon, resulting in inaccurate results. To test for possible KIE influence on delta$^{13}$C, acetic anhydride of graded isotope ratio within the natural abundance range was used under normal derivatization conditions to test for linearity. In all cases, plots of measured steroid acetate delta$^{13}$C versus acetic anhydride delta$^{13}$C were linear and slopes were not significantly different. Regression analysis of the delta$^{13}$C of enriched acetic anhydrides versus delta$^{13}$C of derivatized steroids shows that KIE are similar in all cases. It was concluded that delta$^{13}$C calculated from the mass balance equation is independent of the delta$^{13}$C of the acetic anhydride reagent, and that net KIE under normal derivatization conditions do not bias the final reported steroid delta$^{13}$C [12061].

Gas chromatography-triple quadrupole mass spectrometry

The use of performance enhancing drugs in sports is prohibited. For the detection of misuse of such substances gas chromatography or liquid chromatography coupled to mass spectrometry are the most frequently used detection techniques. In this work the development and validation of a fast gas chromatography tandem mass spectrometric method for the detection of a wide range of doping agents is described. The method can determine 13 endogenous steroids (the steroid profile), 19-norandrosterone, salbutamol and 11-nor-delta9-tetrahydrocannabinol.9-carboxylic acid in the applicable ranges and to detect
qualitatively over 140 substances in accordance with the minimum required performance levels of the World Anti-Doping Agency in 1ml of urine. The classes of substances included in the method are anabolic steroids, beta2-agonists, stimulants, narcotics, hormone antagonists and modulators and beta-blockers. Moreover, using a short capillary column and hydrogen as a carrier gas the run time of the method is less than 8 min [11038].

Gas chromatography-QqQ-MS

A gas chromatography-QqQ-MS method was developed for the detection of over 150 compounds from different classes (steroids, narcotics, stimulants, beta-blockers, beta-2-agonists and hormone antagonists) in a qualitative way. In the quantitative part, the traditional steroid profile with the most important endogenous steroids is expanded with six minor metabolites, which further improves the detection and identification of endogenous steroid abuse. In addition to these, norandrosterone, salbutamol and the major metabolite of cannabis are also quantified. Methods developed for anti-doping purposes should be subjected to the highest level of quality. Here, the addition of a combination of (deuterated) internal standards allows for an accurate quality control of every single step of the methodology: hydrolysis efficiency, derivatization efficiency and microbiological degradation are monitored in every single sample. Additionally, special attention is paid to the relationships between parameters indicating degradation by micro-organisms and the reliability of the steroid profile. The impact of the degradation is studied by evaluation of the quantities and percentages of 5alpha-androstane-3,17-dione and 5beta-androstane-3,17-dione. The concept of measurement uncertainty was introduced for the evaluation of relative abundances of mass-to-charge ratios and the obtained ranges were compared with the World Anti-Doping Agency regulations on tolerance windows for relative ion intensities. The results indicate that the approaches are similar [12063].

Carbon isotope (CIR)-based analyses

The Achilles’ heel of all carbon isotope ratio (CIR)-based assays however is the necessity of a significant difference between the CIR of the administered steroid and the employed endogenous reference compounds (ERCs). As demonstrated earlier various testosterone formulations of mostly illicit origin exhibit CIRs at natural 13C-values. Here, IRMS analyses focusing on carbon isotope signatures only might disallow determining the prohibited administration of a natural steroid. Further to this, the effect of hormones influencing testicular activity such as human chorionic gonadotrophin (hCG) on steroid profiles and CIRs necessitated consideration [12017].

The detection of steroids originating from synthetic precursors against a background of their chemically identical natural analogues has proven to be a significant challenge for doping control laboratories accredited by the World Anti-Doping Agency (WADA). The complementary application of gas chromatography-mass spectrometry (GC-MS) and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) has been demonstrated to provide specific detection of endogenous steroid misuse for improved anti-doping analysis. Markers of synthetically derived steroids are reviewed on the basis of abnormal urinary excretions and low 13C content. A combinatorial approach is presented for the interpretation of GC-MS and GC-C-IRMS data in the anti-doping context. This methodology can allow all relevant information concerning an individual’s metabolism to be assessed in order to make an informed decision with respect to a doping violation [12077].
**IRMS (isotope ratio mass spectrometry)**

Today, many scientists concerned with forensic and environmental analytics will appreciate the introduction of isotope ratio mass spectrometry (IRMS) as a most innovative tool. Significant IRMS-based knowledge about the systematic variation of $^{13}\text{C}/^{12}\text{C}$ ratios in nature was, however, available around 1950. The fundamental depletion of $^{13}\text{C}$ in organic material was even recognized as early as 1939. This development essentially was brought forward by Alfred Nier. Any scientist currently concerned with IRMS will immediately be familiar with Nier's mass spectrometer design from 1947. In fact, significant development of the ion optics, source design, etc., has taken place in the meanwhile. But ever since Nier's inventions, these instruments feature multiple collectors and the characteristic magnetic sector field. Even more striking, the fundamental design of isotope ratio mass spectrometers has not changed since 1940. Nier built his first mass spectrometer in 1936. But on principle, the concept even dates back to 1918, when AJ Dempster advocated the 180° design as “A New Method of Positive Ray Analysis” Five years earlier, Jj Thomson had shown the usefulness of “Positive Rays as a Method of Chemical Analysis”. So, IRMS is a well proven, rather traditional methodology. The term “continuous-flow isotope ratio mass spectrometry” (CFIRMS) was presumably introduced in 1988 when Preston and McMillan coupled an elemental analyzer to an isotope ratio mass spectrometer via a variable leak. Recent improvements in the methodology mainly concern online coupling and analytical peripherals. The breakthrough thus achieved for doping control can hardly be overestimated. If still challenging, $^{13}\text{C}/^{12}\text{C}$ analysis of urinary steroids by gas chromatography coupled to IRMS is now a standard procedure. However, it merely represents one out of a multitude of emerging applications of stable isotope analysis at natural abundance. The relative nature of $d$ scales must always be kept in mind. As a most important consequence, divisions are not feasible. It follows immediately that measurement uncertainties must not be expressed as coefficients of variation. Due to the design of Nier-type mass spectrometers, the analytical error is largely independent of the measured isotope ratio anyway. When emphasis is on quantitation of the respective contributions, the term source apportionment is more appropriate. In doping control, it is implicitly assumed that there are two distinct sources for urinary steroids: Endogenous steroids and/or synthetic steroids. Therefore, low $^{13}\text{C}/^{12}\text{C}$ ratios are often immediately associated with synthetic origin. However, theoretically and practically the situation is more complicated and this should fundamentally be considered. Terms and definitions for possible sources of urinary steroids are not very consistent. For instance, endogenous steroid is mostly used for a compound synthesized physiologically in an organism. But sometimes it is also used to classify pharmaceuticals with chemical structures identical to those of the physiological compounds [12072].

Delta $^{13}\text{C}$ and delta$^{13}\text{C}$ values of endogenous urinary steroids represent physiological random variables. Measurement uncertainty and biological scatter likewise contribute to the variances. The statistical distributions of negative controls are well investigated, but there is little knowledge about the corresponding distributions of steroid-users. For these reasons valid discrimination of steroid users from non-users by ($^{13}\text{C}/^{12}\text{C}$ analysis of endogenous steroids requires elaborate statistical treatment. Corresponding Bayesian approaches are presented following an introduction to the rationale. The use of mixture models appears appropriate. The distribution of routine data has been deconvolved and characterized accordingly. The mixture components, which presumably represent steroid users and non-users, exhibit considerable overlap. The validity of a given result depends on both the analytical uncertainty and the prior probability of doping offenses. Low analytical uncertainties but high prior probabilities facilitate valid detection of doping offenses. Two recommendations can be deduced. First, before starting an $^{13}\text{C}/^{12}\text{C}$ analysis, any initial suspicion should be well-substantiated. This precludes use of permissive criteria derived
from the steroid profile. Secondly, knowledge of relevant $^{13}\text{C}/^{12}\text{C}$ distributions is required. This must cover representative numbers of authentic steroid users. Finally, it is desirable that the conditional probability for steroid administration rather than the measurement uncertainty is calculated and reported. This quantity possesses superior validity and it is largely independent of laboratory bias. The findings suggest and facilitate flexible handling of decision limits [12073].

The detection of steroids originating from synthetic precursors against a background of their chemically identical natural analogues has proven to be a significant challenge for doping control laboratories accredited by the World Anti-Doping Agency (WADA). The complementary application of gas chromatography-mass spectrometry (GC-MS) and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) has been demonstrated to provide specific detection of endogenous steroid misuse for improved anti-doping analysis. Markers of synthetically derived steroids are reviewed on the basis of abnormal urinary excretions and low $^{13}\text{C}$ content. A combinatorial approach is presented for the interpretation of GC-MS and GC-C-IRMS data in the anti-doping context. This methodology can allow all relevant information concerning an individual's metabolism to be assessed in order to make an informed decision with respect to a doping violation [12074].

Isotope ratio mass spectrometry (IRMS) testing is performed to determine if an atypical steroid profile is due to administration of an endogenous steroid. Androsterone (Andro) and etiocholanolone (Etio), and/or the androstanediols (5alpha- and 5beta-androstane-3alpha,17beta-diol) are typically analyzed by IRMS to determine the $^{13}\text{C}/^{12}\text{C}$ ratio. The ratios of these target compounds are compared to the $^{13}\text{C}/^{12}\text{C}$ ratio of an endogenous reference compound (ERC) such as 5beta-pregnane-3alpha,20alpha-diol (Pdiol). Concentrations of Andro and Etio are high so $^{13}\text{C}/^{12}\text{C}$ ratios can easily be measured in most urine samples. Despite the potentially improved sensitivity of the androstanediols for detecting the use of some testosterone formulations, additional processing steps are often required that increase labour costs and turnaround times. Since this can be problematic when performing large numbers of IRMS measurements, we established thresholds for Andro and Etio that can be used to determine the need for additional androstanediol testing. Using these criteria, 105 out of 2639 urine samples exceeded the Andro and/or Etio thresholds, with 52 of these samples being positive based on Andro and Etio IRMS testing alone. The remaining 53 urine samples had androstanediol IRMS testing performed and 3 samples were positive based on the androstanediol results. A similar strategy was used to establish a threshold for Pdiol to identify athletes with relatively $^{13}\text{C}$-depleted values so that an alternative ERC can be used to confirm or establish a true endogenous reference value. Adoption of a similar strategy by other laboratories can significantly reduce IRMS sample processing and analysis times, thereby increasing testing capacity [13095].

Complementary to carbon isotope ratios (CIR), isotope ratio analysis concerning hydrogen and deuterium (HIR) has received increasing attention in doping controls. Particularly the 2-dimensional analysis of urinary steroids, i.e. combined evaluation of CIR and HIR, was considered as a powerful (though time- and cost-intensive) means allowing to lower reference limits in doping controls and to enable the determination of exogenous steroids comprising CIR signatures close to endogenous values. In that respect, potentially confounding factors have to be assessed and the influence of the deuterium content in drinking water on urinary steroid HIR was measured in a recent study. Despite the drastic influence on the HIR of the body water, only shifts of approximately 30 ‰ in urinary steroids were observed. Hence, the HIR analysis proved robust against diet-induced changes, specifically the ingestion of drinking water with different isotopic signature [13009].
Isoelectric focusing

Isoelectric focusing (IEF) is used to detect recombinant EPO in the urine EPO test. Historically, the EPO test at the Olympics (2000 to 2006) was done on paired blood and urine samples collected simultaneously. The blood test is an indirect test because it does not detect the presence of recombinant EPO. Instead, it measures multiple parameters (e.g., hemoglobin, hematocrit, percentage of reticulocytes) and calculates a score that indicates whether the individual is on or recently off recombinant EPO. Since 2002, EPO tests done by United States sports authorities have included only the urine test, a direct test that identifies recombinant EPO. EPO tests are done on only some of all of the urine samples, upon request by the sports authority. Endogenous human EPO is a glycoprotein with a known amino acid sequence and glycosylation pattern. More precisely, it consists of a family of isoforms (molecules that differ only by their degrees of glycosylation). As a result, the pH at which each isoform bears as many negative charges as positive charges (isoelectric point or PI) is different. Recombinant human EPO differs from endogenous human EPO only by its overall glycosylation pattern (i.e., it consists of a different family of isoforms). The difference in overall pattern of isoforms allows differentiation between recombinant and endogenous human EPO. The urine EPO test, also known as the French test or the IEF test, consists of four steps: sample preparation, IEF, double blotting, and visualization. Sample preparation concentrates EPO by multiple ultrafiltrations that leave the proteins of desired molecular weight in the filtration "retentate." Next, the retentate is deposited on a gel with an embedded pH gradient, and a current is applied to achieve electrophoretic separation of the isoforms (IEF). Unknown samples, reference standards, and known positive and negative quality controls are normally run on each gel. Each sample, standard, or control spreads out in its own "lane." Each isoform is charged; therefore, it migrates in the electrical field until it reaches the distance on the gel at which the pH is equal to its PI. There, the isoform is electrically neutral so it stops migrating. Its position or distance up the gel is key, and the goal of the remaining steps is to visualize it. The first blotting step transfers all proteins (erythropoietic and other) to a first membrane. The membrane is incubated with antibodies specific to erythropoietic proteins. The second blot transfers only these specific antibodies to the second membrane, thus transferring the isoform pattern, but leaving behind all proteins, including some that otherwise would obscure the final image. Visualization is based on chemiluminescence; it involves incubation with a second antibody that binds to the first antibody and a chemical reaction that emits light. The image (electropherogram) is captured with a special digital camera. All steps use commonplace molecular biology techniques. The electropherogram contains one lane per sample, standard, or quality control sample. In each lane, the isoform pattern consists of bands. The pattern (number of bands, positions, relative intensities) allows identification. In common language, a negative EPO test often is discussed as if it reflects the absence of EPO, but of course what it means is that there was no recombinant erythropoietic protein in the urine sample, which normally would (hopefully!) contain natural, endogenous EPO [07011].

Electrospray ionization

Electrospray ionization (ESI) mass spectra of 15 anti-estrogenic substances, beta2-agonists and mesocarb were investigated in terms of fragmentation patterns. On the basis of this product ion information, a simultaneous screening method for anti-estrogenic substances, beta2-agonists and mesocarb was developed for doping control purposes. After hydrolysis, liquid-liquid extraction was adopted for the sample preparation. The recoveries for all compounds were 30 and 96 percent. A single liquid chromatography/tandem mass
spectrometry (LC/MS/MS) analysis could be performed in 13 min for the analysis of 15 anti-
estrogenic substances, beta2-agonists and mesocarb. A quantitative analysis was also validated. Inaccuracies were below ± 12 percent and precisions varied from 0 to 16 percent. The limit of detection was below 10 ng/mL except formestane (300 ng/mL) and aminoglutethimide (100 ng/mL). The validated method was applied for the analysis of excretion samples [07054].

**Capillary electrophoresis**

During the past two decades, chiral capillary electrophoresis (CE) emerged as a promising, effective and economic approach for the enantioselective determination of drugs and their metabolites in body fluids, tissues and in vitro preparations. This review discusses the principles and important aspects of CE-based chiral bioassays, provides a survey of the assays developed during the past 10 years and presents an overview of the key achievements encountered in that time period. Applications discussed encompass the pharmacokinetics of drug enantiomers in vivo and in vitro, the elucidation of the stereoselectivity of drug metabolism in vivo and in vitro, and bioanalysis of drug enantiomers of toxicological, forensic and doping interest. Chiral CE was extensively employed for research purposes to investigate the stereoselectivity associated with hydroxylation, dealkylation, carboxylation, sulfoxidation, N-oxidation and ketoreduction of drugs and metabolites. Enantioselective CE played a pivotal role in many biomedical studies, thereby providing new insights into the stereoselective metabolism of drugs in different species which might eventually lead to new strategies for optimization of pharmacotherapy in clinical practice [10320].

At present the role of capillary electrophoresis in the detection of doping agents in athletes is, for the most part, nonexistent. More traditional techniques, namely gas and liquid chromatography with mass spectrometric detection, remain the gold standard of antidoping tests. This Feature will investigate the in-roads that capillary electrophoresis has made, the limitations that the technique suffers from, and where the technique may grow into being a key tool for antidoping analysis [13093].

**Capillary electrophoresis time-of-flight mass spectrometry**

Capillary electrophoresis coupled to orthogonal accelerated time-of-flight mass spectrometry (CE/TOFMS) was used for the analysis of O- and N-glycopeptides of recombinant human erythropoietin (rhEPO), O(126) and N(83) with a tetraantennary complex type glycan (N(83)-4Ant) were selected as glycopeptide models to develop an optimum CE/TOFMS methodology capable of detecting and characterizing the wide variety of glycopeptides present in the glycoprotein digest. Glycopeptide adsorption in the inner surface of the fused-silica capillary was prevented after using a capillary conditioning of 1 M HAc between runs. On the other hand, different acidic conditions in the sheath liquid (SL) and in the background electrolyte (BGE) were tested with the aim of studying their influence in glycopeptide fragmentation. Finally, the fragmentor voltage value of the TOF-MS instrument was optimized to avoid the involuntary fragmentation of the native glycopeptides. Hence, the established method may be regarded as an excellent starting point to obtain reliable glycopeptide maps of complex glycoproteins such as rhEPO by CE/TOFMS [11430].

**Vacuum MALDI-linear ion trap mass spectrometry**

379
Detection of doping agents in urine frequently requires extensive separation prior to chemical analyses. Gas or liquid chromatography coupled to mass spectrometry has produced accurate and sensitive assays, but chromatographic separations require time and, sometimes, chemical derivatization. To avoid such tedious and lengthy procedures, vacuum matrix-assisted laser desorption ionization (vMALDI) coupled with the linear ion trap mass spectrometry (LIT/MS) technique is tested for its applicability as a rapid screening technique. Commonly used doping agents like nandrolone, boldenone, trenbolone, testosterone, and betamethasone were chosen as study compounds. Different MALDI matrixes like alpha-cyano-4-hydroxycinnamic acid (CHCA), dihydroxy benzoic acid (DHB) with and without cetyl trimethyl ammonium bromide (CTAB), a surfactant, and meso-tetrakis(pentafluorophenyl) porphyrin (F20TPP) were tested. Among them, F20TPP (MW 974.57 Da) was selected as the preferred matrix owing to the lack of interfering matrix peaks at the lower mass range (m/z 100-700). Urine samples spiked with study compounds were processed by solid-phase extraction (SPE) and consistently detected through a linear range of 0.1-100 ng/mL. The limit of detection and lower limit of quantification for all five analytes have been determined to be 0.03 and 0.1 ng/mL, respectively, in urine samples. Testosterone-d3 was used as an internal standard, and the quantitative measurements were achieved by the selective reaction monitoring (SRM) mode. The method was validated and showed consistency in the results. Hence, vMALDI-LIT/MS can be used as a rapid screening method to complement the traditional GC/MS and LC/MS techniques for simultaneous identification, confirmation, and quantification of doping agents in urine [06039].

Hyphenated mass spectrometric techniques

Hyphenated mass spectrometric techniques, particularly gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS), are indispensable tools in clinical and forensic toxicology and in doping control owing to their high sensitivity and specificity. They are used for screening, library-assisted identification and quantification of drugs, poisons and their metabolites, prerequisites for competent expertise in these fields. In addition, they allow the study of metabolism of new drugs or poisons as a
basis for developing screening procedures in biological matrices, most notably in urine, or toxicological risk assessment. Concepts and procedures using GC/MS and LC/MS techniques in the areas of analytical toxicology and the role of mass spectral libraries are presented and discussed in this feature article [06040].

**siRNA**

Small interfering ribonucleic acid (siRNA) molecules can effect the expression of any gene by inducing the degradation of mRNA. Therefore, these molecules can be of interest for illicit performance enhancement in sports by affecting different metabolic pathways. An example of an efficient performance-enhancing gene knockdown is the myostatin gene that regulates muscle growth. One study was carried out to provide a tool for the mass spectrometric detection of modified and unmodified siRNA from plasma samples. The oligonucleotides are purified by centrifugal filtration and the use of an miRNA purification kit, followed by flow-injection analysis using an Exactive mass spectrometer to yield the accurate masses of the sense and antisense strands. Although chromatography and sensitive mass spectrometric analysis of oligonucleotides are still challenging, a method was developed and validated that has adequate sensitivity (limit of detection 0.25-1 nmol/mL and performance (precision 11-21 %, recovery 23-67 %) for typical antisense oligonucleotides currently used in clinical studies [10442].

Uncovering manipulation of athletic performance via small interfering (si)RNA is an emerging field in sports drug testing. Due to the potential to principally knock down every target gene in the organism by means of the RNA interference pathway, this facet of gene doping has become a realistic scenario. In the present study, two distinct model siRNAs comprising 21 nucleotides were designed as double strands which were perfect counterparts to a sequence of the respective messenger RNA coding the muscle regulator myostatin of Rattus norvegicus. Several modified nucleotides were introduced in both the sense and the antisense strand comprising phosphothioates, 2'-O-methylation, 2'-fluoro-nucleotides, locked nucleic acids and a cholesterol tag at the 3'-end. The model siRNAs were applied to rats at 1 mg/kg (i.v.) and blood as well as urine samples were collected. After isolation of the RNA by means of a RNA purification kit, the target analytes were detected by liquid chromatography-high resolution/high accuracy mass spectrometry (LC-HRMS). Analytes were detected as modified nucleotides after alkaline hydrolysis, as intact oligonucleotide strands (top-down) and by means of denaturing SDS-PAGE analysis. The gel-separated siRNA was further subjected to in-gel hydrolysis with different RNases and subsequent identification of the fragments by untargeted LC-HRMS analysis (bottom-up, “experimental RNomics”). Combining the results of all approaches, the identification of several 3'-truncated urinary metabolites was accomplished and target analytes were detected up to 24 h after a single administration. Simultaneously collected blood samples yielded no promising results. The methods were validated and found fit-for-purpose for doping controls [13106].

**Mass spectrometric detection of siRNA**

Small interfering ribonucleic acid (siRNA) molecules can effect the expression of any gene by inducing the degradation of mRNA. Therefore, these molecules can be of interest for illicit performance enhancement in sports by affecting different metabolic pathways. An example of an efficient performance-enhancing gene knockdown is the myostatin gene that regulates muscle growth. One study was carried out to provide a tool for the mass spectrometric detection of modified and unmodified siRNA from plasma samples. The oligonucleotides are
purified by centrifugal filtration and the use of an miRNA purification kit, followed by flow-injection analysis using an Exactive mass spectrometer to yield the accurate masses of the sense and antisense strands. Although chromatography and sensitive mass spectrometric analysis of oligonucleotides are still challenging, a method was developed and validated that has adequate sensitivity (limit of detection 0.25-1 nmol/mL) and performance (precision 11-21 %, recovery 23-67 %) for typical antisense oligonucleotides currently used in clinical studies [10322].

High-resolution liquid chromatography-time-of-flight mass spectrometry

A unification of doping-control screening procedures of prohibited small molecule substances – including stimulants, narcotics, steroids, beta2-agonists and diuretics – is highly urgent in order to free resources for new classes such as banned proteins. Conceptually this may be achieved by the use of a combination of one gas chromatography-time-of-flight mass spectrometry method and one liquid chromatography-time-of-flight mass spectrometry method. In this work a quantitative screening method using high-resolution liquid chromatography in combination with accurate-mass time-of-flight mass spectrometry was developed and validated for determination of glucocorticosteroids, beta2 agonists, thiazide diuretics, and narcotics and stimulants in urine. To enable the simultaneous isolation of all the compounds of interest and the necessary purification of the resulting extracts, a generic extraction and hydrolysis procedure was combined with a solid-phase extraction modified for these groups of compounds. All 56 compounds are determined using positive electrospray ionisation with the exception of the thiazide diuretics for which the best sensitivity was obtained by using negative electrospray ionisation. The results show that, with the exception of clenhexyl, procaterol, and reproterol, all compounds can be detected below the respective minimum required performance level and the results for linearity, repeatability, within-lab reproducibility, and accuracy show that the method can be used for quantitative screening. If qualitative screening is sufficient the instrumental analysis may be limited to positive ionisation, because all analytes including the thiazides can be detected at the respective minimum required levels in the positive mode. The results show that the application of accurate-mass time-of-flight mass spectrometry in combination with generic extraction and purification procedures is suitable for unification and expansion of the window of screening methods of doping laboratories. Moreover, the full-scan accurate-mass data sets obtained still allow retrospective examination for emerging doping agents, without re-analyzing the samples [10036].

Ultra-high-pressure liquid chromatography-quadrupole time-of-flight mass spectrometry

For doping control, analyses of samples are generally achieved in two steps: a rapid screening and, in the case of a positive result, a confirmatory analysis. A two-step methodology based on ultra-high-pressure liquid chromatography coupled to a quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS) was developed to screen and confirm 103 doping agents from various classes (e.g., beta-blockers, stimulants, diuretics, and narcotics). The screening method was presented in a previous article as part I (i.e. fast analysis of doping agents in urine by ultra-high-pressure liquid chromatography-quadrupole time-of-flight mass spectrometry. part I: screening analysis). For the confirmatory method, basic, neutral and acidic compounds were extracted by a dedicated solid-phase extraction (SPE) in a 96-well plate format and detected by MS in the tandem mode to obtain precursor and characteristic product ions. The mass accuracy and the elemental composition of
precursor and product ions were used for compound identification. After validation including matrix effect determination, the method was considered reliable to confirm suspect results without ambiguity according to the positivity criteria established by the World Anti-Doping Agency. Moreover, an isocratic method was developed to separate ephedrine from its isomer pseudoephedrine and cathine from phenylpropanolamine in a single run, what allowed their direct quantification in urine [10037].

The urinary steroid profile is constituted by anabolic androgenic steroids, including testosterone and its relatives, that are extensively metabolized into phase II sulfated or glucuronidated steroids. The use of liquid chromatography coupled to mass spectrometry (LC-MS) is an issue for the direct analysis of conjugated steroids, which can be used as urinary markers of exogenous steroid administration in doping analysis, without hydrolysis of the conjugated moiety. In this study, a sensitive and selective ultra high-pressure liquid chromatography coupled to quadrupole time-of-flight mass spectrometer (UHPLC-QTOF-MS) method was developed to quantify major urinary metabolites simultaneously after testosterone intake. The sample preparation of the urine (1 mL) was performed by solid-phase extraction on Oasis HLB sorbent using a 96-well plate format. The conjugated steroids were analyzed by UHPLC-QTOF-MS(E) with a single-gradient elution of 36 min (including re-equilibration time) in the negative electrospray ionization mode. MS(E) analysis involved parallel alternating acquisitions of both low- and high-collision energy functions. The method was validated and applied to samples collected from a clinical study performed with a group of healthy human volunteers who had taken testosterone, which were compared with samples from a placebo group. Quantitative results were also compared to GC-MS and LC-MS/MS measurements, and the correlations between data were found appropriate. The acquisition of full mass spectra over the entire mass range with QTOF mass analyzers gives promise of the opportunity to extend the steroid profile to a higher number of conjugated steroids [11034].

Isotachophoresis sample stacking

A simple and effective method of capillary electrophoresis-amperometric detection (CE-AD) coupled with transient isotachophoresis (tITP) was developed for the trace determination of doping substances. Compared with the conventional capillary electrophoresis method, the maximum enhancement factor in terms of peak heights was up to 5500-fold when the tITP technique was adopted. Under the optimum conditions, detection limit for methylephedrine (MDP), celiprolol (CEL), sotalol (SOT) and indapamide (IDP) were $4.2 \times 10^{-14}$, $6.3 \times 10^{-13}$, $5.8 \times 10^{-14}$ and $9.5 \times 10^{-13}$ mol/L, respectively. The proposed method was successfully applied to determine the contents of SOT and IDP in real urine sample, and the excretion curve of IDP within 48 h was also investigated. The recoveries of the four doping in urine ranged from 90 to 102 percent [10031].

Polar organic chemical integrative samplers (POCIS)

Polar organic chemical integrative samplers (POCIS) were calibrated in situ for selected illicit drugs and their metabolites at a sewage treatment works. Eleven out of 13 target compounds were detected and eight of those exhibited linear uptake kinetics with sampling rates between 0.035 and 0.150 L/d. Subsequently POCIS were deployed for 2 week periods over the course of a whole year, in order to examine trends in drug usage. Amphetamine and methamphetamine showed several similar peaks in concentration during the course of the year as did cocaine and two of its metabolites. Low levels of ecstasy were observed, with a
prominent peak in May and a steady increase toward the end of the year. The antihistamine Cetirizine showed a clear increase in use during the summer months as expected and back calculation of the yearly dosage from POCIS accumulations yielded very similar results to that registered in the Norwegian prescription database. Estimations of cocaine usage using the parent compound averaged between 0.31 and 2.8 g/d per 1000 inhabitants. POCIS is a cost-effective technique for the long-term monitoring of drug usage of a defined population and may overcome the difficulties of representative sampling associated with autosampling equipment [11431].

AICAR

Recently, a new class of prohibited substances reached in the focus of doping control laboratories and their misuse was classified as gene doping. The adenosine monophosphate activated protein kinase activator 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR) was found to significantly enhance the endurance even in sedentary mice after treatment. Due to endogenous production of AICAR in healthy humans, considerable amounts were present in the circulation and, thus, were excreted into urine. Considering these facts, one study was initiated to fix reference values of renally cleared AICAR in elite athletes. Therefore a quantitative analytical method by means of isotope-dilution liquid chromatography (analytical column: C$_6$-phenyl) coupled to tandem mass spectrometry, after a sample preparation consisting of a gentle dilution of native urine, was developed. Doping control samples of 499 athletes were analysed, and AICAR concentrations in urine were determined. The mean AICAR value for all samples was 2,186 ng/mL with a standard deviation of 1,655 ng/mL. Concentrations were found to differ depending on gender, type of sport and type of sample collection (in competition/out of competition). The method was fully validated for quantitative purposes considering the parameters linearity, inter- (12 %, 7 % and 10 %) and intraday precision (14 %, 9 % and 12 %) at low, mid and high concentration, robustness, accuracy (approximately 100 %), limit of quantification (100 ng/mL), stability and ion suppression effects, employing an in-house synthesised $^{13}$C$_5$-labelled AICAR as internal standard [10032].

Microwave assisted extraction

It was described a fast and efficient method for the liquid/liquid extraction from human urine of different classes of drugs, included in the list of prohibited substances published every year by the World Anti-doping Agency, using microwave irradiation. Liquid/liquid extraction was conducted in a temperature controlled single beam microwave oven equipped with an extraction unit and closed vessels. The effects of microwave power and time on the liquid/liquid extraction process were investigated utilizing different organic solvents. The optimum power was found to be 600 W (generating a temperature of 70 degrees C) with an incubation time of 30-60 s for the most thermolable constituents such as triamcinolone, prednisolone, chlorthiazide, chlorthalidone, epi-trembolone and oxandrolone, and 1020 W (generating a temperature of 150 degrees C) with an incubation time of 30-60 s for the other compounds considered in this study. The effectiveness of this approach was evaluated by GC-MS (anabolic steroids, beta$_2$-agonists and narcotics) and by LC-MS/MS (diuretics, glucocorticoids and beta-blockers) analyzing more than 20 different urine samples spiked with the compounds considered in this study. The results showed that the effect of microwave irradiation on the liquid/liquid extraction process was very remarkable: the total sample preparation time can be shortened by 9 min compared to the traditional method (30-60 s instead of 10 min); furthermore, a significant increase in the recovery was recorded for
specific compounds such as terbutaline and several diuretics. In addition to the above the repeatability of the extraction recoveries, the limits of detection and the matrix interferences were comparable with the reference methods, presently accredited under the ISO17025, followed by the World Anti-doping Agency accredited anti-doping laboratory of Rome [10030].

In one contribution it was tested the possibility to use microwave irradiation for the screening and confirmation pre-treatment steps of hydroxyethylstarch, with the aim to speed up gas chromatography-mass spectrometric procedures. Acid hydrolysis and derivatization processes were conducted in a temperature-controlled single beam microwave oven for organic synthesis. The kinetics of hydroxyethylstarch chemical hydrolysis and derivatization were investigated at different microwave power, incubation temperature and incubation time. The best hydrolysis conditions were found at a microwave power value of 1200W (T 100°C) with an incubation time of 2 min; whereas the best derivatization conditions were found at a microwave power value of 1020W (T 100°C) with an incubation time of 5 min. The effectiveness of this approach was evaluated by gas chromatography-mass spectrometry analyzing more than 20 different pools of blank urine samples spiked with hydroxyethylstarch at a concentration of 1mg/mL. The results showed that the effect of microwave irradiation on the chemical hydrolysis process was very remarkable: the total sample preparation time can be shortened by 58 min compared to the reference method (2 min instead of 60 min). In addition to this, the time necessary for the derivatization process can also be drastically shortened with respect to the reference procedure (5 min instead of 30 min). The repeatability of the hydrolysis and derivatization recoveries, the limit of detection and the matrix interferences were comparable to the reference method accredited under the ISO 17025 guidelines and presently followed by the accredited sports anti-doping laboratory of Rome [10321].

Ultrasound and microwave

A comparison between ultrasonication and microwave irradiation as tools to achieve a rapid sample treatment for the analysis of banned doping substances in human urine by means of gas chromatography-mass spectrometry (GC-MS) was performed. The following variables were studied and optimised: time of treatment, temperature, microwave power and ultrasonic amplitude. The results were evaluated and compared with those achieved by the routine method used in the World Anti-Doping Agency (WADA) accredited Antidoping Laboratory of Rome. Only under the effect of the ultrasonic field was it possible to enhance the enzymatic hydrolysis reaction rate of conjugated compounds. Similar reaction yield to the routine method was achieved after 10 min for most compounds. Under microwave irradiation, denaturation of the enzyme occurs for high microwave power. The use of both ultrasonic or microwave energy to improve the reaction rate of the derivatisation of the target compounds with trimethylidosilane/methyl-N-trimethylsilyl trifluoroacetamide (TMSI/MSTFA/NH(4)I/2-mercaptoethanol) was also evaluated. To test the use of the two systems in the acceleration of the reaction with TMSI, a pool of 55 banned substances and/or their metabolites were used. After 3 min of ultrasonication, 34 of the 55 compounds had recoveries similar to those obtained with the classic procedure that lasts for 30 min, 18 increased to higher silylation yields, and for the compounds 13beta,17alpha-diethyl-3alpha,17beta-dihydroxy-5alpha-3alpha,17beta,21-triol (norethandrolone metabolite 1), metoprolol and metipranolol the same results were obtained increasing the ultrasonication time to 5 min. Similar results were obtained after 3 min of microwave irradiation at 1,200 W. In this case, 30 of the 55 compounds had recoveries similar to the classic procedure whilst 18 had higher silylation yields. For the compounds 3alpha-hydroxy-1alpha-methyl-5alpha-androstan-17-one (mesterolone metabolite 1), 17alpha-ethyl-5beta-estrane-3alpha,17beta,21-triol (norethandrolone metabolite 1),
epoxandrolone, 4-chloro-6beta,17beta-dihydroxy-17alpha-methyl-1,4-androstadien-3-one (chlormetandienone metabolite 1), carphedon, esmolol and bambuterol the same results were obtained after 5 min under microwave irradiation [11045].

**Liquid/liquid extraction**

It was described a fast and efficient method for the liquid/liquid extraction from human urine of different classes of drugs, included in the list of prohibited substances published every year by the World Anti-doping Agency, using microwave irradiation. Liquid/liquid extraction was conducted in a temperature controlled single beam microwave oven equipped with an extraction unit and closed vessels. The effects of microwave power and time on the liquid/liquid extraction process were investigated utilizing different organic solvents. The optimum power was found to be 600W (generating a temperature of 70 degrees C) with an incubation time of 30-60s for the most thermolable constituents such as triamcinolone, prednisolone, chlorthiazide, chlorthalidone, epi-trembolone and oxandrolone, and 1020W (generating a temperature of 150 degrees C) with an incubation time of 30-60s for the other compounds considered in this study. The effectiveness of this approach was evaluated by GC-MS (anabolic steroids, beta2-agonists and narcotics) and by LC-MS/MS (diuretics, glucocorticoids and beta-blockers) analyzing more than 20 different urine samples spiked with the compounds considered in this study. The results showed that the effect of microwave irradiation on the liquid/liquid extraction process was very remarkable: the total sample preparation time can be shortened by 9min compared to the traditional method (30-60s instead of 10min); furthermore, a significant increase in the recovery was recorded for specific compounds such as terbutaline and several diuretics [10033].

**Two step derivatization**

Two-step derivatization procedures were developed for the enhancement of the positive ESI in LC-MS detection of anabolic androgenic steroids, a class of prohibited substances with limited ionization efficiency in atmospheric pressure interfaces. The developed procedures are based on the esterification of hydroxyl groups of anabolic steroids with picolinic acid, followed by conversion of carbonyl groups to Schiff bases by either Girard's reagent T or 2-hydrazino pyridin. Ionization efficiency for the model derivatized compounds 19-norandrosterone (nandrolone main metabolite) and methasterone was higher by almost two orders of magnitude compared with the respective efficiency of the underivatized compounds. The obtained derivatives provided a significant improvement in the ESI sensitivity, compared with those of underivatized molecules in positive LC-ESI-ion trap-MS full-scan mode [12082].

**Adsorption to metallic plasmonic nanoparticles**

A comparative study of different plasmonic nanoparticles with different morphologies (nanospheres and triangular nanoprisms) and metals (Ag and Au) was done in this work and applied to the ultrasensitive detection of aminoglutethimide (AGI) drug by surface enhanced Raman spectroscopy (SERS) and plasmon resonance. AGI is an aromatase inhibitor used as an antitumoral drug with remarkable pharmacological interest and also in illegal sport doping. The application of very sensitive spectroscopic techniques based on the localization of an electromagnetic field on plasmonic nanoparticles confirms the previous study of the adsorption of drugs onto a metal surface due to the near field character of these techniques.
The adsorption of AGI on the above substrates was investigated at different pH values and surface coverages, and the results were analyzed on the basis of AGI/metal affinity, considering the interaction mechanism, the existence of two binding sites in AGI, and the influence of the interface on the adsorption in terms of surface charge due to the presence of other ions linked to the surface. Finally, a comparative quantitative detection of AGI was performed on both spherical and triangular nanoprisnm nanoparticles, and a limit of detection lower than those reported so far was deduced on the latter nanoparticles [12083].

Surface plasmon resonance

Within this communication, consistent evidence of a quantitative biosensing principle for steroidal residue analysis is presented. One approach uses a simple method for the quantitative determination of an anabolic agent called stanozolol (Sz). Sz (Mw 328) is widely used in sports, horse racing and as a growth promoter in animals for human consumption. Through the use of localised surface plasmons (LSPs), sustained by three-dimensional noble metal nanostructures, we have developed a highly specific, label-less immunosensor for the detection of this small organic molecule to low levels (nM range). A main practical advantage over conventional flat extended film surface plasmon resonance (SPR) systems is the simplicity of the optical configuration, since there is no need for cumbersome total internal reflection illumination, thus making integration easier. In addition, the active area of the LSP-based sensor is smaller, decreasing the minimum detectable number of molecules involved in the binding event. Assay times are short and the set-up is comprised of relatively cheap instrumentation. Detection levels found here are comparable with SPR, even at this early stage of development and with further modifications, we envisage sensing down to pM (10^{-12}) levels [06042].

Doping analysis relies on the determination of prohibited substances that should not be present in the body of an athlete or that should be below a threshold value. In the case of xenobiotics their mere presence is sufficient to establish a doping offence. However, in the case of human biotics the analytical method faces the difficulty of distinguishing between endogenous and exogenous origin. For this purpose ingenious strategies have been implemented, often aided by state-of-the-art technological advancements such as mass spectrometry in all its possible forms. For larger molecules, i.e. protein hormones, the innate structural complexity, the heterogeneous nature, and the extremely low levels in biological fluids have rendered the analytical procedures heavily dependent of immunological approaches. Although approaches these confer specificity and sensitivity to the applications, most rely on the use of two, or even three, antibody incubations with the consequent increment in assay variability. Moreover, the requirement for different antibodies that separately recognise different epitopes in screening and confirmation assays further contributes to differences encountered in either measurement. The development of analytical techniques to measure interactions directly, such as atomic force microscopy, quartz crystal microbalance or surface plasmon resonance, have greatly contributed to the accurate evaluation of molecular interactions in all fields of biology, and expectations are that this will only increase. Here, an overview is provided of surface plasmon resonance, and its particular value in application to the field of doping analysis [11040].

Isotope ratio mass spectrometry

Detecting the misuse of endogenously occurring steroids, i.e. steroids such as testosterone that are produced naturally by humans, is one of the most challenging issues in doping
control analysis. The established thresholds for urinary concentrations or concentration ratios such as the testosterone/epitestosterone quotient are sometimes inconclusive owing to the large biological variation in these parameters. For more than 15 years, doping control laboratories focused on the carbon isotope ratios of endogenous steroids to distinguish between naturally elevated steroid profile parameters and illicit administration of steroids. A variety of different methods has been developed throughout the last decade and the number of different steroids under investigation by isotope ratio mass spectrometry has recently grown considerably. Besides norandrostosterone, boldenone was found to occur endogenously in rare cases and the misuse of corticosteroids or epitestosterone can now be detected with the aid of carbon isotope ratios as well. In addition, steroids excreted as sulfoconjugates were investigated, and the first results regarding hydrogen isotope ratios recently became available. All of these will were presented in detail within the review together with some considerations on validation issues and on identification of parameters influencing steroidal isotope ratios in urine [11041].

ETD and CID tandem mass spectrometry

Identification of an unknown substance without any information remains a daunting challenge despite advances in chemistry and mass spectrometry. However, an unknown cyclic peptide in a sample with very limited volume seized at a Pennsylvania racetrack has been successfully identified. The unknown sample was determined by accurate mass measurements to contain a small unknown peptide as the major component. Collision-induced dissociation (CID) of the unknown peptide revealed the presence of Lys (not Gln, by accurate mass), Phe, and Arg residues, and absence of any y-type product ion. The latter, together with the tryptic digestion results of the unusual deamidation and absence of any tryptic cleavage, suggests a cyclic structure for the peptide. Electron-transfer dissociation (ETD) of the unknown peptide indicated the presence of Gln (not Lys, by the unusual deamidation), Phe, and Arg residues and their connectivity. After all the results were pieced together, a cyclic tetrapeptide, cyclo[Arg-Lys-N(C(6)H(9))Gln-Phe], is proposed for the unknown peptide. Observations of different amino acid residues from CID and ETD experiments for the peptide were interpreted by a fragmentation pathway proposed, as was preferential CID loss of a Lys residue from the peptide. ETD was used for the first time in sequencing of a cyclic peptide; product ions resulting from ETD of the peptide identified were categorized into two types and named pseudo-b and pseudo-z ions that are important for sequencing of cyclic peptides. The ETD product ions were interpreted by fragmentation pathways proposed. Additionally, multi-stage CID mass spectrometry cannot provide complete sequence information for cyclic peptides containing adjacent Arg and Lys residues. The identified cyclic peptide has not been documented in the literature, its pharmacological effects are unknown, but it might be a "designer" drug with athletic performance-enhancing effects [11042].

Protein chips

Sport and doping are a contradiction in terms, however, doping abuse in sports has been a serious problem for many years. The systematic screening of every athlete for all prohibited drugs should be an indispensable feature of the Olympic Games. The gas chromatography mass spectrometry method is reserved as a reference method, but is limited by its low throughput. The advent of protein chip technology may enable the screening of all athletes for any illegal use of drugs [06043].
SIRT1-activating drugs

The NAD(+) depending enzyme SIRT1 regulates the mitochondrial biogenesis, fat and glucose metabolism through catalyzing the deacetylation of several metabolism-related protein-substrates. Recently, synthetic activators of SIRT1 referred to as STACs (Sirtuin activating compounds, e.g. SRT2104) were identified and tested in clinical studies for the treatment of aging-related diseases such as type 2 diabetes, Alzheimer's and obesity. Although the mechanism of SIRT1 activation by small molecules has caused considerable controversy, STACs demonstrated a significant performance enhancement in mice experiments including an improvement of endurance, muscle strength, and locomotor behavior. Due to their potential to increase exercise tolerance in healthy individuals, SIRT1 activators are currently being monitored by anti-doping authorities. In the present study, the in vivo metabolic clearance of three SIRT1 activators was investigated in rats by the collection of urine, DBS (dried blood spots) and plasma samples following a single oral administration. The resulting metabolic products were studied by positive electrospray ionization - (tandem) mass spectrometry and confirmed by the comparison with in vitro generated metabolites using human and rat liver microsomal preparations. Subsequently, a screening procedure for five SIRT1 activators and the metabolite M1-SRT1720 in DBS specimens was developed. Liquid-liquid-extraction and liquid chromatography/tandem mass spectrometry was employed based on diagnostic ion transitions recorded in multiple reaction monitoring mode and two deuterated internal standards namely d8-SRT1720 and d8-M1-SRT1720 were utilized. The doping control assay was characterized with regard to specificity, limit of detection (10-50 ng/mL), recovery (65-83 %) and imprecision (7-20 %) and ion suppression/enhancement effects (<10 %), demonstrating its fitness-for-purpose for sports drug testing applications [13101].

The efficiency of Sirtuin1, a major target for the treatment of various metabolic disorders such as inflammation and type 2 diabetes mellitus, can be modulated via low molecular mass SIRT1 activators (e.g. resveratrol, SRT1720, and SRT2104). The administration of such compounds results in increased deacetylation of substrates including p53, FOXO1, and PGC1alpha, potentially leading to an improved physical performance. Consequently, proactive and preventive anti-doping measures are required and an assay dedicated to serum and plasma was desirable. Model substances of emerging SIRT1 drug candidates were obtained and synthesized and their mass spectrometric behavior following positive or negative electrospray ionization and collision-induced dissociation was elucidated using low and high resolution/high accuracy (tandem) mass spectrometry. Subsequently, a screening and confirmation procedure necessitating 100 microL of plasma was established employing liquid chromatography/tandem mass spectrometry (LC/MS/MS) based on diagnostic ion transitions recorded in multiple reaction monitoring mode. Sample preparation consisted of the addition of two deuterated internal standards (D(8)-SRT1720 and D(4)-resveratrol) to the plasma specimen and subsequent protein precipitation. Characteristic product ions indicative of the core structures of the model analytes were characterized and utilized for the development of a multi-analyte LC/MS/MS detection method applicable to sports drug testing programs. The doping control assay was validated with regard to specificity, limits of detection (0.1-1 ng/mL), recoveries (90-98 %), intraday and interday precisions (2-18%), and ion suppression/enhancement effects. It was concluded that the fragmentation pathways of SRT1720 and 4 SIRT1 activator models based on a common thiazole-imidazole nucleus as well as two different complementary activators (SIRT1 activator 3 and CAY10602), comprising a quinoxaline core, were studied. The resulting information was used to establish and validate a sports drug testing methodology relevant for an efficient and timely anti-doping procedure, targeting a new class of emerging therapeutics possessing significant potential for misuse in elite and amateur sport [13102].
The enzyme SIRT1 is a metabolic key regulator in mitochondrial biogenesis, fat and glucose metabolism. Its activation through pharmaceutical SIRT1 activators such as SRT2104 results in an increased deacetylation of substrates representing important targets for the treatment of metabolic diseases. Moreover, SRT1720 was found to enhance the physical performance of mice. As SIRT1 activators might therefore be relevant in a doping control context, metabolism studies of target substances need be conducted in order to develop a detection assay for SIRT1 activators in urine. In the present study, the in vitro metabolism of five SIRT1 activators was investigated using human liver microsomes. The mass spectrometric behavior of the resulting metabolites following positive electrospray ionization and collision-induced dissociation was elucidated by high-resolution/high-accuracy (tandem) mass spectrometry, and confirmation of the structure of a major metabolite of SRT1720 was accomplished by chemical synthesis. Subsequently, a screening procedure for urine samples was developed employing liquid-liquid-extraction and liquid chromatography/tandem mass spectrometry based on diagnostic ion transitions recorded in multiple reaction monitoring mode and the use of d8-SRT1720 as deuterated internal standard. The method was validated with regard to specificity, sensitivity (limit of detection 0.5 ng/ml), recovery (88-99 %) and imprecision (7-18 %) as well as ion suppression/enhancement effects (<10 %), demonstrating its fitness-for-purpose for sports drug testing applications [13097].

Bioassay-guided fractionation

Biological tests can be used to screen samples for large groups of compounds having a particular effect, but it is often difficult to identify a specific compound when a positive effect is observed. The identification of an unknown compound is a challenge for analytical chemistry in environmental analysis, food analysis, as well as in clinical and forensic toxicology. In this study bioassay-guided fractionation, ultra high performance liquid chromatography combined with time-of-flight mass spectrometry (UHPLC/TOFMS) and accurate mass database searching was tested to detect and identify unknown androgens. Herbal mixtures and sport supplements were tested using an androgen bioassay and modifications in sample preparations were carried out in order to activate inactive pro-androgens, androgen esters and conjugated androgens to enable their detection in the bioassay. Two of the four herbal mixtures tested positive and bioassay-guided fractionation followed by UHPLC/TOFMS of positive fractions resulted in the identification of nortestosterone phenylpropionate, testosterone cyclohexanecarboxylate and methyltestosterone. Three of the four sport supplements reacted toxic in the bioassay or gave inconclusive results and were further investigated using UHPLC/TOFMS in combination with data processing software and an accurate mass database having approximately 40,000 entries. This accurate mass database was derived from the PubChem database on the internet and coupled to the TOFMS software. This resulted in the tentative identification of several androgens, including methylboldenone, testosterone and the androgen esters methyltestosterone propionate or testosterone isobutyrate, testosterone buciclate and methylenetestosterone acetate. The study showed that bioassay-guided fractionation in combination with UHPLC/TOFMS analysis is a useful procedure to detect, isolate and identify unknown androgens in suspected samples. As an alternative, the use of data processing software in combination with an accurate mass database and coupled on-line with the TOFMS instrument software enabled the identification of androgens and androgen esters in the chromatogram even without bioassay-guided fractionation [10038].
In one study, the use of equine liver/lung microsomes and S9 tissue fractions were used to study the metabolism of the androgenic/anabolic steroid stanozolol as an example of the potential of in vitro technologies in sports drug surveillance. In vitro incubates were analysed qualitatively alongside urine samples originating from in vivo stanozolol administrations using LC-MS on a high-resolution accurate mass Thermo Orbitrap Discovery instrument, by LC-MS/MS on an Applied Biosystems Sciex 5500 Q Trap and by GC-MS/MS on an Agilent 7000A. Using high-resolution accurate mass full scan analysis on the Orbitrap, equine liver microsome and S9 in vitro fractions were found to generate all the major phase-1 metabolites observed following in vivo administrations. Additionally, analysis of the liver microsomal incubates using a shallower HPLC gradient combined with various MS/MS functions on the 5500 Q trap allowed the identification of a number of phase 1 metabolites previously unreported in the equine or any other species. Comparison between liver and lung S9 metabolism showed that the liver was the major site of metabolic activity in the equine. Furthermore, using chemical enzyme inhibitors that are known to be selective for particular isoforms in other species suggested that an enzyme related to CYP2C8 may be responsible for the production of 16-hydroxy-stanozolol metabolites in the equine. In summary, the in vitro and in vivo phase 1 metabolism results reported herein compare well and demonstrate the potential of in vitro studies to compliment the existing in vivo paradigm and to benefit animal welfare through a reduction and refinement of animal experimentation [10039].

Mammalian reporter gene bioassays

Anabolic androgenic steroids (AAS) share the activation of the androgen receptor (AR) as common mechanism of action. The mammalian androgen responsive reporter gene assay (AR CALUX bioassay), measuring compounds interacting with the AR can be used for the analysis of AAS without the necessity of knowing their chemical structure beforehand, whereas current chemical-analytical approaches may have difficulty in detecting compounds with unknown structures, such as designer steroids. One study demonstrated that AAS prohibited in sports and potential designer AAS can be detected with this AR reporter gene assay, but that also additional steroid activities of AAS could be found using additional mammalian bioassays for other types of steroid hormones. Mixtures of AAS were found to behave additively in the AR reporter gene assay showing that it is possible to use this method for complex mixtures as are found in doping control samples, including mixtures that are a result of multi drug use. To test if mammalian reporter gene assays could be used for the detection of AAS in urine samples, background steroidal activities were measured. AAS-spiked urine samples, mimicking doping positive samples, showed significantly higher androgenic activities than unspiked samples. GC-MS analysis of endogenous androgens and AR reporter gene assay analysis of urine samples showed how a combined chemical-analytical and bioassay approach can be used to identify samples containing AAS. The results indicate that the AR reporter gene assay, in addition to chemical-analytical methods, can be a valuable tool for the analysis of AAS for doping control purposes [10040].

Transcriptome analysis

Evolving challenges require evolving responses. The use of illicit performance enhancing drugs by athletes permeates the reality and the perception of elite sports. New drugs with ergogenic or masking potential are quickly adopted, driven by a desire to win and the necessity of avoiding detection. To counter this trend, anti-doping authorities are continually refining existing assays and developing new testing strategies. In the post-genome era,
genetic- and molecular-based tests are being evaluated as potential approaches to detect new and sophisticated forms of doping. Transcriptome analysis, in which a tissue's complement of mRNA transcripts is characterized, is one such method. The quantity and composition of a tissue's transcriptome is highly reflective of milieu and metabolic activity. There is much interest in transcriptional profiling in medical diagnostics and, as transcriptional information can be obtained from a variety of easily accessed tissues, similar approaches could be used in doping control. One article briefly reviewed current understanding of the transcriptome, common methods of global analysis of gene expression and non-invasive sample sources. While the focus of the article is on anti-doping, the principles and methodology described could be applied to any research in which non-invasive, yet biologically informative sampling is desired [10041].

Over the course of the past decade, technical progress has enabled scientists to investigate genome-wide RNA expression using microarray platforms. This transcriptomic approach represents a promising tool for the discovery of basic gene expression patterns and for identification of cellular signalling pathways under various conditions. Since doping substances have been shown to influence mRNA expression, it has been suggested that these changes can be detected by screening the blood transcriptome. In one review, it was critically discuss the potential but also the pitfalls of this application as a tool in doping research. Transcriptomic approaches were considered to potentially provide researchers with a unique gene expression signature or with a specific biomarker for various physiological and pathophysiological conditions. Since transcriptomic approaches are considerably prone to biological and technical confounding factors that act on study subjects or samples, very strict guidelines for the use of transcriptomics in human study subjects have been developed. Typical field conditions associated with doping controls limit the feasibility of following these strict guidelines as there are too many variables counteracting a standardized procedure. After almost a decade of research using transcriptomic tools, it still remains a matter of future technological progress to identify the ultimate biomarker using technologies and/or methodologies that are sufficiently robust against typical biological and technical bias and that are valid in a court of law [11564].

**Compound-specific isotope analysis (CSIA)**

Compound-specific isotope analysis (CSIA) by gas chromatography combustion isotope ratio mass spectrometry (GCC-IRMS) is a powerful technique for the sourcing of substances, such as determination of the geographic or chemical origin of drugs and food adulteration, and it is especially invaluable as a confirmatory tool for detection of the use of synthetic steroids in competitive sport. We review here principles and practices for data processing and calibration of GCC-IRMS data with consideration to anti-doping analyses, with a focus on carbon isotopic analysis ($^{13}$C/$^{12}$C). After a brief review of peak definition, the isotopologue signal reduction methods of summation, curve-fitting, and linear regression were described and reviewed. Considerations for the anti-doping analyst are reviewed [12075].

**Dual-color bioluminescent bioreporter**

Bioassays represent promising complementary techniques to conventional analytical approaches used in doping analysis to detect illicit drugs like anabolic-androgenic steroids (AAS). The fact that all AAS share a common mechanism of action via the human androgen receptor (hAR) enables the use of bioassays, relying on the activation of hAR as antidoping screening tools. Previously, it was developed a dual-color bioreporter based on yeast cells
engineered to express hAR and androgen response elements driving the expression of the bioluminescent (BL) reporter protein Photinus pyralis luciferase. A second reporter protein, the red-emitting luciferase PpyRE8, was introduced in the bioreporter as internal viability control. Here, we report the first forensic application of a straightforward, accurate, and cost-effective bioassay, relying on spectral resolution of the two BL signals, in 96-microwell format. The bioreporter responds to dihydrotestosterone as reference androgen in a concentration-dependent manner from 0.08 to 1,000 nM with intra- and inter-assay variation coefficients of 11.4 and 13.1 percent, respectively. It was also demonstrated the suitability of this dual-color bioreporter to assess (anti)-androgenic activity of pure AAS, mixtures of AAS, and other illicit drugs provided by the Scientific Police. Significant anti-androgenic activity was observed in samples labeled as marijuana and hashish, containing delta(9)-tetrahydrocannabinol as major constituent [13100].

Two-dimensional gas chromatography with heart-cutting

The accuracy and precision of gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) measurements are highly dependent on analyte purity. Reliable analysis of urinary steroids for doping control therefore requires extensive and time-consuming sample preparation (i.e. liquid chromatography fraction collection) prior to GC-C-IRMS analysis. The use of two-dimensional GC (GC-GC) with heart-cutting (Deans Switch) as a possible approach to reduce the sample purification required for IRMS analysis is described herein. The system uses a low thermal mass oven (LTM) incorporated into an existing GC-C-IRMS system. GC-GC allowed the use of a cyanopropyl/phenyl column in the first dimension to optimize the separation of underivatized steroids, while a phenyl-methylpolysiloxane column in the second dimension focuses the selectively cut analytes into narrower peaks for more sensitive and reliable MS analysis. In addition, to confirm analyte identity, eluent from the second GC was split, with 20 percent entering a scanning MS, and 80 percent flowing to the IRMS. As a proof concept, the developed method was then used to analyze a single spot urine (5 ml) from an individual receiving T therapy (2 × 50 mg sachets of Testogel®). The T delta value (-27.8 ‰, [T] = 38 ng/ml) was clearly distinct from 11-ketoetiocholanolone (-22.5 ‰) (used as an endogenous reference compound (ERC)), indicating T as being of exogenous origin. The simultaneous analysis by the scanning MS yielded a full scan mass spectrum of the same chromatographic peak, thus confirming the peak to be T [12078].

RNA sequencing

The abuse of anabolic substances in animal husbandry is forbidden within the EU and well controlled by detecting substance residues in different matrices. The application of newly designed drugs or substance cocktails represents big problems. Therefore developing sensitive test methods is important. The analysis of physiological changes caused by the use of anabolic agents on the molecular level, for example, by quantifying gene expression response, is a new approach to develop such screening methods. A novel technology for holistic gene expression analysis is RNA sequencing. In one study, the potential of this high-throughput method for the identification of biomarkers was evaluated. The effect of trenbolone acetate plus estradiol on gene expression in liver from Nguni heifers was analyzed with RNA sequencing. The expression of 40 selected candidate genes was verified via RT-qPCR, whereby 20 of these genes were significantly regulated. To extract the intended information from these regulated genes, biostatistical tools for pattern recognition were applied and resulted in a clear separation of the treatment groups. Those candidate
genes could be verified in boars and in calves treated with anabolic substances. These results show the potential of RNA sequencing to screen for biomarker candidates to detect the abuse of anabolics. The verification of these biomarkers in boars and calves leads to the assumption that gene expression biomarkers are independent of breed or even species and that biomarkers, identified in farm animals could also act as potential biomarker candidates to detect the abuse of anabolic substances in human sports [12079].

Hydrogen isotope ratio of urinary steroids

The hydrogen isotope ratio (HIR) of body water and, therefore, of all endogenously synthesized compounds in humans, is mainly affected by the HIR of ingested drinking water. As a consequence, the entire organism and all of its synthesized substrates will reflect alterations in the isotope ratio of drinking water, which depends on the duration of exposure. To investigate the effect of this change on endogenous urinary steroids relevant to doping-control analysis the hydrogen isotope composition of potable water was suddenly enriched from -50 to 200 ‰ and maintained at this level for two weeks for two individuals. The steroids under investigation were 5beta-pregnane-3alpha,20alpha-diol, 5alpha-androst-16-en-3alpha-ol, 3alpha-hydroxy-5alpha-androstan-17-one (Andro), 3alpha-hydroxy-5beta-androstan-17-one (Etio), 5alpha-androstane-3alpha,17beta-diol, and 5beta-androstane-3alpha,17beta-diol (excreted as glucuronides) and Etio, Andro and 3beta-hydroxyandrost-5-en-17-one (excreted as sulfates). The HIR of body water was estimated by determination of the HIR of total native urine, to trace the induced changes. The hydrogen in steroids is partly derived from the total amount of body water and cholesterol-enrichment could be calculated by use of these data. Although the sum of changes in the isotopic composition of body water was 150 ‰, shifts of approximately 30 ‰ were observed for urinary steroids. Parallel enrichment in their HIR was observed for most of the steroids, and none of the differences between the HIR of individual steroids was elevated beyond recently established thresholds. This finding is important to sports drug testing because it supports the intended use of this novel and complementary methodology even in cases where athletes have drunk water of different HIR, a plausible and, presumably, inevitable scenario while traveling [12080].

Dried blood spots (DBS)

Although not (yet) a frequent doping control specimen, blood samples are advantageous over urine specimens in a doping control context in at least two ways they commonly contain the intact drug rather than metabolites, which represents a workaround when new or entirely unknown (designer) compounds are misused and metabolism studies are not (or not publicly) available, and they provide information on drug concentrations at the time of sampling, which is of utmost importance concerning those drugs prohibited in-competition only. As a consequence, the option to expand doping controls from urine and (less frequently) plasma or serum to whole blood shortly before or after competition was evaluated and assays for the analysis of minimal-invasively collected dried blood spots (DBS) were reported in 2011 and 2012. DBS, created from a volume of 25 ml, were excised from blood collection cards and consecutively extracted into methanol/ tert.-butyl-methyl ether and acetone. After processing the blood spots, the combined targeted qualitative and quantitative analysis was possible and data for non-target substances for retrospective evaluation or homology searches based on conserved and common molecular structures were recorded [12017].

In one study, a new type of mass spectrometer combining a quadrupole mass filter, a higher
collision dissociation (HCD) cell and an Orbitrap detector, was evaluated for the analysis of dried blood spots (DBS) in doping controls. DBS analysis is characterized by the necessity to detect prohibited compounds in sub-nanogram-per-milliliter levels with high identification capacity. After extraction of DBS with an organic solvent and liquid chromatographic separation (using a regular C18-RP-analytical UHPLC-column) of target analytes, mass spectrometry is performed with a high-resolution full scan in positive and negative mode by means of electrospray ionisation. Single-product ion mass spectra are acquired using the data-dependent analysis mode (employing an inclusion list) for previously selected precursors of known prohibited compounds with fixed retention time ranges. Besides, a sensitive screening in a targeted approach, non-targeted analysis for retrospective data evaluation is thus possible. The chosen experimental design enables the determination of various drugs from different classes with one generic sample preparation which is shown for 26 selected model compounds delta-9-tetrahydrocannabinol (THC), tetrahydrocannabinol-9-carboxylic acid (THC-COOH), methylhexanamine, methylphenidate, cocaine, nikethamide, 3,4-methylenedioxyamphetamine, N-methyl-3,4-methylenedioxyamphetamine, strychnine, mesocarb, salbutamol, formoterol, clenbuterol, metandienone, stanozolol, bisoprolol, propranolol, metoprolol, anastrazole, clomiphene, exemestane, dexamethasone, budesonide, selective androgen receptor modulator (SARM) S4 (andarine), SARM S1, hydrochlorothiazide. Generally, only qualitative result interpretation was focussed upon, but for target analytes with deuterium-labelled internal standards (salbutamol, clenbuterol, cocaine, dexamethasone, THC-COOH and THC) quantitative analysis was also possible. Especially the most challenging analytes, THC and its carboxy metabolite, were detected in DBS at relevant concentrations (<0.5 ng/mL) using targeted HCD experiments. The method was validated for the parameters: specificity, linearity (0-20 ng/mL), precision (<25 %), recovery (mean 60 %), limit of detection/quantification, ion suppression, stability and accuracy (80-120 %). Six isotope-labelled analogues used as internal standards facilitate a quantitative result interpretation which is of utmost importance especially for in-competition drug sports testing [12081].

Whole blood sample collection on cellulose paper has a more than 30-year-long tradition, especially in neonatal screening. The sampling is minimally invasive, fast, discreet, and robust against manipulation. The present approach highlights the potential to determine doping agents in dried blood spots (DBS) after extraction and subsequent analysis by liquid chromatography-mass spectrometry (LC-MS). The assay is focused on selected model compounds of which the circulating target concentration is of particular interest. Here, pre- or post-competition testing with DBS allows probing for the conditions (i.e. presence or absence of relevant drugs) in the athlete’s circulation during competition, which complements earlier approaches towards the identification of urinary indicators for the temporal application of substances prohibited in-competition only. Precise (< 20 %), linear, and robust conditions with limits of detection in low ng/ml range were accomplished by means of LC coupled to high resolution/high accuracy mass spectrometry for the selected model compounds benzoylecgonine, cocaine, pseudoephedrine, amphetamine, salbutamol, and JWH-018. Deuterium-labelled internal standards were used to yield reliable quantitative results. In addition, the non-targeted screening approach (positive/negative switching combined with tandem mass spectrometry (MS/MS) experiments) enables the retrospective qualitative data evaluation for a comprehensive selection of known and unknown substances as exemplarily shown by the extraction of 20 target compounds (corticosteroids, aromatase inhibitors, anabolic steroids, beta-blockers, etc.) at 20 ng/mL. The simple and fast nature of the assay allows for an easy implementation into existing procedures and will potentially enhance the effectiveness of testing by reducing costs and effort of pre-analysis workload. The automation of laboratory processes has continuously been improved, and the simplification and acceleration of pre-analytical steps such as sample collection, transfer, and storage are desirable benefits in most medico-analytical arenas. The collection of whole blood samples,
dried on a piece of paper, offers various advantages over conventional venopuncture-based blood sampling concerning time consumption, workload and costs without compromising the required quality. The use of dried blood spots (DBS) was reported as early as the 1960s when this technique was applied to the sample collection for testing for phenylketonuria in newborns by a simple heel prick. In comparison to conventional venous cannula sampling, the collection of a drop of blood (usually 10-30 microL) from a heel, finger, or ear prick is considerably less invasive. This minimizes the risk of infections especially for sensitive patients such as infants on the one hand, and enables the more frequent collection of samples in pharmacokinetic studies with small laboratory animals (mice, rats, etc) on the other hand. The stability of the cellulose-fixed target analytes is generally described to be superior to plasma, serum, or urine storage conditions due to inactivation of enzymatic degradation processes. All assays follow the common strategy to perform a more or less simple extraction, whereby the extraction conditions (organic/aqueous solvent ratio) strongly depend on the chemical properties of the target analytes. In the present study, an assay for selected prohibited threshold substances by means of liquid chromatographic separation coupled to high resolution/high mass accuracy mass spectrometry (LC-MS) is reported. Samples are manually punched from DBS cards, fortified with labelled internal standards, and extracted with an organic solvent prior to the LC-MS measurement. Target substances implemented in the method are cocaine, benzoylecgonine, salbutamol, ephedrine/pseudoephedrine, amphetamine and JWH-018, and validation for qualitative and quantitative purposes has been performed [11565].

A new hyphenated mass spectrometry

Occasionally, doping analysis has been recognized as a competitive challenge between cheating sportsmen and the analytical capabilities of testing laboratories. Both have made immense progress during the last decades, but obviously the athletes have the questionable benefit of frequently being able to switch to new, unknown and untested compounds to enhance their performance. Thus, as analytical counteraction and for effective drug testing, a complementary approach to classical targeted methods is required in order to implement a comprehensive screening procedure for known and unknown xenobiotics. One study provided a new analytical strategy to circumvent the targeted character of classical doping controls without losing the required sensitivity and specificity. Using 50 microL of plasma only, the method potentially identifies illicit drugs in low ng/mL concentrations. Plasma provides the biological fluid with the circulating, unmodified xenobiotics; thus the identification of unknown compounds is facilitated. After a simple protein precipitation, liquid chromatographic separation and subsequent detection by means of high resolution/high accuracy orbitrap mass spectrometry, the procedure enables the determination of numerous compounds from different classes prohibited by the World Anti-Doping Agency (WADA). A new hyphenated mass spectrometry technology was employed without precursor ion selection for higher collision energy dissociation (HCD) fragmentation experiments. Thus the mass spectra contained all the desired information to identify unknown substances retrospectively. The method was validated for 32 selected model compounds for qualitative purposes considering the parameters specificity, selectivity, limit of detection (<0.1-10 ng/mL), precision (9-28 %), robustness, linearity, ion suppression and recovery (80-112 %). In addition to the identification of unknown compounds, the plasma samples were simultaneously screened for known prohibited targets [10042].

mRNA transcripts
MicroRNAs (miRNAs) are small non-coding RNAs that regulate a variety of biological processes. Cell-free miRNAs detected in blood plasma are used as specific and sensitive markers of physiological processes and some diseases. Circulating miRNAs are highly stable in body fluids, for example plasma. Therefore, profiles of circulating miRNAs have been investigated for potential use as novel, non-invasive anti-doping biomarkers. This review describes the biological mechanisms underlying the variation of circulating miRNAs, revealing that they have great potential as a new class of biomarker for detection of doping substances. The latest developments in extraction and profiling technology, and the technical design of experiments useful for anti-doping, are also discussed. Longitudinal measurements of circulating miRNAs in the context of the athlete biological passport are proposed as an efficient strategy for the use of these new markers. It was also emphasized potential challenges for the translation of circulating miRNAs from research into practical anti-doping applications [13105].

Evolving challenges require evolving responses. The use of illicit performance enhancing drugs by athletes permeates the reality and the perception of elite sports. New drugs with ergogenic or masking potential are quickly adopted, driven by a desire to win and the necessity of avoiding detection. To counter this trend, anti-doping authorities are continually refining existing assays and developing new testing strategies. In the post-genome era, genetic- and molecular-based tests are being evaluated as potential approaches to detect new and sophisticated forms of doping. Transcriptome analysis, in which a tissue's complement of mRNA transcripts is characterized, is one such method. The quantity and composition of a tissue's transcriptome is highly reflective of milieu and metabolic activity. There is much interest in transcriptional profiling in medical diagnostics and, as transcriptional information can be obtained from a variety of easily accessed tissues, similar approaches could be used in doping control. One article briefly reviewed current understanding of the transcriptome, common methods of global analysis of gene expression and non-invasive sample sources. While the focus of the article was on anti-doping, the principles and methodology described could be applied to any research in which non-invasive, yet biologically informative sampling is desired [09040].

**Deuterium/hydrogen ratio**

The hydrogen isotope ratio (HIR) of body water and, therefore, of all endogenously synthesized compounds in humans, is mainly affected by the HIR of ingested drinking water. As a consequence, the entire organism and all of its synthesized substrates will reflect alterations in the isotope ratio of drinking water, which depends on the duration of exposure. To investigate the effect of this change on endogenous urinary steroids relevant to doping-control analysis the hydrogen isotope composition of potable water was suddenly enriched from -50 to 200 permille and maintained at this level for two weeks for two individuals. The steroids under investigation were 5beta-pregnane-3alpha,20alpha-diol, 5alpha-androst-16-en-3alpha-ol, 3alpha-hydroxy-5alpha-androstan-17-one (ANDRO), 3alpha-hydroxy-5beta-androstan-17-one (ETIO), 5alpha-androstane-3alpha,17beta-diol, and 5beta-androstane-3alpha,17beta-diol (excreted as glucuronides) and ETIO, ANDRO and 3beta-hydroxyandrost-5-en-17-one (excreted as sulfates). The HIR of body water was estimated by determination of the HIR of total native urine, to trace the induced changes. The hydrogen in steroids is partly derived from the total amount of body water and cholesterol-enrichment could be calculated by use of these data. Although the sum of changes in the isotopic composition of body water was 150 permille, shifts of approximately 30 permille were observed for urinary steroids. Parallel enrichment in their HIR was observed for most of the steroids, and none of the differences between the HIR of individual steroids was elevated beyond recently established thresholds. This finding is important to sports drug testing.
because it supports the intended use of this novel and complementary methodology even in cases where athletes have drunk water of different HIR, a plausible and, presumably, inevitable scenario while traveling [13099].

The development and application of a combined gas chromatography/thermal conversion/isotope ratio mass spectrometry (GC/TC/IRMS) method for D/H ratio determination of endogenous urinary steroids are presented. The key element in sample preparation was the consecutive cleanup with high-performance liquid chromatography of initially native and subsequently acetylated steroids. This strategy enabled sufficient cleanup of all target analytes for determination of their respective D/H values. Ten steroids (11beta-hydroxyandrostosterone, 5alpha-androst-16-en-3alpha-ol, pregnanediol, androsterone, etiocholanolone, testosterone, epitesto-sterone, 5alpha-androstan-3alpha,17beta-diol, 5beta-androstan-3alpha,17beta-diol and dehydroepiandrosterone) were measured from a single urine specimen. Depending on the biological background, the determination limit for all steroids ranged from 10 to 15 ng/mL for a 20 mL specimen. The method was validated by application of linear mixing models on each steroid and covered repeatability and reproducibility. The specificity of the procedure was ensured by gas chromatography/mass spectrometry (GC/MS) analysis of the sample using equivalent chromatographic conditions to those employed in the GC/TC/IRMS measurement. Within the sample preparation, no isotopic fractionation was observed, and no amount-dependent shift of the D/H ratios during the measurement was noticed. Possible memory effects occurring during IRMS measurements were corrected by applying a simple rule of proportion. In order to determine the naturally occurring D/H ratios of all implemented steroids, a population of 18 male subjects was analyzed. Relevant mean delta values among selected steroids were calculated which allowed us to study the metabolic pathways and production sites of all the implemented steroids with additional consideration of the corresponding $^{13}$C/$^{12}$C ratios [09041].

Quantitative structure-retention relationships

Quantitative structure-retention relationship (QSRR) is a technique capable of improving the identification of analytes by predicting their retention time on a liquid chromatography column (LC) and/or their properties. This approach is particularly useful when LC is coupled with a high-resolution mass spectrometry (HRMS) platform. The main aim of the present study was to develop and describe appropriate QSRR models that provide usable predictive capability, allowing false positive identification to be removed during the interpretation of metabolomics data, while additionally increasing confidence of experimental results in doping control area. For this purpose, a dataset consisting of 146 drugs, metabolites and banned compounds from World Anti-Doping Agency (WADA) lists, was used. A QSRR study was carried out separately on high quality retention data determined by reversed-phase (RP-LC-HRMS) and hydrophilic interaction chromatography (HILIC-LC-HRMS) systems, employing a single protocol for each system. Multiple linear regression (MLR) was applied to construct the linear QSRR models based on a variety of theoretical molecular descriptors. The regression equations included a set of three descriptors for each model: ALogP, BELe6, R2p and ALogP(2), FDI, BLTA96, were used in the analysis of reversed-phase and HILIC column models, respectively. Statistically significant QSRR models indicate a strong correlation between retention time and the molecular descriptors. An evaluation of the best correlation models, performed by validation of each model using three tests (leave-one-out, leave-many-out, external tests), demonstrated the reliability of the models. One paper provided a practical and effective method for analytical chemists working with LC/HRMS platforms to improve predictive confidence of studies that seek to identify small molecules [13089].
Solid phase extraction (SPE) procedure

The development of a generic analytical method remains difficult when a high number of compounds has to be simultaneously considered. One study proposed an innovative strategy for the development of a solid phase extraction (SPE) procedure before liquid chromatography-mass spectrometry analysis of 34 diuretics and beta-blockers in urine samples. These compounds have been selected since they are often encountered in antidoping control. The principle is based on the selection of representative analytes during SPE protocol optimization, allowing a drastic reduction of generated data and development time. To select the representative compounds, all substances were classified based on their SPE behavior with a generic method and groups were formed with the help of a chemometric tool, namely hierarchical cluster analysis (HCA). One representative analyte per group was selected and used for subsequent SPE method development. Once the SPE method was developed, compounds were analyzed by LC-MS and matrix effects were evaluated to determine the influence of the matrix on the SPE process and MS signal alteration due to endogenous compounds. As a result, matrix effects evaluation must be performed on all analytes; representative compounds previously selected for SPE development were unable to predict matrix effects [09042].

17beta-19-nortestosterone (17beta-NT) has been illegally used in antifatigue functional foods to promote muscle growth and improve endurance. A rapid and sensitive solid-phase extraction-enzyme-linked immunosorbent assay (SPE-ELISA) method was developed and successfully applied to analyze the levels of 17beta-NT in antifatigue functional foods. A polyclonal antibody against 17beta-NT was produced from rabbits immunized with the 17beta-NT-BSA conjugate, and a competitive direct enzyme-linked immunosorbent assay was developed for the rapid detection of 17beta-NT. The concentration causing 50 percent inhibition (IC50) and the limit of detection (LOD) were found to be 0.08 and 0.0055 ng/mL, respectively; this was better than methods previously reported that had a LOD of 2.4 ng/mL. 18C cartridges were investigated for use in removing the effects of matrix in foods, and the sample purification protocol was optimized. Using the developed SPE-ELISA method, recoveries of functional food samples were obtained in the range of 71 percent to 92 percent. Moreover, two kinds of antifatigue functional foods were analyzed using the established ELISA and HPLC methods. The correlation coefficient of the results obtained using the 2 methods was greater than 0.98. Thus, the preliminary evaluation of the SPE-ELISA method proved that it is a specific, sensitive, and precise tool that can be used for the practical detection of 17beta-NT in various antifatigue functional food samples [09043].

Solid-phase microextraction

A fully automated, high-throughput method based on thin-film solid-phase microextraction (SPME) and liquid chromatography-mass spectrometry was developed for simultaneous quantitative analysis of 110 doping compounds, selected from ten classes and varying in physical and chemical properties. Among four tested extraction phases, C18 blades were chosen, as they provided optimum recoveries and the lowest carryover effect. The SPME method was optimized in terms of extraction pH, ionic strength of the sample, washing solution, extraction and desorption times for analysis of urine samples. Chromatographic separation was obtained in reversed-phase model; for detection, two mass spectrometers were used: triple quadrupole and full scan orbitrap. These combinations allowed for selective analysis of targeted compounds, as well as a retrospective study for known and unknown compounds. The developed method was validated according to the Food and Drug
Administration (FDA) criteria, taking into account Minimum Required Performance Level (MRPL) values required by the World Anti-Doping Agency (WADA). In addition to analysis of free substances, it was also shown that the proposed method is able to extract the glucuronated forms of the compounds. The developed assay offers fast and reliable analysis of various prohibited substances, an attractive alternative to the standard methods that are currently used in anti-doping laboratories [13090].

Yeast analysis

The classical analytical method for detection of anabolic steroid abuse is gas chromatography followed by mass spectrometry (GC/MS). However, even molecules with a chemical structure typical for this class of substances, are sometimes not identified in routine screening by GC/MS when their precise chemical structure is still unknown. A supplementary approach to identify anabolic steroid abuse could be a structure-independent identification of anabolic steroids based on their biological activity. To test the suitability of such a system, it was analyzed the yeast androgen receptor (AR) reporter gene system to identify anabolic steroids in human urine samples. Analysis of different anabolic steroids dissolved in buffer demonstrated that the yeast reporter gene system is able to detect a variety of different anabolic steroids and their metabolites with high specificity, including the so-called "designer steroid" tetrahydrogestrinone. In contrast, other non-androgenic steroids, like glucocorticoids, progestins, mineralocorticoids and estrogens had a low potency to stimulate transactivation. To test whether the system would also allow the detection of androgens in urine, experiments with spiked urine samples were performed. The androgen reporter gene in yeast responds very sensitive to 5alpha-dihydrotesto-sterone (DHT), even at high urine concentrations. To examine whether the test system would also be able to detect anabolic steroids in the urine of anabolic steroid abusers, anonymous urine samples previously characterized by GCMS were analyzed with the reporter gene assay. Even when the concentration of the anabolic metabolites was comparatively low in some positive samples it was possible to identify the majority of positive samples by their biological activity. In conclusion, the results demonstrated that the yeast reporter gene system detects anabolic steroids and corresponding metabolites with high sensitivity even in urine of anabolic steroid abusing athletes. But most importantly, a biological test system does not require knowledge of the chemical structure of androgenic substances and therefore suitable to detect previously unidentified substances, especially those of the class of so-called designer steroids [08189].

Yeast transactivation system

Anabolic-androgenic steroids are frequently misused compounds in sports, and they belong to the controlled substances according to the requirements of the World Anti-Doping Agency. The classical techniques of steroid detection are mass spectrometry coupled to gas or liquid chromatography. Biological methods that base on the ability of substances to bind the steroid receptor are not applied in routine doping control procedures so far, but they appear to be useful for characterization of steroid androgenic potential. In this study we used the yeast androgen receptor reporter system (YAS), which in the past has already successfully been applied to both various androgenic substances and also urine samples. Giving attention to the androgenic potential of steroidal dietary supplements, we exemplified the analysis using both mass spectrometry techniques and the YAS-based assay on the product "Syntrax Tetrabol" which was a confiscated dietary supplement and marketed as a steroid precursor. Identification, structure and the kinetic behavior of its excreted metabolites were analyzed by NMR, GC-MS and LC-MS/MS. The androgenic potential of the parent compound as well as
its metabolites in urine was evaluated with the help of the YAS. The application of urine samples with a previous deconjugation and the inclusion of urine density values were carried out and led to increased responses on the YAS. Further, the possibility of a complementary application of structure-based instrumental analysis and biological detection of androgenicity with the help of the YAS seems to be desirable and is discussed [12084].

Yeast and mammalian cell-based androgen bioassays

Androgenic steroids marketed online as nutraceuticals are a growing concern in sport doping. The inability of conventional mass spectrometry (MS)-based techniques to detect structurally novel androgens has led to the development of in vitro androgen bioassays to identify such designer androgens by their bioactivity. The objective of this study was to determine the androgenic bioactivity of novel steroidal compounds isolated from nutraceuticals using both yeast and mammalian cell-based androgen bioassays. We developed two new in vitro androgen bioassays by stably transfecting HEK293 and HuH7 cells with the human androgen receptor (hAR) expression plasmid together with a novel reporter gene vector (enhancer/ARE/SEAP). The yeast β-galactosidase androgen bioassay was used for comparison. Our new bioassay featuring the enhancer/ARE/SEAP construct (S) displayed simpler assay format and higher specificity with lower sensitivity compared with the commonly used mouse mammary tumour virus (MMTV)-luciferase. The relative potencies (RP), defined as EC50 of testosterone of steroid, of nutraceutical extracts in the yeast, HEK293-S, and HuH7-S, were 34, 333, and 80,000 for Hemapolin; 208, 250, and 80 for Furazadrol; 0.38, 10, and 106 for Oxyguna; 2.7, 0.28, and 15 for Trena; and 4.5, 0.1, and 0.4 for Formadrol, respectively. The wide discrepancies in rank RP of these compounds was reconciled into a consistent potency ranking when the cells were treated with meclofenamic acid, a nonselective inhibitor of steroid metabolizing enzymes. These findings indicate that steroids extracted from nutraceuticals can be converted in vitro into more or less potent androgens in mammalian but not in yeast cells. It was conclude that the putative androgenic bioactivity of a new compound may depend on the bioassay cellular format and that mammalian cell bioassays may have an added benefit in screening for proandrogens but sacrifice specificity for sensitivity in quantitation [11043].

Laser desorption

New data on sample preparation and matrix selection for the fast screening of androgenic anabolic steroids (AAS) by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) was presented. The rapid screening of 15 steroids included in the WADA prohibited list using MALDI was evaluated. Nine organic and two inorganic matrices were assessed in order to determine the best matrix for steroid identification in terms of ionisation yield and interference by characteristic matrix ions. The best results were achieved for the organic matrices 2-(4-hydroxyphenylazo)benzoic acid (HABA) and trans-3-indoleacrylic acid (IAA). Good signals for all the steroids studied were obtained for concentrations as low as 0.010 and 0.050 microg/mL on the MALDI sample plate for the HABA and IAA matrices, respectively. For these two matrices, the sensitivity achieved by MALDI is comparable with the sensitivity achieved by gas chromatography/mass spectrometry (GC/MS), which is the conventional technique used for AAS detection. Furthermore, the accuracy and precision obtained with MALDI are very good, since an internal mass calibration is performed with the matrix ions. For the inorganic matrices, laser fluences higher than those used with organic matrices are required to obtain good MALDI signals. When inorganic matrices were used in combination with glycerol as a dispersing
agent, an important reduction of the background noise was observed. Urine samples spiked with the study compounds were processed by solid-phase extraction (SPE) and the screening was consistently positive [09045].

Non-target metabolomics

The molecular diversity of the human urinary metabolome is very well reflected by the existing databases displaying thousands of metabolites classified in as much as 70 different structural classes. The majority of these metabolites are small molecular weight compounds having molecular weights between 100 and 800 Dalton (Da) and small peptide fragments with very different physico-chemical properties (solubility, polarity, proton affinity, etc). Despite this astonishing chemo-diversity, for quite some time, mainly targeted studies, often restricted to a particular chemical family or to compounds having similar properties have been applied into doping control studies. Moreover, the metabolites and the changes were often regarded in an univariate manner and the correlations between them were often disregarded. Nowadays, the progresses made in analytical fields like sample preparation, chemical analysis and data processing offer a wider view of the metabolome and greatly contribute to our understanding of the biochemical transformations. Among these, two are believed to have played a key role: the introduction of high (Time of Flight mass spectrometry, TOF) and ultra-high resolution techniques (Fourier Transform Ion Cyclotron Resonance mass spectrometry, FT-ICR/MS) and the development of algorithms capable of handling the thousands of signals generated by such analytical platforms. Indeed, techniques such as FT-ICR/MS are becoming more and more available and their advantages can be now fully exploited. Thus, molecular formulae generation based on exact masses and relevant database search are now possible due to the high resolution and accuracy of this type of technique. Of equal importance, the recent advances in the pre-processing, mathematical modeling and the statistical analysis lead to more comprehensive biological interpretation of the metabolomics data. Up to present, this strategy has been applied in a variety of fields, including drug discovery, nutrition, toxicology, clinical trials and more recently chemical submission. It is of particular interest in areas like doping control as new approaches are needed to fight the ongoing development of performance-enhancing methods. It was therefore detected differences in metabolite levels between doped athletes, clean athletes, and volunteers (non athletes). This outcome is obtained by comparing results of measurements from two analytical platforms: UHPLC-QTOF/MS and FT-ICR/MS. Twenty-seven urine samples tested positive for glucocorticoids or beta-2-agonists and twenty samples coming from volunteers and clean athletes were analyzed with the two different mass spectrometry approaches using both positive and negative electrospray ionization modes. Urine is a highly complex matrix containing thousands of metabolites having different chemical properties and a high dynamic range. It was used multivariate analysis techniques to unravel this huge data set. Thus, the several groups created were studied by Principal Components Analysis (PCA) and Partial Least Square regression (PLS-DA and OPLS) in the search of discriminating m/z values. The selected variables were annotated and placed on pathway by using MassTRIX [13107].

FCMIA

There are a range of applications that require the measurement of multiple drugs such as urine analysis, drug determination in water, and screening for drug contamination on surfaces. Some of the procedures used such as enzyme-linked immunosorbent assay (ELISA) are simple but can only determine one drug at a time, and others such as GC-MS or
LC-MS are complex, time-consuming, and expensive. In one study, fluorescence covalent microbead immunosorbent assay (FCMIA) was investigated as a simple method for the measurement of multiple drugs simultaneously in three matrices: diluted urine, water, and on surfaces. Five different drugs of abuse or their metabolites (methamphetamine, caffeine, benzoylecgonine (a metabolite of cocaine), tetrahydrocannabinol (THC), the active ingredient in marijuana, and oxycodone) were studied over the range 0-15 ng/ml. There was no measureable cross-reactivity among the drugs at the concentrations studied. Urine dilutions from 1/50 to 1/2.5 were studied and dilutions less than 1/20 had a significant effect on the methamphetamine assay but limited effects on the benzoylecgonine and oxycodone assays and almost no effect on the THC assay. For assays performed in 1/20 urine dilution, water, and diluted surface sampling buffer, least detectable doses (LDD) were 1 ng/ml or less for the drugs. Surfaces spiked with drugs were sampled with swabs wetted with surface sampling buffer and recoveries were linear over the range 0-100 ng/100 cm² surface loading for all drugs. FCMIA has potential to be used for the measurement of multiple drugs in the matrices studied [10443].

In hair

Alternative matrices are steadily gaining recognition as biological samples for toxicological analyses. Hair presents many advantages over traditional matrices, such as urine and blood, since it provides retrospective information regarding drug exposure, can distinguish between chronic and acute or recent drug use by segmental analysis, is easy to obtain, and has considerable stability for long periods of time. For this reason, it has been employed in a wide variety of contexts, namely to evaluate workplace drug exposure, drug-facilitated sexual assault, pre-natal drug exposure, anti-doping control, pharmacological monitoring and alcohol abuse. In this article, issues concerning hair structure, collection, storage and analysis are reviewed. The mechanisms of drug incorporation into hair are briefly discussed. Analytical techniques for simultaneous drug quantification in hair are addressed. Finally, representative examples of drug quantification using hair are summarized, emphasizing its potentialities and limitations as an alternative biological matrix for toxicological analyses [13108].

The detection of a single drug exposure in hair (doping offence, drug-facilitated crime) is based on the presence of the compound of interest in the segment corresponding to the period of the alleged event. However, in some cases, the drug is detected in consecutive segments. As a consequence, interpretation of the results is a challenge that deserves particular attention. Literature evaluation and data obtained from the 20-year experience in drug testing in hair of the author are used as the basis to establish a theory to validate the concept of single exposure in authentic forensic cases where the drug is detected in 2 or 3 segments. The gained experience recommends to wait for 4-5 weeks after the alleged event and then to collect strands of hair. Assuming normal hair growth rate (1 cm/mo), it is advisable to cut the strand into 3 segments of 2 cm to document eventual exposure. Administration of a single dose would be confirmed by the presence of the drug in the proximal 2 cm segment (root), whereas not detected in the 2 other segments. However, in the daily experience of the author, it was noticed that sometimes (about 1 case from 10 examinations), the drug can be detected in 2 or 3 consecutive segments. Such a disposition was even observed in volunteer experiments in the literature. As it was also described for cocaine in early 1996, there is considerable variability in the area over which incorporated drug can be distributed in the hair shaft and in the rate of axial distribution of drug along the hair shaft. This can explain why a small amount of drug, as compared with the concentration in the proximal segment, can be measured in the second segment, as a result of an irregular
movement. Another explanation for broadening the band of positive hair from a single dose is that drugs and metabolites are incorporated into hair during formation of the hair shaft via diffusion from sweat and other secretions. The presence of confounding interferences in the hair matrix or changes in the hair structure due to cosmetic treatments might mislead the final result of hair analysis. To qualify for a single exposure in hair, the author proposes to consider that the highest drug concentration must be detected in the segment corresponding to the period of the alleged event (calculated with a hair growth rate at 1 cm/mo) and that the measured concentration be at least 3 times higher than those measured in the previous or the following segments. This must only be done using scalp hair after cutting the hair directly close to the scalp [13109].

In recent years hair has become a fundamental biological specimen, alternative to the usual samples blood and urine, for drug testing in the fields of forensic toxicology, clinical toxicology and clinical chemistry. Moreover, hair-testing is now extensively used in workplace testing, as well as, on legal cases, historical research etc. This article reviews methodological and practical issues related to the application of hair as a biological indicator of drug use/abuse or of chronic exposure to environmental toxicants. Hair structure and the mechanisms of drug incorporation into it are commented. The usual preparation and extraction methods as well as the analytical techniques of hair samples are presented and commented on. The outcomes of hair analysis have been reviewed for the following categories: drugs of abuse (opiates, cocaine and related, amphetamines, cannabinoids), benzodiazepines, prescribed drugs, pesticides and organic pollutants, doping agents and other drugs or substances. Finally, the specific purpose of the hair testing is discussed along with the interpretation of hair analysis results regarding the limitations of the applied procedures [06044].

The monitoring of anabolic steroid residues in hair is undoubtedly one of the most efficient strategies to demonstrate the long-term administration of these molecules in meat production animals. A multi-residue sample preparation procedure was developed and validated for 28 steroids. A 100 mg hair sample was grinded into powder and extracted at 50 degrees C with methanol. After acidic hydrolysis and extraction with ethyl acetate, phenolsteroids, such as estrogens, resorcylic acid lactones and stilbens in one hand, are separated from androgens and progestagens in the other hand. Solid phase extractions were performed before applying a specific derivatisation for each compound sub-group. Detection and identification were achieved using gas chromatography-tandem mass spectrometry with acquisition in the selected reaction monitoring mode after electron ionisation. The method was validated according to the 2002/657/EC guideline. Decision limits (CCalpha) for main steroids were in the 0.1-10 microg/kg range [06045].

Given the limitations of self-reports on drug use, testing for drugs of abuse is important for most clinical and forensic toxicological situations, both for assessing the reality of the intoxication and for evaluation of the level of drug impairment. It is generally accepted that chemical testing of biological fluids is the most objective means of diagnosis of drug use. The presence of a drug analyte in a biological specimen can be used to document exposure. The standard in drug testing is the immunoassay screen, followed by the gas chromatographic-mass spectrometric confirmation conducted on a urine sample. In recent years, remarkable advances in sensitive analytical techniques have enabled the analysis of drugs in unconventional biological specimens such as hair. The advantages of this sample over traditional media, like urine and blood, are obvious: collection is noninvasive, relatively easy to perform, and in forensic situations it may be achieved under close supervision of law enforcement officers to prevent adulteration or substitution. The window of drug detection is dramatically extended to weeks, months or even years when testing hair. It seems that the value of alternative specimen analysis for the identification of drug users is steadily gaining
recognition. This can be seen from its growing use in preemployment screening, in forensic sciences, in clinical applications and for doping control. Hair analysis may be a useful adjunct to conventional drug testing in urine. Methods for evading urinalysis do not affect hair analysis. The aim of one review was to document toxicological applications of hair analysis in drug detection [06046].

The abuse of esters of natural androgenic steroids in cattle fattening and sports is hard to control via routine urine testing. The esters are rapidly hydrolysed in vivo into substances which are also endogenously present in urine. In veterinary control strange findings of 17beta-testosterone and 17alpha-testosterone in urine are often ignored because of the lack of statistically sound reference data of naturally occurring levels. An interesting alternative for inconclusive urine analyses in veterinary control can be provided by the analysis of the administered steroids themselves, i.e. the analysis of intact steroid esters in hair. Unfortunately, the analysis of intact steroid esters is complicated not only by the vulnerability of the esters which precludes alkaline hydrolysis of the hair, but also by the wide polarity range of short and long-chain esters yielding very poor recoveries for either the one or the other. In one study, a multi-steroid esters LC/MS/MS screening method is presented for trace analysis of the synthetic intact esters of 17beta-testosterone and the undecylenate ester of 17beta-boldenone in bovine hair. The method, requiring only 200 mg of pulverised hair, features a mild digestion procedure using tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and the use of four deuterium-labelled steroid esters as internal standards covering the wide polarity range of the analytes. In spiked hair samples for most of the analytes the limit of detection and the accuracy using isotope dilution were 2-5 ng/g and 97-105 percent, respectively. The applicability was demonstrated using hair samples from a controlled experiment in which six bovines were injected intramuscularly with two different doses of two commercial mixtures of testosterone esters, and with two different doses of boldenone undecylenate. Depending on the dose all administered testosterone- and boldenone esters were found to be incorporated in bovine hair following a single intramuscular injection, except testosterone propionate which dose might have been too low [06047].

Sensitive, specific, and reproducible methods for the quantitative determination of eight anabolic steroids in guinea pig hair have been developed using LC/MS/MS and GC/MS/MS. Methyltestosterone, stanozolol, methandienone, nandrolone, trenbolone, boldenone, methenolone and DHEA were administered intraperitoneally in guinea pigs. After the first injection, black hair segments were collected on shaved areas of skin. The analysis of these segments revealed the distribution of anabolic steroids in the guinea pig hair. The major components in hair are the parent anabolic steroids. The time courses of the concentrations of the steroids in hair (except methenolone, which does not deposit in hair) demonstrated that the peak concentrations were reached on days 2-4, except stanozolol, which peaked on day 10 after administration. The concentrations in hair appeared to be related to the physicochemical properties of the drug compound and to the dosage. These studies on the distribution of drugs in the hair shaft and on the time course of their concentration changes provide information relevant to the optimal time and method of collecting hair samples. Such studies also provide basic data that will be useful in the application of hair analysis in the control of doping and in the interpretation of results [09044].

Doping control of anabolic substances is normally carried out with urine samples taken from athletes and horses. Investigation of alternative specimens, e.g. hair samples, is restricted to special cases, but can also be worthwhile, in addition to urine analysis. Moreover, hair material is preferred in cases of limited availability or complicated collection of urine samples, e.g. from horses. In this work, possible ways of interpretation of analytical results in hair samples are discussed and illustrated by practical experiences. The results demonstrate the applicability of hair analysis to detect anabolic steroids and also to obtain further information
about previous abuse. Moreover, the process of incorporation of steroids into hairs is described and the consequences on interpretation are discussed, e.g. on the retrospective estimation of the application date. The chosen examples deal with the detection of the anabolic agent testosterone propionate. Hair samples of an application study, as well as a control sample taken from a racing horse, were referred to. Hair material was investigated by a screening procedure including testosterone, nandrolone and several esters (testosterone propionate, phenylpropionate, decanoate, undecanoate, cypionate; nandrolone decanoate, dodecanoate and phenylpropionate; limits of detection (LODs) between 0.1 and 5.0 pg/mg). Confirmation of testosterone propionate (LOD 0.1 pg/mg) was carried out by an optimised sample preparation. Trimethylsilyl (TMS) and tert-butyl dimethylsilyl derivatives were detected by gas chromatography-high-resolution mass spectrometry (GC-HRMS) and gas chromatography-tandem mass spectrometry (GC-MS/MS) [08190].

Beside the traditionally used body-fluids, defining the abuse-material by the use of hair samples is more and more widespread in the forensic toxicological practice. Using the hair allows the retrospective examination of the abuse-material, and due to the sensitive measuring technics, even one-time use can be proven. A further possibility is the segment-analysis which allows investigation of the abuse-history retroactive for months depending on the length of the hair. The quantitative parameters of the abuse can not always be estimated precisely since the details of the build-up in the hair are complicated and are not clear even today. Furthermore, the sampling, sample preparation and the measuring method will all influence the results. One paper reviewed the opiates, cocaine, amphetamine derivatives, cannabinoids, alcohol-consumption markers and the frequently found drugs in the forensic toxicology as determined by using hair samples [10430].

Hair and saliva

Alternative specimens (e.g. hair and saliva) are well established in forensic toxicology and provide significant benefits as noninvasive, inexpensive alternatives to blood with access to improved long-term retrospection. Based on these experiences, the question of potential applications and limitations of alternative specimens in doping control arose. Compounds prohibited at all times (e.g. clenbuterol, beta2 agonists, estrogen-receptor modulators) may be successfully tested and clearly interpreted in alternative specimens. In contrast, prohibition of certain compounds in sport are limited to time ranges (e.g., stimulants are only prohibited in-competition), dosages or administration routes (e.g. systemic uptake of glucocorticosteroids). This cannot be properly differentiated by semiquantitative tests (e.g. hair analyses), but may be distinguished in saliva. Similarly, proof of external administration of endogenous steroids (e.g. testosterone) only seems to be achievable by quantitative analysis of saliva. Moreover, the retrospective monitoring of the relevance of social drugs or upcoming (unapproved) substances represents promising applications of hair tests in doping control [12086].

The influence on drug incorporation of melanin affinity, lipophilicity, and membrane permeability is of paramount importance. Despite their high lipophilicity, some drugs have quite low incorporation rate into hair, suggesting that the higher incorporation rates of basic drugs (cocaine, amphetamines) than neutral (steroids, benzodiazepines, cannabinoids...) or acidic ones are strongly related to the penetrating ability of the drug to break through the membrane based on the pH gradient between blood and the acidic hair matrix. When using hair analysis as a matrix during investigative analysis, e.g. workplace drug testing, doping, driving under the influence, drug-facilitated crime, the question of importance is to know whether the analytical procedure was sensitive enough to identify traces of drugs; this is particularly important when the urine sample(s) of the subject was positive and the hair
sample(s) was negative. It has been accepted in the forensic community that a negative hair result cannot exclude the administration of a particular drug, or one of its precursors and the negative findings should not overrule a positive urine result. Nevertheless, the negative hair findings can, on occasion, cast doubt on the positive urine analysis, resulting in substantial legal debate and various consequences for the subject. The concept of minimal detectable dosage in hair is of interest to document the negative findings, but limited data is currently available in the scientific literature. Such data includes cocaine, codeine, ketamine, some benzodiazepines and some unusual compounds. Until laboratories will have sensitive enough methodologies to detect a single use of drug, care should be taken to compare urine and hair findings [12087].

Finger nails

In an attempt to obtain alternative doping control matrices, the utility of fingernails as a source of keratinaceous samples (comparable to hair) have been evaluated concerning testosterone, testosterone propionate, and stanozolol. Although the study demonstrated the incorporation of steroidal agents at the proximal nail fold and nail bed, the approach failed to provide the required sensitivity and most likely also the viability in an authentic doping control setting [12016].

In sweat

Sweat is an alternative biological matrix useful to detect drugs of abuse intake. It is produced by eccrine and apocrine glands originating in the skin dermis and terminating in secretory canals that flow into the skin surface and hair follicles. Since many years it has been demonstrated that endogenous and exogenous chemicals are secreted in this biological sample hence its collection and analysis could show the past intake of xenobiotics. From the seventies the excretion of drugs of abuse has been investigated in human skin excretion; later in nineties forensic scientists began to experiment some techniques to trap sweat for analyses. Even if the use of skin excretions for drug testing has been restricted mainly by difficulties in sample recovery, the marketing of systems for the sample collection has allowed successful sweat testing for several drugs of abuse. In the recent years sweat testing developed a noninvasive monitoring of drug exposure in various contexts as criminal justice, employment and outpatient clinical settings. This paper provides an overview of literature data about sweat drug testing procedures for various xenobiotics especially cocaine metabolites, opiates, cannabis and amphetamines. Issues related to collection, analysis and interpretation of skin excretions as well as its advantages and disadvantages are discussed. Moreover the chance to apply the technique to some particular situation such as workplace drug testing, drivers, doping or prenatal diagnosis, the comparison between sweat and other non conventional matrices are also reviewed. According to literature data the analysis of sweat may be usefully alternative for verifying drug history and for monitoring compliance [13103].

Sweat is a biofluid with present scant use as clinical sample. One review tried to demonstrate the advantages of sweat over other biofluids such as blood or urine for routine clinical analyses and the potential when related to metabolomics. With this aim, critical discussion of sweat samplers and equipment for analysis of target compounds in this sample is made. Well established routine analyses in sweat as is that to diagnose cystic fibrosis, and the advantages and disadvantages of sweat versus urine or blood for doping control have also been discussed. Methods for analytes such as essential metals and xenometals, ethanol and
electrolytes in sweat in fact constitute target metabolomics approaches or belong to any metabolomics subdiscipline such as metallomics, ionomics or xenometabolomics. The higher development of biomarkers based on genomics or proteomics as omics older than metabolomics is discussed and also the potential role of metabolomics in systems biology taking into account its emergent implementation. Normalization of the volume of sampled sweat constitutes a present unsolved shortcoming that deserves investigation [13104].

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The potential role of oral fluid in antidoping testing

Currently, urine and blood are the only matrices authorized for antidoping testing by the World Anti-Doping Agency (WADA). Although the usefulness of urine and blood is proven, issues remain for monitoring some drug classes and for drugs prohibited only in competition. The alternative matrix oral fluid (OF) may offer solutions to some of these issues. OF collection is easy, noninvasive, and sex neutral and is directly observed, limiting potential adulteration, a major problem for urine testing. OF is used to monitor drug intake in workplace, clinical toxicology, criminal justice, and driving under the influence of drugs programs and potentially could complement urine and blood for antidoping testing in sports. Content: This review outlines the present state of knowledge and the advantages and limitations of OF testing for each of the WADA drug classes and the research needed to advance OF testing as a viable alternative for antidoping testing. Summary: Doping agents are either prohibited at all times or prohibited in competition only. Few OF data from controlled drug administration studies are available for substances banned at all times, whereas for some agents prohibited only in competition, sufficient data may be available to suggest appropriate analytes and cutoffs (analytical threshold concentrations) to identify recent drug use. Additional research is needed to characterize the disposition of many banned substances into OF; OF collection methods and doping agent stability in OF also require investigation to allow the accurate interpretation of OF tests for antidoping monitoring [13112].
Virtual screening

Parallel ligand- and structure-based virtual screenings of 269 steroids with anabolic activity evaluated in vivo were performed. The quantitative structure-activity relationship (QSAR) model expressed by selected descriptors as the octanol-water partition coefficient, the molar volume and the quantum mechanical calculated charge values on atoms C1, C2, C5, C9, C10, C14 and C17 of the steroid skeleton, expresses structural features of anabolic steroids (AS) contributing to the transport and steroid-receptor interaction. On the other hand, computational simulations of a candidate ligand binding to a receptor study (a “docking” procedure) predict the association of these AS with the human androgen receptor (AR). Fourteen compounds were identified as lead; the most potent was the 7alpha-methylestr-4-en-3, 17-dione. It was concluded that a good anabolic activity requires hydrogen bonding interactions between both Arg752 and Gln711 residues in the cycles A with O3 atom of the steroid and either Asn705 and Thr877 residues in the cycles D of steroid with O17 atom [13110].

Artificial networks

The computational generation of gradient retention time data for retrospective detection of suspected sports doping species in postanalysis human urine sample data is presented herein. Retention data for a selection of 86 compounds included in the London 2012 Olympic and Paralympic Games drug testing schedule were used to train, verify, and test a range of computational models for this purpose. Spiked urine samples were analyzed using solid phase extraction followed by ultrahigh-pressure gradient liquid chromatography coupled to electrospray ionization high-resolution mass spectrometry. Most analyte retention times varied ≤0.2 min over the relatively short runtime of 10 min. Predicted retention times were within 0.5 min of experimental values for 12 out of 15 blind test compounds (largest error: 0.97 min). Minimizing the variance in predictive ability across replicate networks of identical architecture is presented for the first time along with a quantitative discussion of the contribution of each selected molecular descriptor toward the overall predicted value. The performance of neural computing predictions for isobaric compound retention time is also discussed. One work presented the application of neural networks to the prediction of gradient retention time in archived high-resolution urine analysis sample data for the first time in the field of anti-doping [13111].

Appearance and Performance Enhancing Drug Use Schedule (APEDUS)

Appearance-and-performance enhancing drug (APED) use is a form of drug use that includes use of a wide range of substances such as anabolic-androgenic steroids (AASs) and associated behaviors including intense exercise and dietary control. To date, there are no reliable or valid measures of the core features of APED use. One study described the development and psychometric evaluation of the Appearance and Performance Enhancing Drug Use Schedule (APEDUS) which is a semi-structured interview designed to assess the spectrum of drug use and related features of APED use. Eighty-five current APED using men and women (having used an illicit APED in the past year and planning to use an illicit APED in the future) completed the APEDUS and measures of convergent and divergent validity. Inter-rater agreement, scale reliability, one-week test-retest reliability, convergent and divergent validity, and construct validity were evaluated for each of the APEDUS scales. The
APEDUS is a modular interview with 10 sections designed to assess the core drug and non-drug phenomena associated with APED use. All scales and individual items demonstrated high inter-rater agreement and reliability. Individual scales significantly correlated with convergent measures (DSM-IV diagnoses, aggression, impulsivity, eating disorder pathology) and were uncorrelated with a measure of social desirability. APEDUS subscale scores were also accurate measures of AAS dependence. The APEDUS is a reliable and valid measure of APED phenomena and an accurate measure of the core pathology associated with APED use. Issues with assessing APED use are considered and future research is considered [11567].
DETECTION OF DOPING AGENTS IN ENVIRONMENT, FOOD AND FOOD SUPPLEMENTS

As part of a regional screening to evaluate the risk, for the health of populations, to certain classes of emerging substances, several families of pharmaceuticals and hormones were looked for in waters intended to drinking. Thus, 52 substances were investigated in 71 surface waters and 70 groundwaters. Results indicate that no water was free of pollutants, regardless of its origin (surface or groundwater) and the season of collect. The pharmaceuticals most frequently detected and with the highest concentration levels were salicylic acid, carbamazepine and acetaminophen. Among hormones, testosterone, androstenedione and progesterone were detected in almost all the samples. Globally the groundwaters were less contaminated than surface waters in regards pharmaceuticals frequencies and levels. On the other side, androgens and progestagens were present with comparable frequencies and levels in both compartments. The risk linked to the presence of these substances on human health is discussed [11558].

Analytical strategies

Detection of the abuse of synthetic steroids in food production is nowadays relatively straightforward using modern techniques such as gas or liquid chromatography coupled to mass spectrometry (GC-MS/MS or LC-MS/MS, respectively). However, proving the abuse of “endogenous” (or naturally occurring) steroids is more difficult. Despite these difficulties, significant progress in this area has recently been made and a number of methods are now available. The aim of the current review was to systematically review the available analytical approaches, which include threshold concentrations, qualitative marker metabolites, intact steroid esters, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), longitudinal testing and omics biomarker profiling. The advantages/disadvantages of these methods are considered in detail, but the choice of which to adopt is dictated by a number of practical, political, and economic factors, which vary in different parts of the world. These include the steroid/species combination requiring analysis, the matrix tested, whether samples are collected from live or slaughtered animals, available analytical instrumentation, sample throughput/cost, and the relevant legal/regulatory frameworks. Furthermore, these approaches could be combined in a range of different parallel and/or sequential screening/confirmatory testing streams, with the final choice being determined by the aforementioned considerations. Despite these advances, more work is required to refine the different techniques and to respond to the ever increasing list of compounds classified as endogenous. At this advanced stage, however, it is now more important than ever for scientists and regulators from across the world to communicate and collaborate in order to harmonize and streamline research efforts [12355].

Metabolomics

Metabolomics is a science of interest in food analysis to describe and predict properties of food products and processes. It includes the development of analytical methods with the ultimate goal being the identification of so-called 'quality markers', (i.e. sets of metabolites that correlate with, for example, quality, safety, taste, or fragrance of foodstuffs). In turn, these metabolites are influenced by factors as genetic differences of the raw food ingredients (such as animal breed or crop species differences), growth conditions (such as climate, irrigation...
strategy, or feeding) or production conditions (such as temperature, acidity, or pressure). In cases where the routine-based measurement of a food property faces some limitations such as the lack of knowledge regarding the target compounds to monitor, monitoring based on a limited set of crucial biomarkers is a good alternative, which is of great interest for food safety purposes regarding growth promoting practices. Such an approach may be more efficient than using a classic approach based on a limited set of known metabolites of anabolic compounds. In this context, screening strategies allowing detection of the physiological response resulting from anabolic compound administration are promising approaches to detect their misuse. The global metabolomics workflow implemented for such studies is presented and illustrated through various examples of biological matrices profiling (tissue, blood, urine) and for different classes of anabolic compounds (steroids, beta-agonists and somatotropin) [12356].

**Screening for hormone residues in drug residues**

An emerging trend is recognised in hormone and veterinary drug residue analysis from liquid chromatography tandem mass spectrometry (LC/MS/MS) based screening and confirmation towards accurate mass alternatives such as LC coupled with time-of-flight (TOF), Fourier transform ion cyclotron resonance (FTICR) or Fourier transform orbitrap (FT Orbitrap) MS. In this study, mass resolution and accuracy are discussed for LC/MS screening and confirmation of targeted analytes and for the identification of unknowns using the anabolic steroid stanozolol and the designer beta-agonist "Clenbuterol-R" as model substances. It is shown theoretically and experimentally that mass accuracy criteria without proper mass resolution criteria yield false compliant (false negative) results, both in MS screening and MS/MS confirmation of stanozolol. On the other hand, previous medium resolution accurate mass TOFMS/MS data of the designer beta-agonist were fully confirmed by high resolution FT Orbitrap MS(n) experiments. A discussion is initiated through a proposal for additional criteria for the use of accurate mass LC/MS technologies, to be implemented in Commission Decision 2002/657/EC [06041].

**Anabolic steroids**

A method is described for screening and confirmation of synthetic and endogenous steroids in muscle tissue. The method is sensitive, selective, and rapid and the consumption of organic solvents is low, compared to previously published methods. The procedure involves hydrolysis, defattening with heptane and final clean up with SPE using C18 cartridge. After filtration, the analytes are analysed by LC/MS/MS and quantification is performed using deuterated internal standards. Decision limits (CCalpha) varied from 0.02 to 0.33 µg/kg and the detection capabilities (CCbeta) were <0.50 µg/kg. The mean within-laboratory reproducibility ranged 5-22 percent (%RSDm). Endogenous steroids (e.g. testosterone, epistosterone and androstenedione) have been included in the method, to provide an insight into their levels, as the presence of these steroids was detected several times during analysis of imported meat [11046].

For many years anabolic-androgenic steroids (AAS) are by far the most frequently detected pharmacological substances in doping control. In order to improve their performances, professional sportmen are often tempted to take dietary supplements. However, due to the frequent and widespread occurrence of contaminated supplements, the use of such products is not without risk for the athletes involved. In order to minimize the chances of an unattended positive doping test or serious health problems, fast and reliable screening
methods for the detection of anabolic steroids in dietary supplements are needed. A general screening procedure requires the fast and unambiguous detection of a large range of steroids. Gas chromatography-mass spectrometry (GC-MS) has been used intensively in the detection of doping substances for the past 40 years. Over time, many laboratories have delivered spectra to be included in standard reference databases, one of which is maintained by the National Institute of Standards and Technology (NIST) (Gaithersburg, MD, USA). In recent years, however, liquid chromatography coupled to mass spectrometry (LC-MS) has gained popularity. Unfortunately, existing GC-MS libraries are not applicable to LC-MS analysis. In one study, a new mass spectral library of 88 steroids was developed, along with a fast UPLC-MS method. For the construction of this mass spectral library, three different mass spectra were measured for each steroid, with a sample cone voltage of 30, 60 and 100 V, respectively. This method was then successfully tested on contaminated dietary supplements which had previously been tested by means of a targeted LC-MS/MS method. Overall, the library search was shown to identify the same compounds as the MRM method [11047].

The use of steroids as growth-promoting agents in food production is banned under European Union legislation. Detecting the abuse of testosterone, nandrolone, boldenone, oestradiol and progesterone is complicated by the fact that these steroids are known to be endogenous in certain situations. In one study, the concentrations of characteristic metabolites of each of these steroids were quantified in populations of untreated steers and heifers. Steroid concentration population data were then used by a statistical to produce threshold concentrations for screening and confirming the abuse of these steroids in steer and non-pregnant heifer urine. In addition to thresholds based on testing one animal, new methods based on testing multiple animals from a herd allowed threshold concentrations to be significantly reduced and hence false compliances to be minimised. In the majority of cases, the suggested thresholds were found to be capable of confirming the abuse of endogenous steroids in steers and heifers. In the case of estradiol abuse in the female, however, confirmation based on a threshold is not possible and alternative methods such as gas chromatography-combustion-isotope ratio mass spectrometry are required. In addition to the steer and heifer populations, a small number of pregnant animals were also tested, yielding insights into the biosynthetic pathways of some of the steroids [11048].

Recently, the effect of illicit growth promoters (GPs) upon the cattle transcriptome has drawn the increasing attention of the scientific community. In the present study, the pre-transcriptional effects of three different illicit protocols on a set of target genes, including steroidogenic enzymes and three related transcription factors, were estimated in cattle testis. Beef cattle were administered with dexamethasone (DEX) orally (group D1) or intramuscularly in experiment 1 (group DIM). In experiment 2, DEX was orally administered alone (group D2) or with 17beta-estradiol (group DE), and in experiment 3, dehydroepiandrosterone and boldione were orally administered alone (group DHEA and group ADD) or in combination (group DHAD). The GP effects were measured by quantitative real time RT-PCR. The results of our study were significant but not univocal. A GP-dependent effect on target gene mRNA levels was noticed for 3beta-hydroxysteroid dehydrogenase type 1, the cytochrome P450 side chain cleavage, the cytochrome P450 17A1, HSD17beta3, aromatase, the androgen receptor and the mineralocorticoid receptor-like. The results suggest that different GP schedules are likely to affect genes involved in steroid synthesis and regulation in cattle testis. Thus, this tissue might be considered a potential surrogate tissue that warrants further study into its usefulness in the screening of GP abuse [11049].

A sensitive rapid resolution liquid chromatography-tandem mass spectrometry (RRLC-MS/MS) method, combined with solid-phase extraction, ultrasonic extraction and silica gel
cartridge cleanup, was developed for 28 steroids including 4 estrogens (estrone (E1), 17beta-estradiol (E2), 1alpha-ethynyl estradiol (EE2), diethylstilbestrol (DES)), 14 androgens (androsta-1,4-diene-3,17-dione (ADD), 17alpha-trenbolone, 17beta-trenbolone, 4-androstene-3,17-dione, 19-nortestosterone, 17beta-boldenone, 17alpha-boldenone, testosterone (T), epi-androsterone (EADR), methytestosterone (MT), 4-hydroxy-androst-4-ene-17-dione (4-OHA), 5alpha-dihydrotestosterone (5alpha-DHT), androsterone (ADR), stanozolol (S)), 5 progestagens (progesterone (P), ethynyl testosterone (ET), 19-norandrosterone, medroxyprogesterone (MP)), and 5 glucocorticoids (cortisol, cortisone, prednisone, prednisolone, dexamethasone) in surface water, wastewater and sludge samples. The recoveries of surface water, influents, effluents and sludge samples were 91-119 percent (except 5alpha-DHT was 143 %), 44-200 percent, 61-123 percent and 63-138 percent, respectively. The method detection limits for the 28 analytes in surface water, influents, effluents and freeze-dried sludge samples were 0.01-0.24 ng/L, 0.02-1.44 ng/L, 0.01-0.49 ng/L and 0.08-2.06 ng/g, respectively. This method was applied in the determination of the residual stilboidal hormones in two surface water of one river, 12 wastewater and 8 sludge samples from two wastewater treatment plants in Guangdong (China). Ten analytes were detected in surface water samples with concentrations ranging between 0.4 ng/L (17beta-boldenone) and 55 ng/L (5alpha-DHT); twenty analytes in the wastewater samples with concentrations ranging between 0.3 ng/L (progesterone) and 621 ng/L (5alpha-DHT); and 12 analytes in the sludge samples with concentrations ranging between 1.6 ng/g (E1) and 372 ng/g (EADR) [11050].

In the present paper it was reported the LC-MS/MS determination of residues of 12 anabolic steroids in bovine serum, as an expansion of the work protocols for steroids determination in biological matrices. Steroids analyzed included alpha-zearalanol, beta-zearalanol, alpha-trenbolone, beta-trenbolone, methytestosterone, alpha-estradiol, beta-estradiol, ethynylestradiol, alpha-boldenone, beta-boldenone, alpha-nortestosterone, and beta-nortestosterone. Following protein precipitation, serum samples were cleaned up by solid-phase extraction using Oasis HLB and Amino cartridges. Atmospheric pressure chemical ionization (APCI) in both positive and negative ionization modes was used and mass spectrometry detection was carried out in multiple reaction monitoring mode following two or (in most cases) three product ions per precursor ion. The method was validated in accordance with the Commission Decision 2002/657/EC. The decision limit (CCalpha) values obtained, ranged from 0.01 to 0.07 ng/mL and the detection capability (CCbeta) values obtained ranged from 0.02 to 0.12 ng/mL. The recoveries ranged from 70 to 118 percent. The developed method is suitable for routine and confirmatory purposes such as control of illegal use in livestock production [11051].

Anabolic steroids are banned in food producing livestock in Europe. Efficient methods based on mass spectrometry detection have been developed to ensure the control of such veterinary drug residues. Nevertheless, the use of "cocktails" composed of mixtures of low amounts of several substances as well as the synthesis of new compounds of unknown structure prevent efficient prevention. New analytical tools able to detect such abuse are today mandatory. In this context, metabolomics may represent new emerging strategies for investigating the global physiological effects associated to a family of substances and therefore, to suspect the administration of steroids. The purpose of one study was to set up, assess and compare two complementary mass spectrometry-based metabolomic strategies as new tools to screen for steroid abuse in cattle and demonstrate the feasibility of such approaches. The protocols were developed in two European laboratories in charge of residues analysis in the field of food safety. Apart from sample preparation, the global process was different in both laboratories from LC-HRMS fingerprinting to multivariate data analysis through data processing and involved both LC-Orbitrap-XCMS and UPLC-ToF-MS-MetAlign strategies. The reproducibility of both sample preparation and MS measurements
were assessed in order to guarantee that any differences in the acquired fingerprints were not caused by analytical variability but reflect metabolome modifications upon steroids administration. The protocols were then applied to urine samples collected on a large group of animals consisting of 12 control calves and 12 calves administrated with a mixture of 17β-estradiol 3-benzoate and 17β-nandrolone laurate esters according to a protocol reflecting likely illegal practices. The modifications in urine profiles as indicators of steroid administration have been evaluated in this context and proved the suitability of the approach for discriminating anabolic treated animals from control ones. Such an approach may therefore open a new way for the screening of anabolic steroid administration through targeted monitoring of relevant biomarkers highlighted as a result of the metabolomics study [11052].

The presence and metabolism of endogenous steroid hormones in meat-producing animals has been the subject of much research over the past 40 years. While significant data are available, no comprehensive review has yet been performed. Species considered in this review are bovine, porcine, ovine, equine, caprine and cervine, while steroid hormones include the androgenic-anabolic steroids testosterone, nandrolone and boldenone, as well as their precursors and metabolites. Information on endogenous steroid hormone concentrations is primarily useful in two ways: (1) in relation to pathological versus “normal” physiology and (2) in relation to the detection of the illegal abuse of these hormones in residue surveillance programmes. Since the major focus of one review was on the detection of steroids abuse in animal production, the information gathered to date is used to guide future research. A major deficiency in much of the existing published literature is the lack of standardization and formal validation of experimental approach. Key articles are cited that highlight the huge variation in reported steroid concentrations that can result when samples are analysed by different laboratories under different conditions. These deficiencies are in most cases so fundamental that it is difficult to make reliable comparisons between data sets and hence it is currently impossible to recommend definitive detection strategies. Standardization of the experimental approach would need to involve common experimental protocols and collaboratively validated analytical methods. In particular, standardization would need to cover everything from the demographic of the animal population studied, the method of sample collection and storage (especially the need to sample live versus slaughter sampling since the two methods of surveillance have very different requirements, particularly temporally), sample preparation technique (including mode of extraction, hydrolysis and derivatization), the end-point analytical detection technique, validation protocols, and the statistical methods applied to the resulting data. Although efforts are already underway to produce more definitive data and promote communication among the scientific community on this issue, the convening of a formal European Union working party is recommended [09078].

In livestock production, illegal use of natural steroids is hard to prove because metabolites are either unknown or not significantly above highly fluctuating endogenous levels. In one work it was outlined for the first time a metabolomics based strategy for anabolic steroid urine profiling. Urine profiles of controls and bovines treated with the prohormones dehydroepiandrosterone (DHEA) and pregnenolone were analyzed with ultraperformance liquid chromatography in combination with time-of-flight accurate mass spectrometry (UPLC-TOFMS). The obtained full scan urinary profiles were compared using sophisticated preprocessing and alignment software (MetAlign) and multivariate statistics, revealing hundreds of mass signals which were differential between untreated control and prohormone-treated animals. Moreover, statistical testing of the individual accurate mass signals showed that several mass peak loadings could be used as biomarkers for DHEA and pregnenolone abuse. In addition, accurate mass derived elemental composition analysis and verification by standards or Orbitrap mass spectrometry demonstrated that the observed
differential masses are most likely steroid phase I and glucuronide metabolites excreted as a direct result from the DHEA and pregnenolone administration, thus underlining the relevance of the findings from this untargeted metabolomics approach. It is envisaged that this approach can be used as a holistic screening tool for anabolic steroid abuse in bovines and possibly in sports doping as well [09079].

An existing gas chromatography-mass spectrometry-based quantitative screening method for the regulatory analysis of the resorcylic acid lactones zeranol, taleranol, and zearalanone and the stilbene anabolic steroids diethylstilbestrol and dienestrol was extended to include natural precursors of zeranol (zearalenone, alpha-zearalenol, and beta-zearalenol) in veal liver. No changes in sample preparation were required; the instrumental conditions were selected to effect a suitable chromatographic separation and detection of the analytes. Validation experiments were performed to verify the performance and applicability of the extended method for the quantitative screening of the original and additional analytes in veal liver in the concentration range from 0.5 to 2.0 microg/kg. The limits of detection were 0.08-0.19 microg/kg. The limits of quantitation were 0.27-0.64 microg/kg. Recoveries were 29-67 percent. Combined relative measurement uncertainty estimates were 6-21 percent [09080].

The administration of anabolic steroids, for the purposes of growth promotion, to food-producing animals is banned in the EU. Among the compounds covered by this prohibition is ss-nortestosterone (beta-NT). This hormone is known to occur naturally in stallions and boars, and its main bovine metabolite, alpha-nortestosterone (alpha-NT), occurs naturally in pregnant cows and neonatal calves. However, neither compound is believed to occur naturally in male cattle. During 2006, the presence of alpha-NT and, on occasion, beta-NT was confirmed in male cattle (bulls and steers) slaughtered in Northern Ireland on welfare grounds, as a result of acute injury. Subsequent investigations revealed no evidence of abuse at any of the farms involved and revealed that the phenomenon also occurred in three other regions of the EU, in similarly injured animals. A hypothetical link to release of the adrenal steroid, dehydroepiandrosterone (DHEA), in response to the stress of the injury was tested. Following the intravenous administration of DHEA to two normal steers, beta-NT (but not alpha-NT) was confirmed in the urine of one steer. Thus, it may be concluded that both beta-NT and, by implication, alpha-NT can occur naturally in male cattle (or a specific cohort thereof) in contrast to previously accepted scientific knowledge [09081].

A new LC-MS/MS method was developed for the analysis of 29 veterinary drug residues, spanning three different drug classes, in animal tissues. The procedures used to measure the characteristic performance parameters of the method and the results obtained using fortified blank bovine muscle and kidney tissue are described. For a quantitative and confirmatory method, the characteristic performance parameters to be determined are the limits of quantification, trueness, recovery, precision, selectivity, ruggedness, and stability. The characteristic performance parameters defined for the method will be verified during a validation study by an independent experienced analyst to determine whether the method is suitable for use in a regulatory monitoring and control program for residues of the 29 analytes [09082].

The abuse of synthetic esters of natural steroids such as testosterone and estradiol in cattle fattening and sports is hard to detect via routine urine testing. The esters are rapidly hydrolysed in vivo into substances which are also endogenously present in urine. An interesting alternative can be provided by the analysis of the administered synthetic steroids themselves, i.e. the analysis of intact steroid esters in hair by liquid chromatography tandem mass spectrometry (LC/MS/MS). However, retrospective estimation of the application date following a non-compliant finding is hindered by the complexity of the kinetics of the incorporation of steroid esters in hair. In this study, the incorporation of intact steroid esters in
hair following pour-on treatment has been studied and critically compared with results from intramuscular treatment. To this end animals were pour-on treated with a hormone cocktail containing testosterone cypionate, testosterone decanoate and estradiol benzoate in different carriers. The animals were either treated using injection and pour-on application once or three times having 1 week between treatments using injection and pour-on application. Animals were slaughtered from 10-12 weeks after the last treatment. Both hair and blood plasma samples were collected and analysed by LC/MS/MS. From the results, it is concluded that after single treatment the levels of steroid esters in hair drop to CCbeta levels (5-20 microg/kg) after 5-7 weeks. When treatment is repeated two times, the CCbeta levels are reached after 9-11 weeks. Furthermore, in plasma, no steroid esters were detected; not even at the low microgramme per litre level but – in contrast with the pour-on application – after intramuscular injection, significant increase of 17beta-testosterone and 17beta-estradiol were observed. These observations suggest that transport of steroid esters after pour-on application is not only performed by blood but also by alternative fluids in the animal so probably the steroid esters are already hydrolysed and epimerized before entering the blood [09083].

The detection of hormone abuse for growth promotion in food animal production is a global concern. Initial testing for hormones in Canada was directed at the compounds approved for use in beef cattle, melengestrol acetate, trenbolone acetate and zeranol, and the banned compound diethylstilbestrol (DES). No hormonal growth promoters are approved for use in veal production in Canada. However, instances of use of trenbolone and clenbuterol were detected in Canada in the 1990s. During the development of a new analytical method for testosterone and progesterone, there were reports of suspicious injection sites being found in veal calves. Upon implementation of the method, analysis of investigative samples revealed significant residues of testosterone in some injection sites. To prove that the source of these residues was exogenous, a fully validated method for hormone esters was developed to confirm the presence of exogenous hormones in these injection sites. The QUECHERS model was employed in methods development and resulted in a simple, effective extraction technique that consisted of sample pre-homogenization, liquid/liquid partitioning, extract dilution, filtration and use of LC/MS/MS to provide detection selectivity. The result was an adaptable MS/MS confirmation technique that meets the needs of Canadian regulatory authorities to confirm the misuse of injectable testosterone, and potentially other hormones, in food animal production [09084].

**Nandrolone**

17beta-Nandrolone (17beta-NT) is one of the most recurrent forbidden anabolic steroid used in meat producing animals breeding. Because efficient control must both take into account metabolic patterns and associated kinetics of elimination, the metabolism of 17beta-NT in bovines has already been investigated and is well documented, but only focussing on its main metabolites (i.e. 17alpha-nandrolone, 19-noretiocholanolone and 19-norandrostenedione). The goal of one study was to enlarge this panel of 17beta-NT metabolites, especially through the urinary estranediols fraction in order to perform a more global steroid profiling upon 17beta-nortestosterone laureate ester administration in calves. A GC-MS/MS method has been developed to monitor and quantify 5 estranediols isomers including 5alpha-estrane-3beta,17beta-diol (abb), 5beta-estrane-3alpha,17beta-diol (bab), 5alpha-estrane-3beta,17alpha-diol (aba), 5alpha-estrane-3alpha,17beta-diol (aab) and 5beta-estrane-3alpha,17alpha-diol (baa). Their urinary elimination kinetics have been established allowing detection of 4 estranediols up to several days after administration. All animals demonstrated homogeneous patterns of elimination both from a qualitative (metabolite profile) and quantitative point of view (elimination kinetics in urine). 5alpha-Estrane-3beta,17alpha-diol (aba) was found as the major metabolite with concentrations up to 100microg/L [10345].
Trenbolone

Over the last years, extensive research has documented endocrine-disrupting activities for a significant number of substances including, among others, hormones, pharmaceuticals, pesticides and surfactants. Nonetheless, for most endocrine disruptors, toxicological profiles are still incomplete or even lacking. A systematic review has shown that a number of endocrine disruptors with steroid-modulating effects may also exert mutagenic and carcinogenic activities. For trenbolone, an androgenic compound, there is controversy about its genotoxic properties in the literature, apparently with a strong dependence on the choice of the test system. Since fish and other aquatic animals are at risk of exposure to run-offs from cattle feedlots or sewage-discharge sites containing trenbolone, potential consequences to aquatic ecosystems need to be assessed. To this end, the potential genotoxic hazard of trenbolone was tested in vitro in the permanent rainbow trout-liver cell-line RTL-W1, as well as in primary cell cultures derived from zebrafish (Danio rerio) embryos after in vivo exposure. In either test system, a potential genotoxic hazard characterized by biphasic dose-response curves could be documented even at exposure concentrations of 30 microg/L. These results thus confirm the conclusion that the steroid trenbolone may act as a genotoxic substance [11053].

Trenbolone acetate (TbA) is a potent synthetic anabolic steroid that was approved by the FDA as a growth promoter in beef cattle in 1987. Given the endocrine-modulating activity of TbA and its metabolites in all vertebrates, a sensitive and reliable analytical method is needed to detect TbA and related residues in environmental matrices. It was developed a method that incorporates solid phase extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the simultaneous determination of the three major TbA metabolites (trendione, 17beta-trenbolone, 17alpha-trenbolone) in total suspended particulate matter (TSP) samples. Sample preparation involved pressurized liquid extraction followed by cleanup on solid-phase extraction cartridges. The procedure was optimized to obtain maximum recovery and minimum signal suppression/enhancement from matrix effects. Analytes were separated with a Phenomenex Gemini-NX C18 analytical column (150 mm×2.0 mm, 3 microm particle size) using an aqueous methanol gradient at a flow rate of 0.2 mL/min. Column effluent underwent positive electrospray ionization (ESI). Two or more diagnostic product ions were acquired from analyte specific precursor ions for unambiguous confirmation and quantification. The method detection limit was 3.27-4.87 ng/g of particulate matter (PM). Method accuracy, determined with analyte recoveries, ranged between 68 and 117 percent, and method precision, expressed as relative standard deviation, was below 15 percent at spiked levels of 6.67, 33.3, and 167 ng/g PM. Analysis of TSP samples demonstrated the presence of the target species associated with PM in the vicinity of beef cattle feeding operations [11054].

Molecular mechanisms in skeletal muscle associated with anabolic steroid treatment of cattle are unclear and we aimed to characterize transcriptional changes. Cattle were chronically exposed (68 ± 20 days) to a steroid hormone implant containing 200 mg trenbolone acetate and 20 mg estradiol (Revalor-H). Biopsy samples from 48 cattle (half treated) from longissimus dorsi (LD) muscle under local anesthesia were collected. Gene expression levels were profiled by microarray, covering 16,944 unique bovine genes: 121 genes were differentially expressed (DE) due to the implant (99.99 % posterior probability of not being false positives). Among DE genes, a decrease in expression of a number of fat metabolism-associated genes, likely reflecting the lipid storage activity of intramuscular adipocytes, was observed. The expression of IGF1 and genes related to the extracellular matrix, slow twitch fibers, and cell cycle (including SOX8, a satellite cell marker) was increased in the treated muscle. Unexpectedly, a very large 21- (microarray) to 97 (real time quantitative PCR)-fold
higher expression of the mRNA encoding the neuropeptide hormone oxytocin was observed in treated muscle. We also observed an \( \sim 50 \)-fold higher level of circulating oxytocin in the plasma of treated animals at the time of biopsy. Using a coexpression network strategy OXTR was identified as more likely than IGF1R to be a major mediator of the muscle response to Revalor-H. A re-investigation of in vivo cattle LD muscle samples during early to mid-fetal development identified a \( >128 \)-fold increased expression of OXT, coincident with myofiber differentiation and fusion. It was propose that oxytocin may be involved in mediating the anabolic effects of Revalor-H treatment [11055].

**Methenolone acetate in a veal calf**

The use of anabolic steroids has been banned in the European Union since 1981. In this study, the metabolism of the anabolic steroid methenolone acetate, was investigated in a male veal calf. After daily oral administration of methenolone acetate, three main metabolites were detected in both urine and faeces samples. Among these metabolites, alpha-methenolone was apparently the main one, but 1-methyl-5alpha-androstan-3,17-diol and 3alpha-hydroxy-1-methyl-5alpha-androstan-17-one were also observed. The parent compound was still detectable in faeces. As a consequence, abuse of methenolone acetate as growth promoter can be monitored by analysing urine and faeces samples. A few days after the last treatment, however, no metabolites were observed. Alpha-methenolone was detectable in urine until 5 days after the last treatment, but in faeces no metabolites were detectable after 3 days [06240].

**Sexual behavior of fish after trenbolone**

Endocrine disrupting chemicals (EDCs) are a large group of environmental pollutants that can interfere with the endocrine system function of organisms at very low levels. One compound of great concern is trenbolone, which is widely used as a growth promoter in the cattle industry in many parts of the world. The aim of one study was to test how short-term (21-day) exposure to an environmentally relevant concentration of 17beta-trenbolone (measured concentration 6 ng/L) affects reproductive behaviour and fin morphology in the eastern mosquitofish (Gambusia holbrooki). The mosquitofish is a sexually dimorphic livebearer with males inseminating females using their modified anal fin, the gonopodium, as an intromittent organ. Although the species has a coercive mating system, females are able to exert some control over the success of male mating attempts by selectively associating with, or avoiding, certain males over others. It was found that females exposed to trenbolone approached males less and spent more time swimming away from males than non-exposed (control) females. By contrast, it was found no difference in the behaviour of exposed and non-exposed males. Furthermore, exposure did not affect the anal fin morphology of males or females. This is the first study to demonstrate that exposure to an androgenic EDC can impair female (but not male) behaviour. The study illustrates how anthropogenic contaminants can have sex-specific effects, and highlights the need to examine the behavioural responses of environmental contaminants in both sexes [13077].

**Growth hormone**

Black market products of a pharmaceutical nature and nutritional supplements have received substantial and increasing attention because of potential performance enhancement in elite and non-professional sports. In addition, improved general health is claimed for non-competing individuals. The risks and foreseeable dangers of the uncontrolled use of highly potent and non-approved pharmaceutical compounds in healthy individuals are of
considerable concern. In the present case report, the emerging drug candidate GHRP-2 with verified growth-hormone-releasing properties was identified and quantified in tablets offered as an over-the-counter nutritional supplement. The impact of this orally active peptide on the hGH/IGF-axis has been established for several years and its illicit use in elite sports has been assumed. As a releasing factor for hGH, GHRP-2 belongs to the list of substances prohibited by the World Anti-Doping Agency (WADA). Unfortunately, to date there is no routinely performed assay for the determination of these peptides potentially occurring in biological fluids of competing athletes, but the present data will facilitate the implementation by providing principle analytical information on liquid chromatographic and mass spectrometric behaviour. Qualitative identification of the target analyte after extraction from the tablet matrix was performed by high resolution/high accuracy mass spectrometry after liquid chromatographic separation under consideration of the accurate masses and the ratios of the protonated molecules and their fragment ions derived from their collisionally induced dissociation. Quantitative results were obtained by means of liquid chromatography coupled to a triple quadrupole mass spectrometer and linear regression using an external calibration curve (with GHRP-2 reference compound) adjusted via internal standard (Hexarelin). Hereby, the content of GHRP-2 was determined with approximately 50 microg per tablet [10154].

**Growth promoters given to livestock**

In vitro cell-based bioassays are also used to test for illegal substances such as growth promoters that are given to livestock. These androgens or androgen-like molecules enhance the growth of the animals and are used as a means to increase profit. Naturally occurring steroids such as testosterone or synthetic androgens such as 19-nortestosterone, trenbolone acetate, and medroxyprogesterone are used to illicitly augment growth of animal livestock. In addition, prohormones such as dehydroepiandrosterone (DHEA) are being used, and these are often hard to detect if they have been exogenously administered. GC-MS-based screening of material such as meat extracts, feed or urine may fail to detect these substances because deconjugating steps are required prior to GC-MS analysis and these processing steps can destroy the structures. GC-MS may also fail to detect novel structures or those with unknown metabolic profiles. Bioassays can complement the screening of samples as they are capable of detecting hormonally active compounds in prepared extracts and if the appropriate host cell is used for the bioassay such as hepatocytes then such assays may also detect prohormones. For these reasons, bioassays are ideal for the detection of androgens or proandrogens added to nutritional supplements [13084].

**Beta-agonists in pork**

A method was developed to determine 20 illegal residual beta-agonists in pork tissues, including muscle and liver simultaneously. The samples were hydrolyzed by betaglucuronidase, purified by PCX SPE cartridges, and detected by HPLC coupled with electrospray ionization MS/MS operating in the positive ion mode. Matrix-fortified calibration was performed to compensate for the matrix effect and loss in sample preparation. Decision limit ranged from 0.05 to 0.23 microg/kg in muscle and 0.05 to 0.57 microg/kg in liver. Decision capacity ranged from 0.11 to 0.4 microg/kg in muscle and 0.16 to 0.79 microg/kg in liver. In Food Analysis Performance Assessment Scheme proficiency test 0287, a pig liver test material containing 13 beta-agonists was analyzed using the method developed, and clenbuterol and ractopamine were confirmed as being present. Z-scores for clenbuterol and ractopamine were 0.2 and 0.6, respectively [11056].
Clenbuterol

The misuse of the sympathomimetic and anabolic agent clenbuterol has been frequently reported in professional sport and in the livestock industry. In 2010, a team of athletes returned from competition in China and regular doping control samples were taken within the next two days. All urine samples contained low amounts (pg/ml) of clenbuterol, drawing the attention to a well-known problem: the possibility of an unintended clenbuterol intake with food. A warning that Chinese meat is possibly contaminated with prohibited substances according to international anti-doping regulations was also given by Chinese officials just before the Beijing Olympic Games in 2008. To investigate if clenbuterol can be found in human urine, a study was initiated comprising 28 volunteers collecting urine samples after their return from China. For the quantification of clenbuterol at a low pg/ml level, a very sensitive and specific isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay was developed using liquid/liquid re-extraction for clean-up with a limit of detection and quantification of 1 and 3 pg/ml, respectively. The method was validated demonstrating good precision, accuracy, and mean recovery. Clenbuterol was detectable in 22 (79%) of the analyzed samples, indicating a general food contamination problem despite an official clenbuterol prohibition in China for livestock [12358].

Clenbuterol in muscle

A new pretreatment method, solid-phase extraction combined with dispersive liquid-liquid microextraction (SPE-DLLME), was proposed in first time for the determination of clenbuterol (CLB) in porcine tissue samples. The tissue samples were firstly extracted by SPE, then its eluents were used as dispersant of the followed DLLME for further purification and enrichment of CLB. Various parameters (such as the type of SPE sorbent, the type and volume of elution solvent, the type and volume of extractant and dispersant, etc.) that affected the efficiency of the two steps were optimized. Good linearity of CLB was ranged from 0.19 microg/kg to 192 μg/kg with correlation coefficient (r²) of 0.9995. The limit of detection (LOD) was 0.07 μg/kg (S/N=3) and the recoveries at three spiked levels were ranged from 88 percent to 104 percent with the relative standard deviation (RSD) less than 3.9% (n=3). Under the optimized conditions, the enrichment factor (EF) for CLB could up to 62 folds. The presented method that combined the advantages of SPE and DLLME, had higher selectivity than SPE method and was successfully applied to the determination of CLB in tissue samples [11057].

Clenbuterol in pig retina

The aim of one study was to assess the persistence of clenbuterol residues in retinal tissue of pigs after repeated administration in a growth-promoting dose, using enzyme-linked immunosorbent assay (ELISA) as a screening method for quantitative determination. A growth-promoting dose of clenbuterol (20 μg/kg body mass per day) was administered orally to the experimental group (n=6) for 21 days, whereas control animals (n=3) were left untreated. Clenbuterol-treated pigs were randomly sacrificed (n=3) on days 0 and 45 of treatment discontinuation, and clenbuterol residues were determined in retinal tissue dissected from the eye. ELISA was found to be acceptable for quantitative determination of clenbuterol in retinal samples because previous method validation yielded mean recovery values of 84-97 percent with variation coefficients < 14 percent. The mean (± SD) retinal clenbuterol concentration was 1874 ± 114 ng/g immediately upon clenbuterol withdrawal (day 0) and 73 ± 4 ng/g on the last day post-withdrawal (day 45). Study results pointed to a
very high potential of clenbuterol accumulation in retinal tissue and marked persistence of clenbuterol residues upon anabolic dose administration, suggesting retinal tissue to be a very useful matrix for effective control of residual clenbuterol in food-producing pigs [11058].

**Clenbuterol in milk**

A simple and sensitive analytical method was developed for the simultaneous determination of clenbuterol, chloramphenicol and diethylstilbestrol in bovine milk by isotope dilution ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Samples were directly purified through HLB cartridge. The organic phase was dried under nitrogen and residues were redissolved in mobile phase. Samples were analyzed by UPLC-MS/MS on an Acquity UPLC® BEH C(18) column with gradient elution. The samples were quantified using clenbuterol-D(9), chloramphenicol-D(5) and diethylstilbestrol-D(8) as internal standards. The proposed method was validated according to the European Union regulation 2002/657/EC determining specificity, decision limit (CCalpha), detection capability (CCbeta), trueness, precision, linearity and stability. The method is demonstrated to be suitable for the determination of clenbuterol, chloramphenicol and diethylstilbestrol in bovine milk. The total time required for the analysis of one sample was about 50 min [11059].

**Screening of residues in food**

**Egg**

A cheap, reliable and practical high-performance liquid chromatography-tandem mass spectrometric method was developed for the simultaneous determination of seven anabolic steroids in eggs, including trenbolone, boldenone, nandrolone, stanozolol, methandienone, testosterone and methyl testosterone. The analytes were extracted from the egg samples using methanol. The extracts were subjected to the removal of fat by freezing-lipid filtration and then further purified by liquid-liquid extraction using tert-butyl methyl ether. The analytes were separated on a Luna C18 column by a gradient elution program with 0.1% formic acid and acetonitrile. This method was validated over 1.00-100 ng/g for all steroids of interest. The correlation coefficients (r) for each calibration curve are higher than 0.99 within the experimental concentration range. The decision limits of the steroids in eggs ranged from 0.20 to 0.44 ng/g, and the detection capabilities were below 1.03 ng/g. The average recoveries were between 66 and 83 percent in eggs at three spiked levels of 1.00, 1.50 and 2.00 ng/g for each analyte. The between-day and within-day relative standard deviations were in the range of 2-11 percent. High matrix suppression effects were observed for all compounds of interest [12357].

**Bovine**

One paper presents the generation of monoclonal antibodies (mAbs) with high specificity against 19-nortestosterone (NT) through cell fusion procedures, and the development of mAb-based heterologous direct competitive enzyme-linked immunoabsorbent assay (dcELISA) methods to detect NT residue using one of these hybridomas (clone 3B8-E6). Under optimal experimental conditions, this assay exhibited a working range of 0.004 to 19 ng/mL with IC₅₀ and limit of detection values of 0.28 and 0.002 ng/mL, respectively, when it was run in 0.01M phosphate-buffered saline (pH 7.4). Except for minor cross-reactivity with beta-boldenone (6.9 %) and trenbolone (1.2 %), other interference to the assay was negligible. No significant differences were found for IC₅₀ values when the pH of the assay buffer ranged from 6 to 8 and phosphate ion concentration was less than 20 mM. The
dcELISA can tolerate higher concentrations of methanol than other organic solvents tested. When applied to bovine sample, the correlation coefficients of the dcELISA and GC-MS data were 0.9918 in muscle, 0.9834 in liver, and 0.9976 in kidney. Therefore, this assay has the potential to be incorporated into a quantitative monitoring program for the rapid screening of NT residue in food [12359].

Swine

The occurrence and fate of fourteen androgens, four estrogens, five glucocorticoids and five progestagens were investigated by rapid resolution liquid chromatography-tandem mass spectrometry (RRLC-MS/MS) in a typical swine farm with lagoon waste disposal systems, in south China. Nineteen, 22 and 8 of 28 steroids were detected at concentrations ranging from 2.2 ± 0.1 ng/g (androsta-1,4-diene-3,17-dione) to 14,400 ± 394 ng/g (progesterone) in the feces samples, from 6.1 ± 2.3 ng/L (17beta-boldenone) to 10,800 ± 3190 ng/L (norgestrel) in the flush water samples, and from 5.0 ± 0.2 ng/g (progesterone) to 225 ± 79.4 ng/g (5alpha-dihydropregesterone) in the suspended particles, respectively. By comparing the types and concentrations of steroids in different treatment stages of the lagoon systems, it demonstrated that the lagoon systems used in the farm were not effective method to reduce various steroids in wastewater. Among the thirteen synthetic steroids detected in the swine feces and flush water, only seven (methyl testosterone, 17alpha-trenbolone, 17beta-trenbolone, 17alpha-ethynyl estradiol, dexamethasone, medroxyprogesterone, and norgestrel) were regarded as the parent/metabolite compounds of animal exogenous usage. According to the estimated masses of steroids from feces and flush water, the excretion of steroids for sows were mainly from feces, but for piglets or barrows, most excreted steroids were through flush water rather than feces. The total daily excreted masses of androgens, estrogens, glucocorticoids and progestagens in the sow feces were in the range of 91-6310 microg/d, which were up to a thousand fold of those in the feces of other growth stages indicating that the proportion of sow number in the swine farm directly influenced the total excretion mass of steroids. In addition, two natural steroids 4-androstene-3,17-dione and progesterone were worth notice due to their relatively high concentrations per sow excretion, 277 microg/d and 6380 microg/d, respectively, which are approximately equivalent to the daily excretion of 100 persons. Some steroids were also detected in the well water, vegetable field and receiving stream, and may pose potential high risks to some sensitive organisms in the receiving environment [12360].

Fish

A method was developed for the determination of 11 anabolic hormones (boldenone, androstenedione, nandrolone, methandienolone, methyltestosterone, testosterone, testosterone acetate, trenbolone, testosterone propionate, stanozolol, fluoxymesterone) in fish by multi-function impurity adsorption solid-phase extraction-ultrafast liquid chromatography-tandem mass spectrometry. After the sample was extracted by methanol, the extract was cleaned-up quickly by C18 adsorbent, neutral alumina adsorbent and amino-functionalized nano-adsorbent. The separation was performed on a Shim-Pack XR-ODS II column (100 mm x 2.0 mm, 2.2 microm) using the mobile phases of 0.1% (v/v) formic acid in acetonitrile and 0.1% (v/v) formic acid solution in a gradient elution mode. The identification and quantification were achieved by using electrospray ionization in positive ion mode (ESI+) in multiple reaction monitoring (MRM) mode. The matrix-matched external standard calibration curves were used for quantitative determination. The results showed that the calibration curves were in good linearity for the eleven analytes with the correlation coefficients more than 0.999. The limits of detection (LODs, S/N > 3) for the 11 anabolic hormones were from 0.03 microg/kg to 0.4 microg/kg and the limits of quantification (LOQs, S/N > 10) were from 0.1 microg/kg to 1.5 microg/kg. The method is simple, rapid, sensitive,
accurate and suitable for the quantitative determination and confirmation of the 11 anabolic hormones in fish [12361].

**Detection of doping agents in waste water**

The occurrence of 20 human pharmaceutical compounds and metabolites from 10 representative therapeutic classes was analysed from resource and drinking water in two catchment basins located in north-west France. Ninety-eight samples were analysed from 63 stations (surface water and drinking water produced from surface water). Of the 20 human pharmaceutical compounds selected, 16 were quantified in both the surface water and drinking water, with 22 percent of the values above the limit of quantification for surface water and 14 % for drinking water). Psychostimulants, non-steroidal anti-inflammatory drugs, iodinated contrast media and anxiolytic drugs were the main therapeutic classes of human pharmaceutical compounds detected in the surface water and drinking water. The results for surface water were close to results from previous studies in spite of differences in prescription rates of human pharmaceutical compounds in different countries. The removal rate of human pharmaceutical compounds at 11 water treatment units was also determined. Only caffeine proved to be resistant to drinking water treatment processes (with a minimum rate of 5 %). Other human pharmaceutical compounds seemed to be removed more efficiently (average elimination rate of over 50 %) by adsorption onto activated carbon and oxidation/disinfection with ozone or chlorine (not taking account of the disinfection by-products). These results add to the increasing evidence of the occurrence of human pharmaceutical compounds in drinking water that may represent a threat to human beings exposed to a cocktail of human pharmaceutical compounds and related metabolites and by-products in drinking water [11559].

Wastewater analysis can provide estimates of illicit drug consumption in local communities. It was used repeated raw wastewater analysis in urban wastewater treatment plants to estimate loads of cocaine, heroin, methamphetamine, and cannabis consumed daily by the inhabitants of two cities in Northern Italy, Milan and Como, from 2005 to 2009. Daily cocaine loads did not change in Milan from 2005 to 2008 but fell 45 percent in 2009 and there was a similar drop in Como. Heroin also fell from 2008 to 2009 in Milan (66 %) and Como (26 %). However, methamphetamine, which had risen in Milan from 2005 to 2008, rose further in 2009 and cannabis, which was falling from 2005 to March 2009, rose again in September 2009. The results suggest a trend toward a decrease in consumption of costly illicit drug, such as cocaine and heroin. This might be due to a reduction in the number of consumers and/or to a change in their behaviour since there was also an increase in the consumption of less expensive illicit drug. This itself might reflect a drop in consumers' money supply, caused by the economic crisis. Wastewater analysis was useful to estimate illicit drug consumption levels in local communities in real time and promptly identify changes in trends [11560].

A new method was developed for the analysis of natural and synthetic androgenic steroids and their selected metabolites in aquatic environmental matrixes using direct large-volume injection (LVI) high-performance liquid chromatography (HPLC) tandem mass spectrometry (MS/MS). Method accuracy ranged from 87.6 to 108% for analytes with well-matched internal standards. Precision, quantified by relative standard deviation (RSD), was less than 12 percent. Detection limits for the method ranged from 1.2 to 360 ng/L. The method was demonstrated on a series of 1 h composite wastewater influent samples collected over a day with the purpose of assessing temporal profiles of androgen loads in wastewater. Testosterone, androstenedione, boldenone, and nandrolone were detected in the sample
series at concentrations up to 290 ng/L and loads up to 535 mg/h. Boldenone, a synthetic androgen, had a temporal profile that was strongly correlated to testosterone, a natural human androgen, suggesting its source may be endogenous. An analysis of the sample particulate fraction revealed detectable amounts of sorbed testosterone and androstenedione. Androstenedione sorbed to the particulate fraction accounted for an estimated 5 to 7 percent of the total androstenedione mass [11060].

Municipal wastewater has been examined for steroids, beta_{2}-agonists, stimulants, diuretics, and phosphodiesterase type V inhibitors (PDE type V inhibitors), which are "dual-use-drugs" applied either as anabolic, doping, and lifestyle drugs or for treatment of diverse diseases. To identify their origin, fitness centre discharges under suspicion of being point sources and sewage-treatment plant feed and effluents were sampled and concentrations determined. Sensitive and selective methods for determination and quantification based on solid-phase extraction (SPE) followed by high-performance liquid chromatography-high resolution mass and tandem mass spectrometry (HPLC-(HR)MS and HPLC-MS-MS) were developed and established for analysis of these compounds in wastewater and to assess their effect on the environment. The methods developed enabled quantification at trace concentrations (limit of quantification (LOQ): 5 ng/L). Of the steroids and stimulants under investigation, testosterone, methyltestosterone, and boldenone or ephedrine, amphetamine, and MDMA (3,4-methylenedioxy-N-methylamphetamine) were observed at up to 5 microg/L (ephedrine). Of the beta_{2}-agonists salbutamol only, and of the diuretics furosemide and hydrochlorothiazide were confirmed in the extracts. Quite high concentrations of the PDE type V inhibitors sildenafil, tadalafil, and vardenafil and their metabolites were confirmed in fitness centre discharges (sildenafil: 1,945 ng/L) whereas their concentrations in municipal wastewater did not exceed 35 ng/L. The study thus identified anabolic and doping drugs in wastewater for the first time. Results obtained from wastewater treatment plant effluents proved that these "dual-use-drugs", with the exception of hydrochlorothiazide, were mostly eliminated [10342].

A wastewater treatment plant may receive various types of wastewater namely, urban, industrial, agricultural, washout from the streets, wet or dry atmospheric deposition. As such, scientists have detected in wastewaters all major categories of pollutants like persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs) and pesticides, but also substances that are widely used as pharmaceuticals and cosmetics, classified as "PPCPs" (pharmaceuticals and personal care products). Finally, the latest categories of compounds to be looked upon in these types of matrices are illicit drugs (drugs of abuse, like cocaine, etc.) and doping substances. This review article summarises major categories of organic microcontaminants that have been detected in wastewaters and studies their fate during the wastewater treatment process. Occurrence of these compounds in the influents and effluents are reported, as well as percents of removal, mass balances and phase distributions [12089].

It was reported a monitoring study analysing wastewater and associated suspended particulate matter (SPM) to determine the concentration of drugs of abuse and metabolites in wastewater influent. The monitoring of SPM is crucial for target analytes because, depending on their physico-chemical properties, they may partition to particulates; thus, analysis of wastewater only will result in under-reporting of the concentration of target analytes in the sample. A daily one week monitoring study was carried out at a WWTP serving one of the largest cities in the Czech Republic; representing the first comprehensive application of the sewage epidemiology approach in the Czech Republic. In total, 60 analytes were targeted in the monitoring programme including stimulants, opioid and morphine derivatives, benzodiazepines, antidepressants, dissociative anaesthetics, drug precursors and their metabolites. Analysis of SPM determined that significant proportions of some compounds
were present on the solids. For example, 21-50 percent of the total concentration of EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine) in the sample was determined on SPM and 11-20 percent of methadone. The highest proportion on SPM was determined for fluoxetine in the range 68-80 percent, norfluoxetine 47-62 percent and amitriptyline 22-51 percent. In contrast, some compounds presented very little partitioning to SPM. Less than 5 percent was determined partitioned to SPM over the week period for analytes including cocaine, benzoylecgonine, cocaethylene, amphetamine, methamphetamine, MDMA (3,4-methylenedioxymethamphetamine), codeine, dihydrocodeine, tramadol, nortramadol, oxazepam and ephedrine. Determined concentrations in wastewater influent were subsequently utilised in the sewage epidemiology approach to estimate drug consumption, in the community from which the wastewater was derived. This back-calculcation was updated for the first time to include the concentration of analytes present on SPM. The consumption of methamphetamine and MDMA was determined to be especially high in the studied community in relation to other European countries, while cocaine and methadone consumption was relatively low. This manuscript shows that in order to apply the sewage epidemiology approach, SPM analysis is required for some compounds; whereas for others the partitioning is small and one may regard this as negligible [12090].

Fate and occurrence of fourteen androgens, four estrogens, five glucocorticoids and five progestagens were investigated in three swine farms and three dairy cattle farms with different farming scales and wastes disposal systems in China. Twenty-one, 22, and 12 of total 28 steroids were detected in feces samples with concentrations ranging from below method limit of quantitation (<LOQ for estrone) to 8100 ± 444 ng/g (progesterone), in wastewater samples with concentrations ranging from <LOQ (estrone) to 20,700 ± 1490 ng/L (androsterone), in suspended particles with concentrations ranging from <LOQ (17beta-trenbolone) to 778 ± 82.1 ng/g (5alpha-dihydrotestosterone) in the six farms, respectively. The steroids via swine farms and human sources were mainly originated from wastewater into the receiving environments while those steroids via cattle farms were mainly from cattle feces. The total contributions of steroids to the environment in China are estimated to be 139, 65.8 and 60.7 t/year from swine, dairy cattle and human sources, respectively [12091].

The occurrence and fate of fourteen androgens, four estrogens, five glucocorticoids and five progestagens were investigated in two different types of wastewater treatment plants (Plant A: activated sludge with chlorination, and Plant B: oxidation ditch with UV) of Guangdong province, China. 14, 14, and 10 of 28 target compounds were detected in the influent, effluent and dewatered sludge samples with the concentrations ranging from below 1.2 ± 0.0 ng/L (stanozolol) to 1368 ± 283 ng/L (epi-androsterone), below 1.0 ± 0.0 ng/L (progesterone) to 23.1 ± 1.0 ng/L (5a-dihydrotestosterone), 1.0 ± 0.1 ng/g (estrone) to 460 ± 4.4 ng/g (5alpha-dihydrotestosterone), respectively. The concentrations of total androgens (1554-1778 ng/L in influent, 13.3-47.8 ng/L in effluent, 377-923 ng/g in dewatered sludge) were much higher than those of total estrogens (41.5-60.2 ng/L in influent, 5.6-13.5 ng/L in effluent, 13.9-57.8 ng/g in dewatered sludge), glucocorticoids (171-192 ng/L in influent, 2.2-6.3 ng/L in effluent, N.D.-4.4 ng/g in dewatered sludge), and progestagens (39.6-40.5 ng/L in influent, 6.9-12.1 ng/L in effluent, N.D. in dewatered sludge) in these two WWTPs. According to mass balance analysis, the removal rates of most target steroids in Plant A had exceeded 90 percent, while those in Plant B for nearly half of detected target steroids were lower than 80 percent. It is obvious that the treatment capacity of the activated sludge system (Plant A) is superior to the oxidation ditch (Plant B) in the degradation of steroids in sewage treatment systems. Androgens, estrogens and progestagens were mainly removed by sorption and degradation, while the reduction of glucocorticoids was primarily due to degradation [12362].

Urine from urinals
Analysis of urine samples collected across a city centre, for the detection of novel psychoactive substances (NPS) was done in a cross-sectional study of anonymized urine samples used for the analysis of classical recreational drugs, NPS and metabolites. Pooled urine samples collected from portable stand-alone four-person urinals across a city centre were analysed using full-scan accurate-mass high-resolution liquid chromatography coupled to tandem mass spectrometry. Data were processed against compound databases containing >1700 drug compounds and metabolites. Seven established recreational drugs (3,4-methylenedioxyamphetamine, cocaine, cannabis, ketamine, 3,4-methylenedioxy-\(N\)-methylamphetamine, methamphetamine and amphetamine) and six potential NPS [hordenine (all 12 urinals), cathine (11), methylhexaneamine (9), 4-methylmethylcathinone (6), methiopropamine and metabolites (2) and methoxetamine and metabolites (1)] were detected. Methylhexaneamine, methiopropamine and hordenine are currently uncontrolled in the UK, whereas methoxetamine is currently subject to a Temporary Class Drug Order. Metabolites of the anabolic steroid nandrolone were found in two urinals and trenbolone metabolites and clenbuterol in one urinal. Thus, analysis of pooled urine samples collected anonymously from stand-alone urinals in a large inner city can detect the use of recreational drugs, NPS and anabolic steroids. Metabolite detection indicates actual drug use, metabolism and elimination rather than simply discarded drugs in the urinals. This technique by confirming the actual drug(s) used has the potential to be additive to currently used datasets/key indicators providing more robust information for healthcare authorities, legislative and law enforcement on the drugs actually being used [12092].

A cross-sectional study of anonymized urine samples collected across a city centre was used for the analysis of classical recreational drugs, novel psychoactive substances (NPS) and metabolites. Pooled urine samples collected from portable stand-alone four-person urinals across a city centre were analysed using full-scan accurate-mass high-resolution liquid chromatography coupled to tandem mass spectrometry. Data were processed against compound databases containing >1700 drug compounds and metabolites. Seven established recreational drugs (3,4-methylenedioxyamphetamine, cocaine, cannabis, ketamine, 3,4-methylenedioxy-\(N\)-methylamphetamine, methamphetamine and amphetamine) and six potential NPS [hordenine (all 12 urinals), cathine (11), methylhexaneamine (9), 4-methylmethylcathinone (6), methiopropamine and metabolites (2) and methoxetamine and metabolites (1)] were detected. Metabolites of the anabolic steroid nandrolone were found in two urinals and trenbolone metabolites and clenbuterol in one urinal. It was concluded that analysis of pooled urine samples collected anonymously from stand-alone urinals in a large inner city can detect the use of recreational drugs, NPS and anabolic steroids. Metabolite detection indicates actual drug use, metabolism and elimination rather than simply discarded drugs in the urinals. This technique by confirming the actual drug(s) used has the potential to be additive to currently used datasets/key indicators providing more robust information for healthcare authorities, legislative and law enforcement on the drugs actually being used [12093].

Rests in other parts of nature

Land application of manure may contribute endocrine disrupting compounds (EDCs) such as steroid hormones to the environment. Little attention has been paid to the potential for degradation of steroid hormones by manure-borne bacteria and their degradation kinetics and pathways. In a laboratory study, the potential for biodegradation of testosterone, 17beta-estradiol (E\(_2\)) and progesterone by swine (Sus scrofa) manure-borne bacteria was examined. In addition, the impact of temperature, pH (6, 7, and 7.5), glucose amendments (0, 3, and 22 mmol L\((-1))\), and presence of oxygen on testosterone degradation kinetics was determined.
Testosterone, 17beta-estradiol and progesterone were biodegraded within 25 h of reaction initiation under aerobic conditions. The degradation of testosterone followed pseudo first-order and zero-order reaction kinetics under aerobic and anaerobic conditions, respectively, in tryptic soy broth (TSB) pre-enriched systems. The half-life ($t_{1/2}$) for the degradation of testosterone under anaerobic conditions was six times longer than aerobic conditions. Testosterone degradation was found to significantly increase (-17 %) when incubated at 37 degrees C versus 22 degrees C. The impact of pH ($t_{1/2}$ ranged from 4.4-4.9 h) and glucose amendments ($t_{1/2}$ ranged from 4.6-5.1 h) on the testosterone degradation rate were found to be small. Testosterone was transformed to dehydrotestosterone (DHT) (major degradation product), androstenedione (AD), and androstadienedione (ADD) under aerobic conditions as revealed by liquid chromatography-time-of-flight mass spectrometry (LC/TOF-MS). These results indicate that testosterone is rapidly degraded by manure-borne bacteria under a wide range of environmentally relevant conditions. However, the formed degradation products are still of potential concern due to their endocrine disrupting potential [10343].

**Beef palatability**

The use of anabolic implants has a long-standing place in the cattle feeding industry, due to their positive impact on growth performance and subsequent profitability. However, implants can have adverse effects on carcass quality, shear force, and eating quality depending on the dose and frequency, or what some refer to as the aggressiveness of the implant regimen administered. Within the past decade, a new class of growth promotants, known as beta-adrenergic agonists (beta-AA), has emerged in the beef feeding industry in the United States. Currently, 2 have gained U.S. Food and Drug Administration approval for use in beef finishing diets to improve performance and carcass yields. Much like anabolic implants, these repartitioning agents can have negative effects on Warner-Bratzler shear force (WBSF), but the differences do not necessarily translate directly to consumer responses for palatability and acceptance in some instances, especially when tenderness is managed through proper postmortem aging. As researchers continued to investigate the mechanisms responsible for the impact of beta-AA, inevitably this led to consideration of the interaction between beta-AA and anabolic implants. Early work combining zilpaterol hydrochloride (ZH) with anabolic implants improved performance, carcass yield, and meat yield with additive negative effects on WBSF. Similar results were produced when pairing ZH with anabolic steroids equipped with various release patterns. As with any tool, the key to success is proper management. Certain cattle populations may be better suited to receive growth promotants such as implants and beta-AA, and postmortem management of subprimal cuts becomes vital when producers take more aggressive approaches to improve performance and yield. The objective of one review is to overview research findings related to the impact of growth promotant technologies on beef palatability, focusing specifically on the role of implants and beta-AA on carcass quality, beef tenderness, and consumer responses for meat palatability [13115].

**Laboratory techniques**

*In meat*

Within the scope of the European Community member states' residue monitoring plan, illicit administration of anabolic steroids is monitored at slaughterhouse level as well as on living animals. At farm level, urine is one of the target matrices to detect possible abuse of anabolic steroid growth promoters. Optimisation of the routinely applied analysis method resulted in a
A gas chromatography/mass spectrometry (GC/MS) method was developed for the determination of multi-residues of steroid anabolic hormones epitestosterone (ETS), testosterone 17-propionate (PTS), nandrolone (17beta-NT), 17alpha-methyltestosterone (MTS), 17beta-estradiol (17beta-ES), estriol (EST), 17alpha-ethinylestradiol (EES), estrone (ESN) and 17beta-estradiol 3-benzoate (BES) in the muscle tissues of various animal species. Homogenized tissue samples were enzymatically digested in acetate buffer (pH 5.0). Consequently, methanol was added and the mixtures were extracted under ultrasonication incubation. Clean-up was carried out for at least two times with methyl tert-butyl ether (MTBE) liquid-liquid partitioning followed by a reversed-phase solid phase extraction (SPE) cartridge purification. The eluate with methanol was evaporated to dryness by N2 at 40 degrees C and derivatization was achieved with N-methyl-N-(trimethylsilyl) trifluoroacetamide/iodotrimethylsilane/dithioerythritol (MSTFA-TMIS-DTE) at 60 degrees C for 45 min. The reaction mixture was injected into a gas chromatograph with a DB-1 capillary column coupled with a mass spectrometer. The samples were tested by different selected ion monitoring modes with electron impact (EI) source for the androgens and estrogens. The limits of quantitation (LOQ) for the above 9 hormones were in the range of 1.0 - 2.0 microg/kg. At the 2.0 microg/kg LOQ spiked level, the mean recoveries were within 63-81 percent, and the relative standard deviations were within 13-27 percent. The real sample tests showed this method can be used for the sensitive and accurate determination of multi-steroid anabolic hormones residues in biological muscle samples [06330].

**Proteomics**

The use of beta-agonists, sexual steroids, and corticosteroids as growth-promoting agents (GPAs) in veal calves is forbidden in the European Union (EU) and subjected to restrictions in the US because it may be potentially noxious for both treated animals and the consumer. Although official controls performed in the EU have revealed a limited number of positive samples, the analysis of seized preparations indicate that the use of illegal GPAs is far from being abandoned. The presence of these compounds in matrixes of biological origin often goes unnoticed because of the use of very low dosages and/or of molecules of unknown chemical structure. It is therefore necessary to develop screening methods based on the biological effects of these substances that allow the simultaneous screening of many components, as proteome analysis. When hepatic cytosols and microsomes from calves treated with a combination of GPAs were analyzed by 2-DE, it was found changes in the expression of two proteins, which we identified as adenosine kinase and reticulocalbin. The aim was not to speculate about molecular mechanisms, but to show the ability of the proteomic approach to find biomarkers of illicit treatments and to use it as a basis to develop large-scale screening methods [06331].

**In faeces**

Feces are a possible medium to be used for horse doping control. Efficient methods for detecting drugs in feces collected from various animals are routinely applied in institutes of
food safety in Belgium. It has already tested whether they are applicable to horse feces. In this report, accelerated solvent extraction (ASE), an efficient method for extracting compounds from solid material, has been tested. ASE has been used to replace the diethyl ether liquid-liquid extraction step present in the method initially set up. This technique has been optimized for detecting several non-steroidal anti-inflammatory drugs (NSAIDs) in horse feces. Extraction recovery and limit of detection have been determined for several NSAIDs, such as meclofenamic acid, flunixin, vedaprofen, celecoxib, carprofen, diclofenac, and ketoprofen. The method has been successfully applied to meclofenamic acid, flunixin, and phenylbutazone post-administration feces samples, and the main metabolites identified in urine were also detected in feces. In the case of meclofenamic acid, the detection profile in feces presented in this report is in accordance with our previous finding in feces obtained with the original method. The use of ASE decreases the time necessary for sample preparation. This method is applicable on a large scale, which is useful for horse doping control [06332].

**Liquid chromatography tandem mass spectrometry**

A method had been developed for determination of residues of 10 anabolic steroids (ASs) in animal muscle tissues by liquid chromatography tandem mass spectrometry (LC/MS/MS). After enzymolysis, the sample was extracted with tert-butyl methyl ether, cleaned up through reverse solid-phase extraction and further determined by LC/MS/MS under multiple reaction monitoring (MRM) mode. The limits of detection (LOD) of LC/MS/MS method used for testing epitestosterone (ETS), nandrolone (17 beta-NT), 17 alpha-methyl-testosterone (MTS), testosterone 17-propionate (PTS), medroxyprogesterone (MED), progesterone (PG), estrone (ESN), 17 beta-estradiol (17 beta-ES), 17alpha-ethynylestradiol (EES) and estriol (EST) in animal muscle ranged from 0.06 to 0.22 microg/kg, and the limits of quantification (LOQ) were from 0.12 to 0.54 microg/kg. Experiments on spiked samples of pork, beef, chicken and fish showed that at addition level of 1.0 microg/kg, the average recoveries of the ASs ranged from 64 to 77 percent, and coefficients of variation from 7 to 20 percent, while at addition level of 2.0 microg/kg, the average recoveries ranged from 70 to 89 percent, and coefficient of variation from 7 to 19 percent [06241].

**High- and low-resolution mass spectrometry**

Within the European Union the use of anabolic steroids for promoting growth and improving meat-to-fat ratio in food-producing animals has been banned since 1988. For the unequivocal identification of hormone residues in a complex matrix such as meat we have developed a rapid, specific and sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method, in combination with a simple extraction procedure based on the matrix solid-phase dispersion (MSPD). The performances of a triple quadrupole (QqQ) and a quadrupole/time-of-flight (QqTOF) were compared: the QqQ mass spectrometer was found to be more sensitive for almost all studied analytes, but the selectivity was superior using the QqTOF system; the full-scan spectra (acquired without losing sensitivity), mass accuracy and resolution of the hybrid instrument enabled a more probatory analyte identification than that obtained selecting two multiple-reaction monitoring (MRM) transitions with a QqQ. Average recoveries ranged from 80 to 100 percent, and the detection capabilities (CCbetas) were less than 1.06 ppb with the QqQ instrument and less than 5.20 ppb with the QqTOF instrument for the bovine meat, which proved to be the most complex matrix [06242].
ANABOLIC ANDROGENIC SUBSTANCES

Overviews

Ever since the ancient Olympic Games, athletes have long sought the ability to enhance their performance in sports and continue to do so in the modern era of elite competition. Over the past few decades, athletes have attempted to enhance their performance with the use of exogenous hormones including androgens, erythropoietin and growth hormone. Androgens are the most effective form of sports doping and are the most common type of performance enhancing substances detected in screening tests. It was not until the 1972 Munich Olympic Games that the International Olympic Committee (IOC) introduced screening tests for exogenous androgens. Since then, the World Anti-Doping Agency (WADA) enforces the banning of androgens through urine screening to detect even trace amounts of an extensive list of banned and prohibited substances which athletes are screened for prior, and during, competition. This list relies on screening urine samples with highly specific and sensitive techniques such as gas chromatography-mass spectrometry (GC-MS). For some athletes, the prospect of success outweighs the health and unethical concerns of sports doping. In an attempt to bypass screening methods, designer androgens have been created that have different chemical structures to known androgens and, therefore, cannot be easily detected by GC-MS. However, because designer androgens have biological activity they activate the androgen receptor (AR), and therefore can be detected by androgen bioassays. Therefore, androgen bioassays may prove to be a suitable tool for routine screening of nutraceutical or biological samples suspected to contain an androgen [13084].

Androgens are the class of sex steroids responsible for male sexual characteristics, including increased muscle mass and decreased fat mass. Illicit use of androgen doping can be an attractive option for those looking to enhance sporting performance and/or physical appearance. The use of in vitro bioassays to detect androgens, especially designer or pro-androgens, is becoming increasingly important in combating androgen doping associated with nutritional supplements. The nutritional sports supplement market has grown rapidly throughout the past decade. Many of these supplements contain androgens, designer androgens or pro-androgens. Many designer or pro-androgens cannot be detected by the standard highly-sensitive screening methods such as gas chromatography-mass spectrometry because their chemical structure is unknown. However, in vitro androgen bioassays can detect designer and pro-androgens as these assays are not reliant on knowing the chemical structure but instead are based on androgen receptor activation. For these reasons, it may be advantageous to use routine androgen bioassay screening of nutraceutical samples to help curb the increasing problem of androgen doping [13084].

Anabolic steroids are widely used for doping, in professional and non-professional sports. The mechanism of action may differ somewhat depending on the specific molecule due to structural differences that influence the specificity of binding with steroid receptors. When used by athletes in training, they can improve performance to levels that cannot be attained by almost any combination of sophisticated nonchemical support by modern sport science. The severity of the undesired effects of anabolic steroids depends on a variety of factors, from the type and combination of them, the dose and duration of administration, as well as the gender of the person taking the drug. Younger individuals and women show greater effects caused by anabolic steroids in terms of performance, but are also at greater risk of side effects [09047].

The anabolic-androgenic steroids (AAS) have been used by elite athletes since the 1950s,
but they did not become widespread drugs of abuse in the general population until the 1980s. Thus, knowledge of the medical and behavioral effects of illicit AAS use is still evolving. Surveys suggest that many millions of boys and men, primarily in Western countries, have abused AAS to enhance athletic performance or personal appearance. AAS use among girls and women is much less common. Taken in supraphysiologic doses, AAS show various long-term adverse medical effects, especially cardiovascular toxicity. Behavioral effects of AAS include hypomanic or manic symptoms, sometimes accompanied by aggression or violence, which usually occur while taking AAS, and depressive symptoms occurring during AAS withdrawal. However, these symptoms are idiosyncratic and afflict only a minority of illicit users; the mechanism of these idiosyncratic responses remains unclear – the personality of the victim before taking the drugs seems to be of importance. AAS users may also ingest a range of other illicit drugs, including both "body image" drugs to enhance physical appearance or performance, and classical drugs of abuse. In particular, AAS users appear particularly prone to opioid use. There may well be a biological basis for this association, since both human and animal data suggest that AAS and opioids may share similar brain mechanisms. Finally, AAS may cause a dependence syndrome in a substantial minority of users. AAS dependence may pose a growing public health problem in future years but remains little studied [09048].

Anabolic Androgenic Steroids (AASs) are chemical and pharmacological derivatives of the male hormone testosterone which are widely used for increasing burst and sprinting activities in sports. Although AASs are thought to be transversal to the plurality of sports disciplines, they are principally misused by bodybuilders, weightlifters, shot, hammer, discus or javelin throwers, rugby and American football players as well as by swimmers and runners. AAS exert a kaleidoscope of effects on human biology, principally through the 5-alpha-reductase-mediated conversion into dihydrotestosterone, the aromatase-mediated conversion into female sex hormones, a competitive antagonism to the glucocorticoid receptors, the potential stimulation of erythropoietin secretion as well as psychoactive effects on the brain. The influence of AASs on physical performance is still undefined, since the large numbers of studies published so far have described discordant and often contradictory outcomes. Nevertheless, animal and human investigations support the hypothesis that the administration of AASs might increase lean body mass, muscle mass, and maximal voluntary strength especially in men, so that they would represent an appealing form of doping for increasing power capacity, sustaining intensive training periods and, last but not least, as a cosmetic muscle makeover [11061].

The World Anti-Doping Agency regulations governing anti-doping in elite sports found in 2005 anabolic compounds as the most frequently detected doping agents, accounting for about 43 percent of positive results in 2005. Among these testosterone, nandrolone and stanozolol were predominant [08011].

With increasing availability of designer androgens, significant efforts are needed by antidoping authorities to develop sensitive methods to detect their use. The PubMed and Google Scholar search engines were used to identify publications addressing various forms of doping, methods employed in their detection, and adverse effects associated with their use. It appears that the use of AAS continues to be associated with premature mortality (especially cardiovascular) in addition to suppressed spermatogenesis, gynecomastia, and virilization. The attention that androgen abuse has received lately should be used as an opportunity to educate both athletes and the general population regarding their adverse effects. The development of sensitive detection techniques may help discourage (at least to some extent) the abuse of these compounds. Investigations are needed to identify ways to hasten the recovery of the gonadal axis in anabolic androgenic steroids users and to determine the mechanism of cardiac damage by these compounds [10043].
Anabolic steroids regulate the building blocks for the adolescent growth spurt and body composition. Even the body uses the combination of testosterone, hGH and IGF-I to subserve the adolescent growth spurt and the "partitioning" of food energy into the various compartments of body composition. As noted previously the early attainment of pubertal development confers some advantage on adolescent athletes compared to their "on-time" peers, although this is likely less important later in puberty given that sport-specific skills become more important as the majority of athletes will have entered and progressed through pubertal development [10001].

An anabolic steroid is a sex hormone that promotes the development and maintenance of the male sex characteristics. Testosterone is the principal secreted androgen in men. Androgens have both masculinizing (development of male secondary sex characteristics, including hair growth) and anabolic effects (increase in skeletal muscle mass and strength). For decades, pharmaceutical companies have attempted to develop androgens that have preferential anabolic activity and reduced or no androgenic activity; these compounds have been referred to as anabolic steroids. There is feedback control of androgen synthesis and secretion involving the hypothalamus (GnRH) and the pituitary. During adolescence there are remarkable alterations in both secretion and feedback sensitivity that underpin male pubertal development. Testosterone is strongly bound to sex-hormone binding globulin (SHBG), loosely bound to albumin, and a small proportion circulates as the free hormone. The biological activity is thought to reside in the free and loosely bound (albumin-bound) fractions [10001].

Androgens mediate their action through their binding to the androgen receptor (AR) which is mainly expressed in androgen target tissues, such as the prostate, skeletal muscle, liver and central nervous system. There is a wide spectrum of testosterone and synthetic AAS structure modifications related to the intended enhancement in anabolic activity [08124].

Anabolic androgenic steroids are the most abused class of prohibited substances, with testosterone accounting for many positive cases. Testosterone abuse is problematic because synthetic testosterone is indistinguishable from endogenous testosterone by routine screening methods such as gas chromatography–mass spectrometry. In the 1980s, it was discovered that testosterone use alters the ratio of testosterone glucuronide to epitestosterone glucuronide (T/E ratio) in urine. Epitestosterone is a naturally occurring biologically inactive epimer of testosterone that remains relatively constant in urine. A population-based T/E ratio cutoff of 6.0 was initially used to indicate synthetic testosterone use; the T/E ratio cutoff was lowered to 4.0 in 2005. Based on data from several laboratories, the average T/E ratio ranges from 0.9 to 1.6 for healthy male adolescents and men. At the UCLA Olympic Analytical Laboratory, it was found that the average T/E ratio during a 31-month period was 1.1 (median 0.9 %). Approximately 99.6 percent of urine samples have a T/E ratio <4.0, and 99.8 percent have a T/E ratio <6.0 [08124].

The T/E ratio is typically used as a screening test, and urine samples with a ratio >4.0 are submitted for confirmation testing by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). GC/C/IRMS has excellent specificity and can measure very small differences in the $^{13}\text{C}$ to $^{12}\text{C}$ ratio of testosterone and steroid metabolites. The $^{13}\text{C}$ content of natural (endogenously produced) testosterone is influenced by plant and animal sources consumed in food. In contrast, synthetic (pharmaceutical) testosterone is produced from plant precursors (stigmasterol) containing less $^{13}\text{C}$. This results in smaller $^{13}\text{C}/^{12}\text{C}$ ratios for synthetic testosterone, compared to natural testosterone. Although GC/C/IRMS is used to confirm testosterone use, the technique has low analytical sensitivity and is labor-intensive (owing to extensive sample cleanup) and costly to perform, and thus cannot be used as a
screening test [08124].

An androgen (anabolic-androgenic steroid) is a derivative of the major sex steroid in men and most mammals, testosterone (T). Others might answer in the more generic sense: any compound that is an agonist (or partial agonist) at the androgen receptor (AR). There are seemingly enough forms of T activity available to satisfy the needs of the medical population with oral, buccal, cutaneous patches (really, drug delivery devices) and gels, injectables of various durations (days to months), and implantable forms with a duration of action of 6 months or more. It seems that performance enhancement for human (and at least equine) athletes provide the backbone for the design of new anabolic steroids to circumvent the sophisticated testing procedures that surround major national and international competitions [12113].

Anabolic steroids are synthetic derivatives of testosterone, modified to enhance its anabolic actions (promotion of protein synthesis and muscle growth). They have numerous side effects, and are on the International Olympic Committee's list of banned substances. Gas chromatography-mass spectrometry allows identification and characterisation of steroids and their metabolites in the urine but may not distinguish between pharmaceutical and natural testosterone. Indirect methods to detect doping include determination of the testosterone/epitestosterone glucuronide ratio with suitable cut-off values. Direct evidence may be obtained with a method based on the determination of the carbon isotope ratio of the urinary steroids. One paper aimed to give an overview of the use of anabolic-androgenic steroids in sport and methods used in anti-doping laboratories for their detection in urine, with special emphasis on doping with testosterone. It was made a review of the recent literature of anabolic steroid testing, athletic use, and adverse effects of anabolic-androgenic steroids. Procedures used for detection of doping with endogenous steroids are outlined. The World Anti-Doping Agency provided a guide in August 2004 to ensure that laboratories can report, in a uniform way, the presence of abnormal profiles of urinary steroids resulting from the administration of testosterone or its precursors, androstenediol, androstenedione, dehydroepiandrosterone or a testosterone metabolite, dihydrotestosterone, or a masking agent, epitestosterone. Technology developed for detection of testosterone in urine samples appears suitable when the substance has been administered intramuscularly. Oral administration leads to rapid pharmacokinetics, so urine samples need to be collected in the initial hours after intake. Thus there is a need to find specific biomarkers in urine or plasma to enable detection of long term oral administration of testosterone [06048].

Androgens remain the most effective and widely abused ergogenic drugs in sport. The use of particularly anabolic androgenic steroids has changed from being a problem restricted to sports to one of public-health concern. In a review the prevalence of misuse, the evidence that some drugs improve performance in sport, their side-effects, and the long-term consequences of AAS misuse for society at large were discussed. There is substantial under-reporting of the side-effects of AAS to health authorities. It was described neuropsychiatric side-effects of AAS and their possible neurobiological correlates, with particular emphasis on violent behaviour. Analytical methods and laboratories accredited by the World Anti-Doping Agency can detect the misuse of all doping agents; although the analysis of testosterone requires special techniques, and recently discovered interethnic differences in testosterone excretion should be taken into account. The prevention of misuse of doping agents should include random doping analyses, medical follow-ups, pedagogic interventions, tougher legislation against possession of AAS, and longer disqualifications of athletes who use AAS [08061].

Although androgen doping has been prohibited for over 3 decades with a ban enforced by mass spectrometric-based urine testing for synthetic and exogenous natural androgens,
attempts continue to develop increasingly complex schemes to circumvent the ban. A prominent recent approach has been the development of designer androgens. Such never-marketed androgens evade detection because mass spectrometry relies on identifying characteristic chemical signatures requiring prior knowledge of chemical structure. Although once known, designer androgens are readily detected and added to the Prohibited List. However, until their structures are elucidated, designer androgens can circumvent the ban on androgen doping. To combat this, in vitro androgen bioassays offer powerful new possibilities for the generic detection of unidentified bioactive androgens, regardless of their chemical structure. Another approach to circumvent the ban on androgen doping has been the development of indirect androgen doping, the use of exogenous drugs to produce a sustained increase in endogenous testosterone production. Apart from estrogen blockers, however, such neuroendocrine active drugs mostly provide only transient increases in blood testosterone. Finally the ban on androgen doping must allow provision for rare athletes with incidental, proven androgen deficiency who require testosterone replacement therapy. The Therapeutic Use Exemption (TUE) mechanism makes provision for such necessary medical treatment, subject to rigorous criteria for demonstrating a genuine ongoing need for testosterone and monitoring of testosterone dosage. Effective deterrence of sports doping requires novel, increasingly sophisticated detection options calibrated to defeat these challenges, without which fairness in sport is tarnished and the social and health idealization of sporting champions devalued [08062].

Anabolic-androgenic steroids (AASs) are synthetic derivatives of testosterone. The term “androgenic” indicates masculinizing. Androgens are responsible for stimulating the growth of the male reproductive tract and secondary sex characteristics. The term “anabolic” indicates tissue building and is the component of a steroid which is responsible for thickening of the vocal cords, enlargement of larynx, increasing libido, linear growth acceleration before epiphyseal plate closure, increasing muscle bulk and strength through dose-dependent hypertrophy, and decreasing body fat. The anabolic action is mediated by androgen receptors on skeletal muscle. Testosterone increases muscle protein synthesis, thus increasing the cross-sectional area of the fibers themselves, as well as increasing the myonuclear number [07007].

Research activity

The consumption of anabolic steroids (AS) has been growing continuously in recent years. It has gone beyond the sports world; AS are now widely used as drugs of abuse in connection with bodybuilding. This study sets out to assess the state of scientific research in the area. Bibliometrics were employed to evaluate the literature retrieved from the principal relevant bibliographic databases: MEDLINE, SportDiscus, the Science Citation Index Expanded and the Social Sciences Citation Index. The core journals were identified along with the leading authors and research groups and their institutional affiliations. Techniques based on social network analysis were applied in order to build up a concept map of research. 1325 documents were retrieved. They were produced by 3131 different researchers giving a Collaboration Index of 3.32. The institutions with the most productive authors were Ball State University (Muncie, IN, USA), the Ecole Nationale Vétérinaire de Nantes (ENVN), the Institut Municipal d’Investigació Mèdica (IMIM) (Barcelona, Spain), the Institute of Biochemistry of the German Sport University Cologne (DSHS), Iowa State University, Maastricht University and the University of Iowa. It was concluded that there has been an upward trend in the number of research projects. The sources used complemented one another, as 78 percent of the documents retrieved were unique to one source. The productivity ranking was headed by sports medicine journals, followed by journals of chemistry, physiology, endocrinology and
substance abuse. Besides sporting activities, the most important research clusters were those connected with bodybuilding and with youth groups [07057].

**Clinical use**

Anabolic-androgenic steroids were developed to treat hypogonadism, a condition in which the testes do not produce sufficient testosterone for normal growth, development, and sexual functioning. Clinical studies suggest that the use of AASs (e.g., nandrolone) or testosterone improves lean body mass in patients with mild to moderate cachexia secondary to chronic illness (e.g., human immune deficiency virus, HIV). The use of testosterone and AAS has not been effective for the treatment of the catabolic states associated with severe burns or muscle-wasting diseases. Historical uses of AASs include anemia, hereditary angioedema, metastatic breast cancer, protein deficiencies following trauma or severe infections, and certain psychiatric disorders (involutional psychoses and depression) [13002].

Androgens are used clinically to treat a range of different human disorders. Among these are several catabolic conditions such as obstructive pulmonary disease, severe burn injuries, and also HIV-related muscle wasting. It can also be used to treat a number of conditions resulting from deficiencies in androgen production, such as constitutional growth retardation and hypogonadism. Androgen therapy can be administered orally, by intramuscular injection, and as gels and creams. Synthetic alkyl esters of androgens have been used therapeutically for decades due to their high potency and prolonged action. The realization that androgen administration can augment muscle hypertrophy has led to the abuse of androgens to increase muscle size, strength and sport performance [13084].

The anabolic properties of AASs have proven beneficial for some therapeutic applications. They have been used in clinical practice since the 1940s for the treatment of trauma, burns, extensive surgery, radiation therapy, and chronic debilitating illnesses. Before the advent of bone marrow transplantation and synthetic erythropoietin, AASs were used often in the treatment of various types of anemias. AASs have shown promise in treating short stature, as in Turner's syndrome, or constitutional growth and puberty delay. 1985-2006, the clinical use of AASs has increased 400 percent, mostly due to the management of AIDS-associated wasting syndrome. AASs may enhance the effects of the increased caloric intake and exercise regimen. A pilot study in malnourished HIV-infected children as young as 4 years old showed that oxandrolone treatment was well-tolerated and improved nutritional status. After 3 months of treatment, the study subjects experienced an accelerated rate of weight gain, increased body mass index, increased muscle mass, and decreased fat stores as compared with pretreatment values. The results were supported further by the improved serum albumin levels noted during the course of treatment. Future studies using a larger study population and longer- or higher-dose AAS administration would strengthen the current data. In patients with severe burns, AASs may play an important role in reversing the catabolic state. A small prospective randomized study of patients who had burns showed that those receiving oxandrolone in addition to a high-protein diet experienced a significantly greater increase in weight and physical therapy index than did patients who were treated with diet alone. AAS therapy seems to be promising in the treatment of malnutrition and muscle wasting seen in patients who have end-stage renal disease. In addition to the increase in lean body mass, these patients also benefit from a stimulated erythropoiesis resulting from the administration of AASs [07008].

*Hereditary angioedema*
Androgen derivatives are regarded as standard in the long-term prophylaxis of swelling attacks in patients with hereditary angioedema (HAE). Because of their relatively slow onset of action, they are not suitable for acute therapy. Long-term prophylaxis with androgen derivatives must be regarded critically, especially on account of their androgenic and anabolic effects, some of which are severe. The risk of adverse events increases with the daily dose and the duration of treatment. Thus, treatment always calls for close monitoring of patients with regard to potential adverse events. In addition, androgens are subject to numerous contraindications and they show interactions with a large number of other drugs. Off-label use, doping issues, clarification of reimbursement and the need to import the androgen derivatives, which are no longer marketed in Germany, result in additional effort for the treating physician in terms of logistics and time involved. In symptomatic treatment of acute attacks the intravenous substitution of C1-INH and – since 2008 – subcutaneous administration of icatibant are available. The two substances are well tolerated and their effect occurs rapidly and, when the diagnosis has been confirmed, reliably. In the light of these two treatment options for controlling acute attacks, prophylactic treatment of HAE patients with androgen derivatives such as danazol should be reassessed. Patients might benefit from a dose reduction or the withdrawal of androgen prophylaxis and attacks can be controlled with demand-oriented acute treatment using C1-INH or icatibant [11064].

**Effect of androgen deprivation**

Indices of body composition and muscular strength were compared between men with prostate cancer (PCa) treated with androgen deprivation therapy (ADT) and asymptomatic matched men. Nine subjects aged 63-83 years with PCa who received ADT (PCa+ADT; duration 6-180 months) and 11 asymptomatic aged-matched eugonadal men (HM) aged 59-80 years were assessed for prostate-specific antigen (PSA) and total testosterone (TT). Total body non-osseous lean mass (TBLM) and right thigh non-osseous fat-free mass (RTLM) were assessed using dual-energy X-ray absorptiometry. Peak torque of the right knee extensors at 0° s⁻¹ and 60° s⁻¹, maximal handgrip strength of the dominant hand (MHS) and whole-body strength (WBS) were assessed. ISO and CON per unit mass of RTLM and MHS and WBS per unit mass of TBLM were calculated. Age, height, mass, body mass index and prostate-specific antigen were comparable between groups, while TT was lower in PCa+ADT. RTLM was similar between groups. Absolute peak torque of the right knee extensors were lower for PCa+ADT as were extensor effects per unit of RTLM. Absolute MHS, WBS and MHS per unit of TBLM and WBS per unit of TBLM were lower for PCa+ADT. Men with PCa who receive ADT experience significant losses in whole-body muscular strength compared with asymptomatic age-matched men, which may impair functional capacity. These losses in muscular strength appear to involve neuromuscular mechanisms that are yet to be identified [13124].

**Abuse**

One of the earliest accounts of androgen abuse dates back to the 1950s where Soviet weightlifters were allegedly taking testosterone. While the abuse of androgens is more commonly associated with the weightlifting industry, their use is widespread amongst many sports, both at the elite and amateur levels. Alarmingly, androgen use is not limited to adults, with reports that school-aged children use androgens. For bodybuilding and enhanced athletic performance enhancement, it is common that very high doses of androgens are consumed. Moreover, it is common practice to employ “stacking” regimes where a number of different androgens and/or metabolism inhibitors are simultaneously consumed. This can
lead to serious clinical consequences such as abnormal liver function, gynecomastia, severe psychological or psychiatric disorders, increased risk of cardiovascular disease; and in females, menstrual disorders and virilization. Many of the androgens that are used are 17-methylated compounds and are associated with high-liver toxicity [13084].

**Methods of abuse**

The pattern of AAS abuse among athletes is quite variable, and the dosing intervals are not usually regular. These patterns include stacking, tapering, plateauing, cycling, and pyramiding. Many AAS abusers are poly-drug users including the abuse of traditional recreational drugs and misuse of prescription drugs. Athletes typically administer AAS intramuscularly with or without oral preparations in cycles lasting 6-12 weeks with periods of abstinence between these cycles as a mean store due adverse effects. The abuse of transdermal patches, sublingual tablets, dermatologic gels, and nasal sprays is rare. In an attempt to maximize anabolic gains and minimize side effects, some AAS users start with low doses at the beginning of a cycle and then steadily increase the dose until a gradual “tapering” phase at the end of a cycle (i.e. “pyramid” regimen). Frequently, these athletes use more than 1 steroid simultaneously (i.e. “stacking”) or use several AASs in overlapping patterns to avoid the development of tolerance (i.e. “plateauing”). In an internet study of 207 weight lifters and bodybuilders using AASs, the steroid regimens included a mean of 3 agents with cycles ranging from 5 to 10 weeks and AAS doses 5-29 times above physiologic replacement doses. Often these athletes ingest other drugs (i.e. “array”) as a mean store due adverse effects and enhance the effect of AASs. These additional medications include human chorionic gonadotrophin, anti-acne drugs, oral hypoglycemic agents, analgesics, ketoconazole shampoo, stimulant aminoacids, erythropoietin, aminoglutethimide, diuretics, and estrogen antagonists. The abuse of AASs continues, in part, because of the effectiveness of these regimens with training programs and the perceived difficulty detecting AAS use, particularly in weight lifters. Commonly abused steroid supplements (i.e. precursors of testosterone and related hormones) include androstenedione and DHEA. The latter compound is an endogenous hormone secreted by the adrenal cortex in response to adrenocorticotropic (ACTH) [13003].

**Abuse dosage**

Athletes usually consume supraphysiologic doses of AAS. Steroids are generally taken in 4- to 12-week cycles. Athletes often “stack” multiple steroids simultaneously and “pyramid” the dosing schedule, beginning with low dosage and increasing amounts during the middle and end of a cycle. Between dosing cycles, it is typical for users to have a period of abstinence, known as a “drug holiday,” which usually occurs during the competition phases. This method provides 50 to 100 times the physiologically needed dose of steroids, resulting in levels that are far higher than physiological levels [12119].

Anabolic steroids may be taken orally or injected intramuscularly and are grouped into three main classes. Testosterone esters, such as testosterone propionate, are injected compounds and constitute class I. Class II agents include the nortestosterone derivatives (eg, nandrolone decanoate and nandrolone phenpropionate). Class I and II AASs exert effects at androgen receptors as well as at estrogen receptors by way of aromatization to estradiol. The third class of AASs are those alkylated at C-17 and are the orally administered compounds oxymetholone, methandrostenolone, and stanozolol. Alkylation of these compounds involves the addition of a methyl or ethyl group to the carbon at position 17 of the steroid backbone. The alkylation slows the hepatic metabolism of these agents. A typical pattern of use consists of a combination of injectable and oral steroids taken during 6- to 12-week cycles.
Injectable forms tend to be favored by users because they are less hepatotoxic than the oral forms. Because oral preparations are cleared from the system more quickly, they are the preferred form of steroids when drug testing is anticipated. The simultaneous use of multiple steroids is referred to as "stacking." A pattern of increasing a dose through a cycle is called "pyramiding." Pyramiding can lead to doses 10 to 40 times greater than the dose recommended for medical indications. By stacking and pyramiding doses, the user hopes to maximize steroid receptor binding, thereby reducing toxic side effects. These patterns have remained popular, despite the lack of scientific evidence of a benefit. Some users take other drugs concurrently in an effort to minimize side effects. These "accessory" medications include clomiphene and human chorionic gonadotropin and are administered to reverse the endogenous testosterone production. Additionally, tamoxifen and antiaromatase drugs can prevent or decrease gynecomastia by limiting estrogenic effects and the metabolism of excess testosterone derivatives to estradiol. It is not uncommon for users to take other legal performance-enhancing substances and dietary supplements, such as creatine, glutamine, and protein, while using AASs [07008].

It is worthy to note that the classification of anabolic steroids (AS) covers a number of structural variants. Classically, AS are classified as water-soluble orally active forms (17-alpha-alkylated) and lipid-soluble parenteral forms (17-beta-esterified). In addition, they are often also classified as either testosterone-based, dihydrotestosterone-based (DHT) or 19-nortestosterone-based (Nandrolone) all of which have differing properties and expected side effects. The situation is further complicated by belief among users, often stemming from anecdotal advice, that some AS are better for predominantly "bulking" (e.g. Deca-Durabolin) while others are better suited to losing body fat or "cutting" (e.g. Winstrol). Users will often use these different forms of AS in varying quantities. The use of AS is also characterised by periods of use followed by periods of abstinence, or "cycles". This helps to maximise the effects of the drugs while also limiting the negative consequences and allowing the body to normalise following an "on" cycle. Furthermore, users will often supplement their cycles with additional pharmaceutical agents both when bulking (e.g. insulin, human growth hormone) and when losing body fat (clenbuterol, cytomel, 2,4, dinitrophenol). Finally, there are a surprising number of drugs used to attempt to limit side effects of AS use or normalise the hypothalamo-pituitary-gonadal (HPG) axis following an AS cycle. These include estrogen receptor antagonists (tamoxifen), selective estrogen receptor inhibitors (clomifene), aromatase inhibitors (arimidex), 5-alpha reductase inhibitors (finesteride) and HPG axis stimulators such as HCG [12114].

**Dose effects**

The dose of AAS used by athletes depends on individual needs and the athletic requirements of the particular sport. Endurance athletes use AAS doses near or slightly below physiologic replacement concentrations (i.e. about 7 mg testosterone daily) as a means to block catabolism, while sprinters typically use 1.5-2 times replacement concentrations. Traditional strength athletes use much higher doses (i.e. 10-100 times replacement concentrations) to "bulk up." Generally, the dose of AASs is lower in women than in men. Data from human test participants indicate that AAS produce a dose-dependent and gender-dependent increase in lean body mass and strength, but the changes are highly variable and relatively small without an accompanying conditioning and strength program. The administration of AAS to men participating in weight training consistently produces increased strength when compared with controls (i.e. weight training alone). The endogenous testosterone production during male adolescence reproduces a sex-differential in lean body mass similar to the increment in lean body mass caused by the administration of exogenous AAS to adults. However, different androgen-dependent processes have different testosterone-dose-response relationships. In a study of 61 eugonadal men receiving a long-acting gonadotropin-releasing hormone agonist to suppress endogenous testosterone
secretion, changes in leg press strength, leg power, thigh and quadriceps muscle volumes, hemoglobin, and insulin-like growth factor 1 (somatomedin C) positively correlated to testosterone concentrations. Changes in fat mass and plasma high-density lipoprotein (HDL) cholesterol were negatively correlated to the testosterone dose. Although adverse effects following AAS administration are usually dose related, there are few data on the long-term physiologic effects of chronic AAS use, particularly in women. In addition to dose and duration of use, long-term toxicity depends on the age of initiation, gender, steroid structure, and concurrent illicit use of other drugs. The daily production of testosterone in healthy men is about 4-10 mg compared with about 1 mg in healthy women. Psychotic symptoms associated with AAS abuse typically occur in individuals using 41 g testosterone weekly, but the development to psychologic changes is highly variable. In a randomized, placebo-controlled, crossover trial of 56 healthy men aged 20-50 years, the administration of testosterone cypionate for 6 weeks in doses increasing to 600 mg/week caused little psychologic change in most participants (i.e. 84 %). The regimen produced mild hypomania in 12 percent and marked hypomania in 4 percent of the men [13003].

Different actions due to ways of administration

The route of administration has been found to have an effect on the detection of anabolic steroids since quantification of testosterone in urine samples is reliable only when the drug has been administered intramuscularly. Oral administration, by contrast, is more problematic since it results in rapid pharmacokinetics, this requiring urine samples to be collected as soon as possible following administration to enable reliable quantification. AAS bind with variably affinity in the cytoplasm to the androgen receptor (AR), a member of the steroid hormone receptor family, where they exert potent anabolic and endocrine activities. The AR’s binding to the androgen response element triggers its potential to act as a transcriptional modifier of various genes. The enzyme 5-alpha-reductase seems to possess an essential role by converting AAS into dihydrotestosterone (androstanoelone), which acts in the cell nucleus of target organs, while the enzyme aromatase converts AAS into female sex hormones (estradiol and estrone). By displacing cortisol from its receptors, they antagonize the catabolic effects of glucocorticoids. AAS increase strength (by about 5-20 %) and body weight (by about 2-5 kg) due to an increase of the lean body mass without reduction of fat mass, although no effects have been observed on endurance performance [12011].

Different anabolic androgenic steroids with different specific actions

Stanozolol is an anabolic steroid compound particularly favoured among athletes and body builders since it boosts strength without weight gain, while it is not converted to estradiol. Metribolone (methyltrienolone) is a potent anabolic steroid, a non-aromatizable androgen, the 17-methylated derivative of trenbolone, which is characterized by high potential for hepatotoxicity [12011].

Recognizing steroid abuse

Early recognition and intervention may prevent adverse and potentially irreversible consequences. New-onset acne on the back and chest, temporal hair loss, and alopecia are common signs. Subtle personality or mood changes are sometimes the only manifestation [07031]:

- Cardiac disease in absence of risk factors
- Thrombotic events in absence of risk factors
- Alopecia
Possible use in children and adolescents

The dietary supplements androstenedione, dehydroepiandrosterone, and androstenediol are precursors in the endogenous production of testosterone. The efficacy and safety of these prohormones are not well established but are promoted to have the same androgenic effects on building muscle mass and strength as anabolic-androgenic steroids. Studies have demonstrated repeatedly that acute and long-term administration of these oral testosterone precursors does not effectively increase serum testosterone levels and fails to produce any significant changes in lean body mass, muscle strength, or performance improvement compared with placebo. Testosterone precursors are banned by most major sports organizations [07083].

Clinical effects

Children seem to be the most susceptible to the adverse effects of AAS use. Children and adolescents experience accelerated maturation associated with changes in physique and earlier development of secondary sexual characteristics. An additional concern with adolescents is premature closure of growth plates in long bones, leading to a decrease in final height; this likely is due to aromatization to estrogens. Precocious puberty in boys and contrasexual precocity in girls also can occur. With adolescents, some of the effects may become irreversible with chronic use, particularly the virilizing effects in young women [07058].

One article was part of a Special Issue "Puberty and Adolescence". Puberty is a critical period for brain maturation that is highly dependent on gonadal sex hormones. Modifications in the gonadal steroid environment, via the use of anabolic androgenic steroids (AAS), have been shown to affect brain development and behavior. Studies in both humans and animal
models indicate that AAS exposure during adolescence alters normal brain remodeling, including structural changes and neurotransmitter function. The most commonly reported behavioral effect is an increase in aggression. Evidence has been presented to identify factors that influence the effect of AAS on the expression of aggression. The chemical composition of the AAS plays a major role in determining whether aggression is displayed, with testosterone being the most effective. The hormonal context, the environmental context, physical provocation and the perceived threat during the social encounter have all been found to influence the expression of aggression and sexual behavior. All of these factors point toward an altered behavioral state that includes an increased readiness to respond to a social encounter with heightened vigilance and enhanced motivation. This AAS-induced state may be defined as emboldenment. The evidence suggests that the use of AAS during this critical period of development may increase the risk for maladaptive behaviors along with neurological disorders [13123].

Aggresiveness
In a double-blind, placebo-controlled, crossover trial, it was given injections of testosterone in increasing doses to 35 boys and oral doses of conjugated estrogens to 14 girls with delayed puberty. Both treatment groups had more physical aggressive behavior and aggressive impulses than those receiving placebo. In another study it was compared plasma levels of hormones in 15 boys with conduct disorders and in 25 normal controls. The boys with conduct disorders had significantly higher levels of dehydroepiandrosterone sulfate, marginally significantly higher levels of androstenedione, and no differences in testosterone levels [07031].

Epiphyseal plate closure in adolescents
Premature closure of the epiphyseal plates during chronic AAS use can stunt bone growth [13003].

Prevalence of adolescent anabolic-androgenic steroid use
The first reported adolescent use of AASs was in 1959 by a high school football player. Estimates of high school steroid usage in 2007 ranged from 4 to 11 percent in boys and up to 3.3 percent in girls. A landmark study of prevalence involved a nationwide survey of more than 3000 boys. They found that 6.6 percent of male high school seniors had tried steroids, with 67 percent initiating use by 16 years of age and 40 percent using multiple cycles. These results have been confirmed in later studies and a 2003 Centers for Disease Control and Prevention report finding of a 6.4 percent use of steroids by 12th-grade boys. The largest nationwide cohort of nearly 50,000 students is being examined in the Monitoring the Future study. As of 2004, results of this ongoing study indicated a 1.3, 2.3, and 3.3 percent annual prevalence of male AAS users in the eighth, 10th, and 12th grades, respectively. Girls in the 12th grade had a 1.7 percent use rate in this study, whereas the Centers for Disease Control and Prevention reported a 3.3 percent lifetime prevalence in 12th-grade girls. AAS use by adolescents is not limited to the United States. Three Canadian studies, two Swedish surveys, two South African investigations, one British study, and one Australian investigation reported an overall prevalence range between 1 and 3 percent. Although slightly lower, these rates approximate those reported in the United States, demonstrating that the impact of AASs on athletic performance and physical appearance reaches across cultures. A considerable percentage of adolescents turn to AAS use to help them achieve an attractive physique. This is the second most popular reason for using AASs. One study of bodybuilders suggests that the drive for a muscular physique sometimes reaches an unhealthy extreme and likens the use of AAS to the “unhealthy extremes” that are characteristic of anorexic and bulimic individuals. Just as eating disordered women see their bodies as smaller than they actually are, some men perceive themselves as smaller than they actually are. This
phenomenon has been referred to as “bigamerexia” and suggests that this misperception may be a contributory factor in AAS use. This misperception is likely evident in many ninth-grade boys, who – in the early stages of puberty – are impatient with their muscular development. Perceiving themselves smaller than their peers, these boys may engage in AAS use as a shortcut to increasing muscle strength and size. Exposure to the media may intensify this body dysmorphia. Adolescent AAS use has been associated with the use of other harmful drugs, including cigarettes, smokeless tobacco, marijuana, alcohol, cocaine, and injected drugs. Thus, AAS use would be considered a part of this cluster rather than an isolated behavior [07008].

It was examined the prevalence, persistence, secular and longitudinal trends, and predictors of steroid use in a diverse sample of adolescents. Data are from Project EAT-II (Eating Among Teens), a 5-year longitudinal study of eating, activity, weight, and related variables in 2516 middle and high school students. Data were collected in 1999 (time 1) and 2004 (time 2). Approximately 1.5 percent of adolescents reported steroid use at time 2. Use differed by ethnicity but not socioeconomic status. Steroid use was not stable across time, although the risk of use at time 2 was higher for girls and (marginally) for boys who used steroids at time 1. No secular trends were noted in middle adolescents' steroid use between 1999 and 2004. Developmentally, steroid use decreased as adolescents grew older. Predictors of use for male adolescents included wanting to weigh more and reporting higher use of healthy weight-control behaviors. Female time 2 steroid users had higher BMIs and were less satisfied with their weight, had poorer nutrition knowledge and concern for health, and were marginally more likely to have participated in weight-related sports at time 1. It was concluded that the prevalence of steroid use in adolescents was low but of concern. Although use was not persistent over 5 years, time 1 use was a risk factor for time 2 use in female adolescents. There was no change in the prevalence of steroid use by middle adolescents between 1999 and 2004 despite a great deal of public interest in steroids during this time period. Steroid use decreased as adolescents grew older. Weight-related variables predicted adolescents' steroid use 5 years later, and health and nutrition knowledge and concern and (marginally) participation in weight-related sports further predicted use in female adolescents. These findings suggest that early preventive efforts may be most useful [07008].

**Girls**

Recent media reports have portrayed an alarming increase in apparent anabolic-androgenic steroid (AAS) use among American teenage girls; Congress even held hearings on the subject in June 2005. It was questioned whether AAS use among teenage girls was as widespread as claimed. It was reviewed four large national surveys and many smaller surveys examining the prevalence of AAS use among teenage girls. Virtually all of these surveys used anonymous questionnaires. It was asked particularly whether the language of survey questions might generate false-positive responses among girls who misinterpreted the term "steroid." It was also reviewed data from other countries, together with results from the only recent study in which investigators personally interviewed female AAS users. The surveys produced remarkably disparate findings, with the lifetime prevalence of AAS use estimated as high as 7.3 percent among ninth-grade girls in one study, but only 0.1 percent among teenage girls in several others. Upon examining the surveys reporting an elevated prevalence, it appeared that most used questions that failed to distinguish between anabolic steroids, corticosteroids, and over-the-counter supplements that respondents might confuse with "steroids." Other features in the phrasing of certain questions also seemed likely to further bias results in favor of false-positive responses. It was concluded that many anonymous surveys, using imprecise questions, appear to have greatly overestimated the lifetime prevalence of AAS use among teenage girls; the true lifetime prevalence may well be as low as 0.1 percent. Future studies can test this impression by using a carefully phrased
question regarding AAS use [07085].

Use of testosterone precursors by adolescents

The extent of the use of testosterone precursors, such as androstenedione and DHEA, in the pediatric and adolescent population is unknown. The initial over-the-counter dietary supplement status and availability more than likely led to a large increase in the number of adolescents using testosterone precursors. A 2002 survey of 475 high school students by revealed that 4 percent of athletes and nonathletes admitted to using steroid precursors in the past year. Surveys by the National Collegiate Athletic Association (NCAA) revealed that 5 percent of athletes admitted to using DHEA or androstenedione, 33 percent admitted to using nutritional supplements, and 1 percent admitted to using anabolic steroids. With the passage of the Anabolic Steroid Control Act of 2004, androstenedione became illegal to purchase; the only available source is through the black market or acquaintances. One would surmise that the use of testosterone precursors among adolescents would be higher than the use of AASs [07085].

Use in women

Prevalence

Anabolic-androgenic steroids (AAS) are synthetic derivatives of testosterone that maximize anabolic and minimize androgenic effects. Exogenous testosterone is rapidly metabolized and has no significant effect on performance. Men have traditionally used AAS for muscle gain. Women have also used AAS for increased strength and lean muscle mass. AAS use by women was first sensationalized when East German athletes routinely received steroids from team doctors. Surveys analyzing AAS prevalence in adolescent girls have found a range from 0.1 to 4.8 percent. The Centers for Disease Control and Prevention (CDC) reported an overall prevalence of lifetime steroid use in females, grades 9 to 12, of 3.2 percent. In another study surveying boys and girls between 9 and 13 years old, found that 2.8 percent of girls and 2.6 percent of boys reported AAS use. Steroid use by girls was found across various sports, with the highest prevalence in weight training. Steroid users were more likely to think that steroids improve athletic performance or enhance appearance Steroid use prevalence has also been studied in women collegiate athletes. A National Collegiate Athletic Association (NCAA) survey of more than 19,000 collegiate men and women found that between 2001 and 2005 AAS use increased in women's ice hockey, gymnastics, and volleyball. Between 2001 and 2005, AAS use increased in women's ice hockey from 0.8 to 2.4 percent, in women's gymnastics from 0 to 1 percent, and in volleyball from 0.1 to 0.6 percent [07086].

Effects

Studying AAS effects is difficult due to an incomplete understanding of how athletes use steroids. Athletes often combine AAS with other supplements, or use doses several times higher than recommended. Data on the performance-enhancing effect of AAS on women is limited. However, most studies in men found an increase in muscle mass and body weight by an average of 2 to 5 kg. Steroids have also been shown to increase strength, with improvements ranging from 5 to 20 percent. Clinical trials have not demonstrated improved endurance performance. A double-blind, placebo-controlled trial gave AAS to men 12 times over 1 month, showing no improvement in treadmill testing [07086].
**Side effects**

Women taking AAS are at risk for androgenic side effects, including deepened voice, acne, male pattern baldness, clitoromegaly, menstrual irregularities, and increased facial hair. A survey of steroid-using women bodybuilders found that 64 percent reported an adverse psychological effect, including labile mood, irritability, or aggressive behavior most frequently. More serious side effects include liver damage. Transient increases in hepatic enzymes that return to normal after steroids are discontinued have occurred. Other significant adverse effects include cholestasis, hepatic peliosis, and hepatocellular hyperplasia that may lead to hepatic carcinoma [07086].

In one study it was evaluated 75 female bodybuilders and weightlifters and found that 33 percent reported current or past anabolic-androgenic steroid use. Among steroid users, 56 percent reported hypomanic symptoms during use, and 40 percent reported depression when the steroids were discontinued. Some users developed a body image distortion similar to “reverse anorexia,” in which they felt they were too small. Ten of the 75 weightlifters had been raped as teenagers or adults, and most started or increased their weightlifting activities as a defense strategy. Seven of the 10 rape victims used anabolic steroids [07031].

**Steroid precursors (androstenedione and dehydroepiandrosterone)**

Steroid precursors include androstenedione and dehydroepiandrosterone (DHEA). These substances are closely related to testosterone. Due to poor surveillance, the prevalence of steroid precursor use by girls and women is difficult to determine. The NCAA’s study of substance use habits of college student-athletes found that 4.7 percent of men and women athletes surveyed had tried DHEA or androstenedione products at least once in the past year. The survey data, however, did not organize the percentages in terms of men and women. The CDC’s survey on youth risk behavior does not ask specifically about steroid precursors. One study of high school students found that androstenedione was the second most popular supplement, with 4 percent of students using it at least once in the past year. Again, the authors did not isolate a number for girls using androstenedione. It is also believed that these substances may have an androgenizing effect in female athletes. Androstenedione, produced in the adrenal glands and gonads, is more potent than DHEA, which is naturally produced in the adrenal cortex. The actual physiologic effects and effects on athletic performance have not been consistent. Several studies in women have shown increased serum androstenedione and testosterone after androstenedione supplementation. No trials have been published examining steroid precursors’ performance-enhancing effects in women. Further, studies in men have not consistently found an improvement in athletic performance with androstenedione or DHE. Steroid precursor use carries the same risks as testosterone use. Common adverse effects in women include virilization. There is also a risk of testing positive for testosterone or androstenedione [07086].

**Anabolic steroids in young females**

Women who take AAS can experience androgenic effects including changes in libido, male-pattern baldness, deepening of the voice, acne vulgaris, and other masculinizing effects in early use. Longer-term use causes clitoromegaly, changes in pubic hair growth, menstrual irregularities, and even breast reduction [06031].

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the prevalence of AAS use among teenage girls. Virtually all of these surveys used anonymous questionnaires. It was asked particularly whether the language of survey questions might generate false-positive responses among girls who misinterpreted the term "steroid." It was also reviewed data from other countries, together with results from the only recent study (to our knowledge) in which investigators personally interviewed female AAS users. The surveys produced remarkably disparate findings, with the lifetime prevalence of AAS use estimated as high as 7.3 percent among ninth-grade girls in one study, but only 0.1 percent among teenage girls in several others. Upon examining the surveys reporting an elevated prevalence, it appeared that most used questions that failed to distinguish between anabolic steroids, corticosteroids, and over-the-counter supplements that respondents might confuse with "steroids." Other features in the phrasing of certain questions also seemed likely to further bias results in favor of false-positive responses. It was concluded that many anonymous surveys, using imprecise questions, appear to have greatly overestimated the lifetime prevalence of AAS use among teenage girls; the true lifetime prevalence may well be as low as 0.1 percent. Future studies can test this impression by using a carefully phrased question regarding AAS use [06097].

**Estrogen antagonists**

Estrogen antagonists, such as tamoxifen, clomiphene, and anastrozole, have been used to block estrogen and increase testosterone effects. In the 1970s, the first reports of men using tamoxifen along with AAS occurred. Bodybuilders claimed that it decreased fat over muscle and produced a leaner appearance. An Internet-based survey of 500 bodybuilders found that 53 percent of AAS users were also using tamoxifen. This study included 494 men and 6 women. One case report and one survey have captured tamoxifen use for performance enhancement in women. All published reports have been in bodybuilders. In 2002, it was published a case report detailing a female competitive bodybuilder using tamoxifen based on a recommendation from another bodybuilder. She claimed it was easier to lose fat but noted side effects such as night sweats, hot flashes, and breast-mass loss. A recent survey of health club members to determine the prevalence of nontherapeutic medication use found that 22 percent had used tamoxifen. Seven percent of respondents to the survey were women. Tamoxifen use had increased from its previous levels. Tamoxifen's effect on athletic performance is unknown. Anecdotal reports of fat loss and leaner physique exist, but no studies have evaluated their validity. This medication involves multiple adverse effects for women, including endometrial cancer, thromboembolic events, teratogenic effects, ovarian cysts, bone-density loss, cataracts, hot flashes, and vaginal discharge [07086].

**Genetic influence**

In 1982, the International Olympic Committee accepted a T/E ratio greater than six as proof of testosterone doping, based on the log-normal distribution of the ratio established from the first population studies. A testosterone over epitestosterone (T/E) ratio exceeding 4.0 has later been considered as suspicious of testosterone administration, irrespectively of individual heterogeneous factors such as the athlete’s ethnicity. In antidoping laboratories, the urinary steroid profile usually encompasses the concentration levels of testosterone (T); its inactive epimer, epitestosterone (E); four testosterone metabolites, androsterone (A), etiocholanolone (Etio), 5α-androstane-3α,17β-diol (α-diol) and 5β-androstane-3α,17β-diol (β-diol); and a testosterone precursor, dehydroepiandrosterone (DHEA). The following cut-off concentration levels of endogenous steroids equivalent to the glucuronide: T>200 ng/ml, E>200 ng/ml, A>10 000 ng/ml, Etio>10 000 ng/ml and DHEA>100 ng/ml are considered as putative markers of androgen administration. In contrast to absolute steroid concentrations,
ratios such as T/E, A/Etio, A/T, α-diol/E and α-diol/β-diol are robust to circadian rhythm or changes in physiological conditions such as exercise workload for athletes. On the other hand, these parameters may be significantly altered according to the administered steroid and its application mode. However, a T/E higher than 4.0 no longer constitutes proof of testosterone misuse, but requires a subsequent confirmation analysis by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). GC/C/IRMS allows measurement of slight differences in $^{13}$C/$^{12}$C ratio of testosterone metabolites. A discrimination is feasible since exogenous testosterone or its precursors contain less $^{13}$C than their endogenous homologues. A deletion polymorphism in the UGT2B17 gene was demonstrated to account for a significant part of the interindividual variability in the T/E between Caucasians and Asians. The variability of urinary steroid profiles was examined in a widely heterogeneous cohort of professional soccer players. The steroid profile of 57 Africans, 32 Asians, 50 Caucasians and 32 Hispanics was determined by gas chromatography–mass spectrometry. Significant differences were observed between all ethnic groups. After estimation of the prevalence of the UGT2B17 deletion/deletion genotype (African: 22 %; Asian: 81 %; Caucasian: 10 %; Hispanic: 7 %), ethnic-specific thresholds were developed for a specificity of 99 percent for the T/E (African: 5.6; Asian: 3.8; Caucasian: 5.7; Hispanic: 5.8). Finally, another polymorphism could be hypothesised in Asians based on specific concentration ratio of 5α-/5β-androstane-3α,17β-diol in urine. These results demonstrate that a unique and non-specific threshold to evidence testosterone misuse is not fit for purpose. An athlete's endocrinological passport consisting of a longitudinal follow-up together with the ethnicity and/or the genotype would strongly enhance the detection of testosterone abuse. Finally, additional genotyping studies should be undertaken to determine whether the remaining unexplained disparities have an environmental or a genetic origin [09049].

**Doping tests and genetic confounders**

So far it is known of only genetic variations in three enzyme (UGT2B17, CYP17 and PDE7B) genes with a substantial influence on the T/E ratio. The major part of testosterone is excreted as the glucuronide but it was found a 100- or more -fold variation in excretion rate, both in a Caucasian and an Asian study group. A majority (75 %) of Asian subjects falls within the “low excretion” group compared with 9 percent among the Caucasians, and the widely different distribution into the two modes indicated a monogenic background of the excretion pattern. The conspicuous intra- and interethnic differences in testosterone excretion gave cause for concern about how to interpret the classical urinary T/E screening test as an indicator of exogenous administration of the hormone. The future test programme for testosterone ought to adopt a Bayesian inference technique for analysis of consecutive T/E samples in the individual, as has been applied in the detection of blood doping with the “blood pass” [12192].

In summary, large genetic variation in the disposition and effects of anabolic androgenic steroids is a reality that must be considered in the doping control strategy in sports as well as in society. Polymorphisms in three crucial enzyme genes have hitherto been shown to have an impact on the disposition of testosterone and the T/E ratio. Further genetic confounders in other genes cannot be excluded at present time. False negative and false positive results with the current principles for interpretation of the T/E screening test as described will lead to unnecessary and expensive follow-up analyses of many samples [12192].

**UDP-glucuronosyltransferase and UGT2B17 genotype**

Recently, it was demonstrated that genotype-based cutoff values may improve the sensitivity
and specificity of the test, this demonstrating that genetic variation in androgen disposition is of importance in those instances when androgen urinary excretion profile should be tested. A deletion polymorphism in the UGT2B17 gene can lead to misinterpretation of T/E ratio, this accounting for a significant part of ethnic interindividual variability. UGT2B17 genotype information is therefore crucial to the decision as to which initial cutoff ratio is to be employed for an individual as well as for enhancement of the sensitivity of the Bayesian analysis (a method of interpretation of probabilities). On the basis of these data, the proposition has been made that a Bayesian interpretation of consecutive tests in the same individual should be adopted to replace the epithestosterone ratio [12011].

The anabolic steroid testosterone can be used by athletes to enhance athletic performance and muscle growth. UDP-glucuronosyltransferase (UGT2B17) is the key enzyme involved in the glucuronidation of testosterone to testosterone glucuronide, which also serves as a marker for the testosterone/epitestosterone (T/E) ratio used to detect testosterone abuse in sport. Inhibitors of testosterone glucuronidation could have an impact on circulating testosterone levels, thus aiding performance, as well as potentially affecting the urinary T/E ratio and therefore masking testosterone abuse. Previous reports have revealed that non-steroidal, anti-inflammatory drugs, diclofenac and ibuprofen, inhibit the UGT2B17 enzyme. The aim of one study was to analyse dietary tea samples for inhibition of testosterone glucuronidation and, where inhibition is present, to identify the active compounds. Analysis of testosterone glucuronidation was conducted by performing UGT2B17 assays with detection of un-glucuronidated testosterone using high performance liquid chromatography. The results from this study showed that testosterone glucuronidation was inhibited by the green and white tea extracts, along with specific catechin compounds, notably: epicatechin, epigallocatechin gallate (EGCG) and catechin gallate. The IC50 inhibition value for EGCG was determined, using a Dixon plot, to be 64 μM, equalling the most active NSAID inhibitor diclofenac. Thus, common foodstuffs and their constituents, for the first time, have been identified as inhibitors of a key enzyme involved in testosterone glucuronidation. Whilst these common compounds are not substrates of the UGT2B17 enzyme, we showed that they inhibit testosterone glucuronidation which may have implications on current doping control in sport [12193].

The large variation in disposition known for most drugs is also true for anabolic androgenic steroids. Genetic factors are probably the single most important cause of this variation. Further, there are reasons to believe that there is a corresponding variation in efficacy of doping agents. Doped individuals employ a large variety of doping strategies in respect of choice of substance, dose, dose interval, duration of treatment and use of other drugs for enforcement of effects or correction of side effects. Metabolic steps up-stream and down-stream of testosterone are genetically variable and contribute substantially to the variation in disposition of testosterone, the most common doping agent in sports and in society. Large inter- and intra-ethnic variation in testosterone glucuronidation and excretion is described as well as the pitfalls in evaluation of testosterone doping test results. The hydrolysis and bioactivation of testosterone enanthate is also genetically variable yielding a 2-3 fold variation in excretion rate and serum concentration, thereby implicating a substantial variation in "efficacy" of testosterone. Given this situation it is logical to adopt the new findings in the doping control programme. The population based cut-off level for the testosterone:epitestosterone ratio should be replaced by a Bayesian interpretation of consecutive tests in the same individual. When combined with the above genetic information the sensitivity of the test is considerably improved. The combination of the three approaches should reduce the rate of falsely negative or positive results and the number of expensive follow-up tests, stipulated by the World Anti-Doping Agency [12192].
In the light of the continuously growing knowledge on the relevance of steroid conjugation (i.e. phase-II metabolism reactions), various new studies were initiated and reported concerning steroid glucuronides and sulfates, factors arguably confining their urinary concentrations, and the accurate and sensitive determination of these steroid conjugates for potential evaluation as a complement to the current steroid profiling approaches. It was investigated the utility of combining analytical results of 7 steroid glucuronides and 5 steroid sulfates for the detection of transdermal and oral administrations of testosterone and testosterone undecanoate, respectively. A total of 19 volunteers were subjected to genotyping concerning the insertion/deletion of the UGT2B17 gene yielding 7 ins/ins, 7 ins/del, and 5 del/del genotypes. All participants underwent a transdermal testosterone application (via patches providing 2.4 mg of testosterone/24 h) and, after washout, oral testosterone undecanoate administration (2 x 40 mg), and urine samples were collected over a period of 96 h. By means of LC-MS/MS, relevant steroid conjugates were quantified, corroborating the issue of common GC-MS-based steroid profile approaches that population-based reference ranges barely allow the identification of topical and oral testosterone administration. Employing an intra-individual profiling strategy, the administration of testosterone via patches was identified, particularly by means of the ratios of testosterone glucuronide (TG)/epitestosterone glucuronide (epiTG) as well as androsterone glucuronide (AG)/etiocholanolone glucuronide (EG). The ingestion of testosterone undecanoate was detectable predominantly by means of etiocholanolone sulfate (ES), especially in UGT2B17 del/del genotypes [13009].

UGT2B17 deletion genotypes are especially prevalent among Asian athletes, and the traceability of intramuscularly administered testosterone enanthate to female Japanese volunteers was therefore investigated with a cohort consisting of six del/del, three ins/del, and one ins/ins genotype. As expected, the T/epiT ratio of the del/del group did not exceed the limit of 4 at any time of the 16 days of the study period, thus no follow-up analyses by IRMS would have been triggered. Consequently, the authors suggested employing subject-based reference ranges and/or genotype-specific thresholds for steroid profile parameters such as the T/epiT ratio [13009].

Emphasis was put for instance on UDP-glucuronosyltransferase UGT2B17, a key enzyme in testosterone glucuronidation. In an in vitro experiment it was shown that UGT2B17 was be negatively influenced by catechins (epicatechin, epigallocatechin gallate, and catechin gallate) commonly found in dietary green and white teas. Since tea consumption can lead to pharmacologically relevant concentrations of these catechins, it is conceivable that steroid profiles can vary due to such licit dietary products; however, in vivo data remain to support this assumption and to assess the relevance for sports drug testing. Concerning the same key enzyme UGT2B17, the role of androgen sulfation was studied in volunteers with two, one, or no allele of the respective gene, who received a single oral dose of testosterone enanthate. While sulfates of urinary steroids were found to be inadequate for monitoring purposes in this scenario, the increased excretion of androsterone (A) glucuronide was considered helpful (especially when evaluated in relation to epitestosterone (EpiT) glucuronide), which is in agreement with earlier studies outlining the relevance of the A/EpiT ratio in steroid profiling. Deletion polymorphism concerning UGT2B17 is of great importance when interpreting steroid profile data; hence, the availability of a test assay for its determination from doping control urine sample was desirable and established in 2011. A total of 674 urine samples was phenotyped, corresponding T/EpiT ratios were determined and significant correlations between homozygote gene-deletion and low T/EpiT ratios confirmed [13012].

It was investigated the androgen receptor (AR) bioluminescence response in serum and urine before and after testosterone challenge in different genotypes of the UGT2B17
enzyme, which catalyses testosterone glucuronidation. The androgen receptor activity was determined using a yeast-based bioluminescence assay. The androgens were analysed using LC-MS/MS, and the individuals were genotyped for UGT2B17 deletion polymorphism using real-time polymerase chain reaction. The serum concentrations of testosterone and dihydrotestosterone (DHT) were markedly elevated on days 2 and 4 and were still above baseline on day 15 after a dose of 500 mg testosterone enanthate. The androgenic activity in serum increased in parallel and correlated with the hormone concentrations and remained above baseline on day 15. The urinary androgenic activity increased 4-5-fold and was closely related to the unconjugated testosterone and independent of the UGT2B17 genotype. The AR assay may serve as a complement to the urinary testosterone/epitestosterone (T/E) doping test, because this is profoundly influenced by the UGT2B17 deletion polymorphism. It may also be useful for detection of other illicit androgens in sports, or in the society, or for monitoring and diagnostics of androgen-related disorders [13217].

*Japan*

Ethnicity has been found to influence urinary testosterone glucuronide to epitestosterone glucuronide (T/E) ratios among athletes. Uridine diphospho-glucuronosyltransferase 2B17 (UGT2B17) is the most active enzyme in testosterone glucuronidation. UGT2B17 polymorphism analysis is rarely performed in Japanese athletes, and the influence of testosterone administration on steroid profiles and carbon isotope ratios, according to gene polymorphisms, in Asians remains unknown. The prevalence of UGT2B17 genotypes and urinary androgenic steroid profiles, classified according to UGT2B17 genotypes, was investigated in Japanese athletes (255 male and 256 female). Testosterone enanthate (100 mg) was administered intramuscularly to Japanese female volunteers (del/del: n=6, del/ins: n=3, ins/ins: n=1). The distribution rates of the UGT2B17 del/del genotype in Japanese male and female athletes were 75 percent and 60 percent, respectively. The ins/ins genotype was detected in only three male (1.2 %) and seven female (2.7 %) athletes. The prevalence of the UGT2B17 deletion genotype was extremely high in Japanese athletes. The T/E ratio in the del/del group was significantly lower than that in the other groups. After testosterone was administered to female volunteers, the T/E ratios for the del/del individuals failed to reach the positivity criterion of 4. By contrast, in all of the del/del subjects, the gas chromatography/combustion/isotope ratio mass spectrometry (GC-C-IRMS) analysis successfully fulfilled the positivity criterion. The overall result has demonstrated the limited effectiveness of population-based T/E ratios in screening tests for testosterone use. Subject-based steroid profiling with UGT2B17 genotyping will be an effective strategy for detecting testosterone misuse [12194, 13218].

*Genetic polymorphism*

Emphasis has been put on UDP-glucuronosyltransferase UGT2B17, a key enzyme in testosterone glucuronidation. In an in vitro experiment it was shown that UGT2B17 was negatively influenced by catechins (epicatechin, epigallocatechin gallate, and catechin gallate) commonly found in dietary green and white teas. Since tea consumption can lead to pharmacologically relevant concentrations of these catechins, it is conceivable that steroid profiles can vary due to such licit dietary products; however, in vivo data remain to support this assumption and to assess the relevance for sports drug testing. Concerning the same key enzyme UGT2B17, the role of androgen sulfation was studied in volunteers with two, one, or no allele of the respective gene, who received a single oral dose of testosterone enanthate. While sulfates of urinary steroids were found to be inadequate for monitoring purposes in this scenario, the increased excretion of androsterone (A) glucuronide was considered helpful (especially when evaluated in relation to epitestosterone (EpiT) glucuronide), which is in agreement with earlier studies outlining the relevance of the A/EpiT ratio in steroid profiling. Deletion polymorphism concerning UGT2B17 is of great importance.
when interpreting steroid profile data [12017].

Aiming at the identification of new, complementary biomarkers for endogenous steroid abuse, the utility of a steroidomic approach using UHPLC-HRMS was assessed. In a controlled elimination study with orally administered testosterone undecanoate (80 mg), urine samples were subjected to a holistic steroid analysis followed by chemometric/statistical data evaluation. Here, numerous glucuronidated or sulfated steroids, the deconjugated analogs, of which mostly constitute the established steroid profile, were found to support the discrimination of the groups having received either placebo or testosterone undecanoate. The study demonstrated the principle of modern analytical approaches commonly referred to as “-omics” strategies and its potential application to issues of doping controls; in order to consider the whole (holistic) picture of such approaches, complementary analyses (e.g. by means of GC-HRMS) might be required to strengthen the outcome and value [12017].

Urinary steroid profiling is used in doping controls to detect testosterone abuse. A testosterone over epitestosterone (T/E) ratio exceeding 4.0 is considered as suspicious of testosterone administration, irrespectively of individual heterogeneous factors such as the athlete's ethnicity. A deletion polymorphism in the UGT2B17 gene was demonstrated to account for a significant part of the inter-individual variability in the T/E between Caucasians and Asians. Now it was examined the variability of urinary steroid profiles in a widely heterogeneous cohort of professional soccer players. The steroid profile of 57 Africans, 32 Asians, 50 Caucasians and 32 Hispanics was determined by gas chromatography-mass spectrometry. Significant differences have been observed between all ethnic groups. After estimation of the prevalence of the UGT2B17 deletion/deletion genotype (African 22%; Asian 81%; Caucasian 10%; Hispanic 7%), ethnic-specific thresholds were developed for a specificity of 99 percent for the T/E (African 5.6; Asian 3.8; Caucasian 5.7; Hispanic 5.8). Finally, another polymorphism could be hypothesized in Asians based on specific concentrations ratio of 5α-/5β-androstan-3α,17β-diol in urine. These results demonstrate that a unique and nonspecific threshold to evidence testosterone misuse is not fit for purpose. An athlete’s endocrinological passport consisting of a longitudinal follow-up together with the ethnicity and/or the genotype would strongly enhance the detection of testosterone abuse. Finally, additional genotyping studies should be undertaken to determine if the remaining unexplained disparities have an environmental or a genetic origin [09050].

Genetic variation has a large impact on androgen disposition. This variation is of the utmost importance for the interpretation of doping test results and may modulate the effects of testosterone replacement therapy and testosterone doping [09051].

**Association with renal disease and gene polymorphism**

With prolonged use of anabolic androgenic steroids (AAS), occasional incidents of renal disorders have been observed. Independently, it has also been established that there are considerable inter-individual and inter-ethnic differences, in particular with reference to the uridine diphosphate-glucuronosyltransferase 2B17 (UGT2B17) gene, in metabolising these compounds. One report postulated the association of deletion polymorphism in the UGT2B17 gene with the occurrence of renal disorders on chronic exposure to AAS. The major deactivation and elimination pathway of AASs is through glucuronide conjugation, chiefly catalyzed by the UGT2B17 enzyme, followed by excretion in urine. Excretion of steroids is affected in individuals with a deletion mutation in the UGT2B17 gene. It was hypothesize that UGT2B17 deficient individuals are more vulnerable to developing renal disorders with prolonged use of AAS owing to increases in body mass index and possible direct toxic effects of steroids on the kidneys. Elevated serum levels of biologically active steroids due to inadequate elimination can lead to prolonged muscle build up. An increase in body mass
index may cause renal injuries due to sustained elevated glomerular pressure and flow rate. In the absence of controlled clinical trials in humans, observational studies can be carried out. Real time PCR with allelic discrimination should be employed to examine the prevalence of different UGT2B17 genotypes in patients with impaired renal function and AAS abuse. In individuals with the UGT2B17 deletion polymorphism, blood tests, biofluid analyses, urinalysis, and hair analyses following the administration of an anabolic steroid can be used to determine the fate of the substance once in the body. If the hypothesis is upheld, anabolic steroid users with a deletion mutation in the UGT2B17 gene may be exposed to an increased risk of developing renal disorders. In the current detecting – sanctioning anti-doping system, athletes motivated by the potential to evade detection owing to their unique genetic make-up could subject themselves to a serious health consequence. More research on AAS metabolism in the presence of UGT2B17 gene deletion is required. Benefit or harm evaluations in therapeutic use of anabolic steroids should also consider this potential link between UGT2B17 gene deletion polymorphism and renal disorders [10057].

Chemistry

Androgenic and anabolic steroids (AASs) are a class of chemical substances closely related to testosterone in molecular structure. They can be abused to enhance performances in human and equine athletes, and are banned by the sports authorities. To assist with method development for doping analyses of AASs, investigations were conducted to correlate their product ion profiles with the molecular structures. Although very similar in chemical structure, AASs generated noticeably different product ion profiles from collision-induced dissociation (CID). On the basis of both outlines of the product ion profiles and molecular structures, AASs studied were classified into six subclasses. In each subclass, the product ion profiles were identical or similar. However, the product ion profiles in one subclass were remarkably different from those in another. The classification reveals that the position and number of double bond(s) in conjugation with the 3-carbonyl group in the molecular structure of an AAS have significant effects on product ion profile. The presence or absence of the 19-methyl group in an AAS also has a remarkable influence on its product ion profile. A substitution in the A-, B- or D-ring of an AAS may cause a shift in mass value of the product ions. The correlation of product ion profiles with molecular structures of AASs has the implication that each AAS can be characterized by a combination of its [M + H]+ ion and product ion profile and as a result be identified with specificity. The classified product ion pattern may be useful in the identification of unknown AASs [10323].

Steroid hormones

Steroid hormones include the sex hormones, glucocorticoids, and mineralocorticoids. Within the family of sex hormones are the androgens, estrogens, and progestogens. All of the steroid hormones bind to their own specific receptor, which may be cytosolic or nuclear, to induce changes within a cell. All natural steroid hormones are synthesized from cholesterol in the adrenal glands and/or gonads. Some steroid hormones are further metabolized in the liver, peripheral and/or target tissues. As their precursor is cholesterol, they are hydrophobic in nature which allows them to pass easily through cell membranes. Once synthesized, the steroid hormones are carried in the blood stream bound to carrier proteins such as albumin, steroid hormone-binding globulin (SHBG) or corticosteroid-binding globulin to target tissues. The androgen produced in the highest concentration in the body is testosterone (T). This is a 19-C steroid that has androgenic and anabolic effects within the body. T is primarily produced in the gonads but a small amount is produced in the adrenal cortex or from the peripheral conversion of androstenedione. T production is much greater in males than in
females (5000-7000 microg/day vs 300 microg/day). In males, T is primarily produced by the Leydig cells in the testes whereas in females, the primary production of T occurs in the Theca cells of the ovaries. In both sexes, small amounts come from the adrenal cortex and the peripheral conversion of androstenedione. T acts in the body by acting directly through the AR or indirectly via metabolism to other sex steroids. T can be aromatized to estradiol (E2) which activates ER-alpha and/or ER-beta. Alternatively, T can be irreversibly converted to the more potent 5alpha-dihydrotestosterone (5alpha-DHT) by the enzyme 5alpha-reductase. T has many physiological actions in the body. It acts on muscles to stimulate growth and maintenance, it promotes bone development while inhibiting bone resorption, and it increases red blood cell and hemoglobin levels, augments libido and erectile function, enhances mood and cognition, and induces lipolysis. Low testosterone levels or deficit in androgen action induces frailty, sarcopenia, poor muscle quality, muscle weakness, hypertrophy of adipose tissue and impaired neurotransmission [13084].

**Testosterone and its modifications**

Testosterone is the most important androgen in the human body. The effects of androgens are most evident during puberty, as they elicit dramatic physiological changes in the male body, including the onset of secondary male characteristics, hair growth pattern, sebaceous gland activity and maturation of sperm and libido. These are considered the virilizing or “androgenic” effects. Daily testosterone synthesis ranges from 2.1 to 11.0 mg in individual males, with normal plasmatic levels of 300-1000 ng/dL, which progressively decline with age. Testosterone has several possible metabolic fates. First, it binds to the androgen receptor (AR) in target tissues to exert its effects. Second, it is reduced to 5alpha-dihydrotestosterone (5DHT), which also acts on the AR. Following a different path, testosterone may be aromatized to estradiol to exert estrogenic effects, typically water retention, breast tissue growth and an increase in body fat deposition. Along with the androgenic changes comes the “anabolic” effect. Anabolism is defined as any state in which nitrogen is differentially retained in lean body mass through the stimulation of protein synthesis and/or a reduction in protein breakdown. It includes growth promotion, protein and collagen synthesis and an increase in muscle size and bone metabolism. Characteristically, steroids that are more anabolic present weaker AR bindings, and those that are more androgenic strongly bind the AR, exerting a more potent effect. A “myotrophic-androgenic index”, based on the association between anabolic and androgenic bioassays in rats has been previously described. Since testosterone is the basic AAS, it has a 1:1 anabolic-androgenic ratio. Structural modifications have been made to the testosterone molecule in an attempt to maximize the anabolic effects and minimize the androgenic ones; however, all AASs are virilizing if administered for long enough, at high enough dosages. AASs therefore include synthetic derivatives of testosterone, and not only testosterone itself. The AAS structural base is the steran nucleus, consisting of three condensed cyclohexan rings, in nonlinear junction, and a cyclopentane ring. The anabolic effects are dose-dependent, and usually occur when supraphysiological testosterone levels (>1000 ng/dL) are found, which generally requires weekly doses of 300 mg or more. Traditionally, AASs are classified according to the route of administration and their carrier solvent and fall into two categories: oral and parenteral. Alkyl substitution prevents deactivation of the steroid by hepatic first-pass metabolism (necessitating hepatic monitoring), which promotes oral activity. They usually have short half-lives, making several daily doses necessary to maintain appropriate blood levels. This class includes the very common stanozolol and oxandrolone, as well as methyltestosterone and others. If the 17beta-hydroxyl group is esterified with an acid moiety it prevents rapid release from the oily vehicle. Roughly, the longer the chain length of the acid moiety, the more slowly the preparation is released into the blood stream. Once in the circulation, hydrolysis rapidly occurs yielding the active compound. They usually have a longer half-life and a slower absorption rate, bringing much less hepatic stress than the orally taken steroids. Pain at
injection sites is common, because of the oily base. There are several basic active compounds: testosterone, bound to esters such as undecanoate, cypionate, propionate and others; and 19-nortestosterone (or nandrolone), also bound to different esters. Nandrolone is extremely popular, owing to its high anabolic to androgenic ratio. In contrast to testosterone, nandrolone is converted to a less potent metabolite after 5alpha-reduction. This, in addition to nandrolone’s lesser affinity to AR, explains the higher myotrophic to androgenic ratio. Other group of compounds is headed by boldenone, bound to ester undecylenate; and trenbolone, bound to ester acetate [11028].

Two modifications made to the testosterone molecule alter the androgenic/anabolic profile, and thus the type of side effects seen from usage of the various AAS. Class A modifications are formulated by esterification of the 17-alpha-hydroxyl group, which increases the lipophilic properties, allowing a slow, delayed absorption as an injectable form. This is the most common form of modification because it allows the injection to be administered as infrequently as once every 2 to 6 weeks. This pharmacokinetic alteration also results in increased and unwanted androgenic effects. Class B modifications result from alkylation of the 17-alpha portion of the molecule, which decreases hepatic metabolism. This allows increased oral absorption and slower hepatic degradation. Slower clearance from the liver results in greater hepatic toxicity [06031].

**General metabolism**

The major elimination and deactivation pathway of AAS and their phase I metabolites is through glucuronide conjugation (phase II metabolism), mainly catalysed by the enzyme UGT2B17, followed by excretion in urine. However, inter-individual and inter-ethnic variations in the prevalence of deletion polymorphism in the gene coding of the UGT2B17 enzyme have been reported, which eventually influence the urinary excretion of AAS and potentially lead to false-negative doping results. It has also been reported that the glucuronidation activity of UGT2B17 and other UGTs towards AAS is inhibited by commonly used anti-inflammatory drugs like diclofenac and ibuprofen, in vitro. Common dietary substances such as red wine, white tea and green tea have also shown similar inhibitory effects in in vitro studies. Although the inhibitory effect is yet to be examined and reported in vivo, these in vitro results indicate that concomitant use of such over-the-counter medication or common dietary products with AAS may lead to impaired urinary excretion of AAS and their metabolites [12116].

Considering that such genetic and metabolic variations may limit the efficacy of urinalysis in testing doping, it can be suggested that urinalysis, if used as a stand-alone test, is susceptible to confounding doping results. Owing to the growing number of doping cases with AAS, there is an ever-increasing need to develop new methods to detect drug doping. The current anti-doping regime can be reinforced by employing additional biological samples like blood and hair analysed in tandem with urine. Since impaired glucuronidation leads to reduction in the urinary excretion rate of AAS, it can be assumed that the levels of unconjugated AAS and their phase I metabolites in the systemic circulation will be elevated and thus higher levels of AAS and their phase I metabolites will be available to get incorporated into hair and other body tissues. Hair analysis has been used in the past for detecting drug use as it predominantly favours the direct detection of parent AAS and determines a retrospective history of drug use. Thus, hair analysis and blood analysis can provide complementary information to urinalysis to prevent false doping results [12116].

**Effect of oxidizing adulterants on human urinary steroid profiles**

Steroid profiling is the most versatile and informative technique adapted by doping control laboratories for detection of steroid abuse. The absolute concentrations and ratios of
endogenous steroids including testosterone, epitestosterone, androsterone, etiocholanolone, 5alpha-androstane-3alpha,17beta-diol and 5beta-androstane-3alpha,17beta-diol constitute the significant characteristics of a steroid profile. In one study we report the influence of various oxidizing adulterants on the steroid profile of human urine. Gas chromatography-mass spectrometry analysis was carried out to develop the steroid profile of human male and female urine. Oxidants potassium nitrite, sodium hypochlorite, potassium permanganate, cerium ammonium nitrate, sodium metaperiodate, pyridinium chlorochromate, potassium dichromate and potassium perchlorate were reacted with urine at various concentrations and conditions and the effect of these oxidants on the steroid profile were analyzed. Most of the oxidizing chemicals led to significant changes in endogenous steroid profile parameters which were considered stable under normal conditions. These oxidizing chemicals can cause serious problems regarding the interpretation of steroid profiles and have the potential to act as masking agents that can complicate or prevent the detection of the steroid abuse [12117].

Steroids from musk deer

The administration of musk extract, that is, ingredients obtained by extraction of the liquid secreted from the preputial gland or resulting grains of the male musk deer (e.g. Moschus moschiferus), has been recommended in Traditional Chinese Medicine (TCM) applications and was listed in the Japanese pharmacopoeia for various indications requiring cardiovascular stimulation, anti-inflammatory medication or androgenic hormone therapy. Numerous steroidal components including cholesterol, 5alpha-androstane-3,17-dione, 5beta-androstane-3,17-dione, androsterone, etiocholanolone, epiandrosterone, 3beta-hydroxyandrost-5-en-17-one, androst-4-ene-3,17-dione and the corresponding urea adduct 3alpha-ureido-androst-4-en-17-one were characterised as natural ingredients of musk over several decades, implicating an issue concerning doping controls if used for the treatment of elite athletes. In the present study, the impact of musk extract administration on sports drug testing results of five females competing in an international sporting event is reported. In the course of routine doping controls, adverse analytical findings concerning the athletes' steroid profile, corroborated by isotope-ratio mass spectrometry (IRMS) data, were obtained. The athletes' medical advisors admitted the prescription of TCM-based musk pod preparations and provided musk pod samples for comparison purposes to clarify the antidoping rule violation. Steroid profiles, IRMS results, literature data and a musk sample obtained from a living musk deer of a local zoo conclusively demonstrated the use of musk pod extracts in all cases which, however, represented a doping offence as prohibited anabolic-androgenic steroids were administered [12120].

Physiology

Testosterone supplementation acts via numerous mechanisms as a highly potent anabolic agent to skeletal muscle. Although growth hormone (GH) strongly affects collagen synthesis and lipolysis, as well as increasing lean body mass, it is not anabolic toward the contractile (ie, myofibrillar) muscle tissue in healthy individuals. However, there is a persistent belief (both in scientific literature and among recreational weightlifters) that exercise-induced release of GH and testosterone underpins muscular hypertrophy with resistance training. This is a premature assumption because although pharmacological GH supplementation can increase muscle strength or size in individuals with clinical GH deficiency, there is no evidence that transient exercise-induced changes in GH have the same effects in individuals with normal GH levels. Exercise paradigms are designed based on the assumption (not necessarily evidenced-based mechanisms) that GH and testosterone facilitate anabolic processes that lead to skeletal muscle protein accretion and hypertrophy. Recent work
disputes this assumption. Instead, data indicate that exercise-induced hormonal elevations do not enhance intracellular markers of anabolic signaling or the acute postexercise elevation of myofibrillar protein synthesis. Furthermore, data from our training study demonstrate that exercise-induced increases in GH and testosterone availability are not necessary for and do not enhance strength and hypertrophy adaptations. Instead, our data lead us to conclude that local mechanisms that are intrinsic to the skeletal muscle tissue performing the resistive contractions (ie, weightlifting) are predominant in stimulating anabolism. Clarifying both the role of hormones in regulating muscle mass as well as the underlying basis for adaptation of skeletal muscle to resistance exercise will hopefully enhance and support the prescription of resistance exercise as an integral component of a healthy lifestyle [10444].

Dimethandroline (DMA: 7α,11β-dimethyl-19-nortestosterone) and 11β-methyl-19-nortestosterone (MNT) are potent androgens in development for hormonal therapy in men. As 5α-reduced androgens, such as 5α-dihydrotestosterone (DHT), may raise the risk of benign prostate hyperplasia, accelerate the development of prostate carcinoma, and increase male pattern baldness and acne, we investigated the role of 5α-reduction in the androgenic activity of DMA and MNT. The authentic 5α-reduced metabolites, 5α-dihydroDMA (5α-DHDMA) and 5α-dihydroMNT (5α-DHMNT), were prepared by chemical synthesis and compared in vitro and in vivo to the parent compounds. Both 5α-reduced androgens bind with high affinity to the rat androgen receptor (AR) and were potent inducers of transactivation of 3XHRE-LUC in CV-1 cells cotransfected with a human AR expression plasmid. To examine in vivo androgenic (stimulation of ventral prostate and seminal vesicle weights) and anabolic (stimulation of levator ani muscle weights) activity, 22-day-old castrate male rats were treated sc for 7 days with various doses of DMA, 5α-DHDMA, or testosterone (T) or MNT, 5α-DHMNT, or T and necropsied on day 8. 5α-DHDMA was at least threefold more potent than T in stimulating growth of the ventral prostate but only 30-40 percent as potent as DMA. 5α-DHMNT was four- to eightfold more potent than T, whereas MNT was approximately equipotent to T. To assess the possible role of 5α-reduction in ventral prostate and seminal vesicle growth, castrate immature rats were treated with maximally effective doses of T, DHT, DMA, MNT, or the related 19-norandrogen, 7α-methyl-19-nortestosterone (MENT), or vehicle, with or without dutasteride (DUT), an inhibitor of 5α-reductases types 1 and 2. In rats treated with T+DUT, serum T was significantly higher than in rats treated with T alone, and serum DHT was decreased to levels observed in castrate vehicle-treated rats. DUT significantly reduced both ventral prostate and seminal vesicle weights in T-treated rats, whereas there was no significant effect of DUT on weights of these accessory sex glands in rats treated with DMA, MNT, DHT, or MENT. These results indicate that inhibition of 5α-reductase activity in vivo does not affect the androgenic potency of DMA, MNT, or MENT [10445].

Over a period of 10 to 20 weeks, a supraphysiologic dose of testosterone administered to healthy young men can increase lean body mass, as well as muscle size and strength with or without exercise. These significant increases are dose dependent and only occur with doses of 300 mg per week and higher. The most profound effects are noted when supraphysiologic doses accompany a training program and are used in conjunction with a diet adequate in protein and calories. Testosterone-induced muscle hypertrophy and increases in muscle strength are the result of increases in the cross-sectional area of muscle fibers and myonuclear number. Research suggests that these anabolic effects are mediated by testosterone-influenced increases in muscle protein synthesis, creating a positive nitrogen balance. Androgen receptors in skeletal muscle regulate the transcription of the target genes that control the accumulation of DNA needed for muscle growth. Complementary effects include glucocorticoid antagonism, which minimizes the catabolic actions of corticosteroids released during the stress of athletic activity. Similarly, stimulation of the growth hormone
insulin-like growth factor-1 axis and enhanced collagen synthesis and bone mineral density are additional anabolic effects [07008].

Testosterone is produced in several areas within the human body. In men, most of it is synthesized in the Leydig cells of testes and in the adrenal glands; in women, testosterone is produced in the ovaries and adrenal glands, with a smaller fraction produced in other peripheral sites. Its synthesis involves a cholesterol precursor and a series of enzymatic reactions. Secretion is determined by a negative feedback mechanism that involves the anterior pituitary gland. It is here that luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are stored. Elevated levels of testosterone affect the hypothalamus and pituitary. At high levels of testosterone, LH tends to be reduced (affecting sperm and endogenous testosterone production). Studies by the World Health Organization examined the use of anabolic steroids as a form of male birth control, but the results were not promising [07007].

Mechanism of action

Testosterone is both an active hormone and a prohormone for the formation of the more active androgen, 5alpha-dihydrotestosterone (DHT) via 5alpha-reductase...The production of testosterone occurs predominantly (i.e. 95 %) in the Leydig cells of the testes and, to a lesser, extent in the adrenal glands. These creations of androgens from the adrenal cortex are insufficient to maintain male sexuality. Women also secrete small amounts of testosterone from the ovaries and adrenal glands including DHEA and androstenedione. Testosterone acts as an androgen either directly by binding to the androgen receptor or indirectly by conversion to DHT. This latter compound binds more avidly to the androgen receptor than testosterone. Dihydrotestosterone amplifies the action of androgen and conveys specific function to the androgen–androgen receptor complex. The conversion of testosterone to DHT is especially important for the appearance of virilization in female AAS users because high levels of 5alpha-reductase activity in the male accessory sex glands limits the effects of DHT. Activation of the intracellular, ligand-dependent androgen receptor complex by testosterone and AAS results in the production RNA, DNA, and the subsequent enhancement of protein synthesis, including increased amounts of actin and myosin in skeletal muscles. The androgen receptor belongs to the nuclear receptor family that contains a DNA-binding domain, a ligand-binding domain, and at least 2 transcriptional activation domains. The enzyme, aromatase controls the androgen-estrogen ratio by catalyzing the conversion of testosterone to estradiol in other tissues (e.g. adipose tissue and brain). Anabolic-androgenic steroids (e.g. methandienone, nandrolone, stanozolol) with the most potent anabolic effects also possess the relatively greatest androgenic effects. The interactions of AAS with the androgen receptors in various tissues vary between compounds in this group, and these variations account for differences in the anabolic andandrogenic effects of these compounds. The use of AAS increases skeletal muscle mass and strength when used in combination with intensive strength training and high-protein, high-caloriediets. Endogenous testosterone is responsible for sexual maturation at all stages of development throughout the life of males. Increased AAS plasma concentrations suppress gonadotropin-releasing hormone, endogenous testosterone secretion, luteinizing hormone, and follicle-stimulating hormone by a negative-feedback mechanism. Endogenous androgens stimulate RNA-polymerase, resulting in an increased production of proteins. These proteins are responsible for normal male sexual development, including the growth and maturation of the prostate, seminal vesicle, penis, and scrotum. During puberty, androgens cause a sudden increase in growth hand development of muscle along with redistribution of body fat and deepening of the voice.
Continued endogenous testosterone secretion produces increased hair (beard, and body hair), fusion of the epiphyses, termination of growth, and the maintenance of spermatogenesis. Testosterone also affects the formation of erythropoietin, the balance of calcium, and blood glucose concentrations [13002].

**Chemical structure versus function**

Steroids are organic molecules with a tetracyclic ring system; all steroids with the exception of retinoic acid are derived from cholesterol. There are 4 major classes of natural steroid hormones (androgens, corticoids, estrogens, and progestogens) with testosterone being the principal male androgenic steroid. AASs are synthetic compounds similar in chemical structure to testosterone (molecular weight 288 g/mol). There are 3 major classes of AASs (oral, injectable-oil-based, and injectable-water-based) and at least 30 anabolic-androgenic steroid compounds. Abuse of other forms of AASs includes the use of buccal (Striant), sublingual (tetrahydrogestrinone), and transdermal (testosterone cream) preparations; these short-acting preparations are typically testosterone-based. The advantage of the buccal, sublingual, and transdermal preparations is the rapid clearance within 1 week after even large doses compared with 2-14 days for oral preparations and 4 weeks for water-soluble parenteral preparations. Non-steroidal selective androgen receptor modulators (SARMs) are experimental substances (e.g. bicyclic hydantoin, analogs of ary/propionamide, quinoline, tetrahydroquinoline) that may offer better dissociation of the biologic and anabolic effects of steroids; although these substances are not routinely available, the World Anti-Doping Agency (WADA) added these substances to the prohibited list in 2008. Up-to-date information on the list of banned substances is available on the WADA website (http://www.wada-ama.org/en/). Testosterone is the prototype for all AASs. Modification of testosterone during the synthesis of AASs involves the following three methods: alkylation of the 17beta-hydroxyl group, esterification at the 17alpha-position, or modification of the steroid nucleus to enhance anabolic properties. Although all currently available anabolic steroids have androgenic properties, the anabolic properties are greater for synthetic AASs than for testosterone. Some structural modifications improve bioavailability and prolong the duration of action, whereas other modifications reduce anabolic effects while enhancing androgenic effects. Esterification at the 17beta-hydroxy position increases lipophilic and androgenic properties while improving intramuscular bioavailability. In general, alkylation of the 17alpha-hydroxy position retards hepatic degradation and improves oral bioavailability, but these compounds typically are less potent than 17beta-esters. Weaker formulations of AASs are often marketed as prohormones in dietary supplements, particularly dehydroepiandrosterone (DHEA), 19-norandrostenediol, androstenedione, 19-norandrostenedione, 1-testosterone, and prostandol. The lack of the 17alpha-alkyl moiety results in extensive first-pass metabolism, and the anabolic effect of DHEA and androstenedione are limited by their weak binding to the androgen receptor despite some conversion to testosterone. The effect of these AASs is substantially greater on women than men because of the relatively larger increase in testosterone in the former. Tetrahydrogestrinone (THG;13-ethyl-17hydroxy-18,19-dinor-17alphapregn-4,9,11-trien-3-one), norbolethone, and madol (17alpha-methyl-5alpha-androst-2-en-17beta-ol) are designer anabolic steroids, which were synthesized to avoid detection during use. However, THG is a nonspecific androgen agonist that binds many steroid hormone receptors, particularly the glucocorticoid receptors [13002].

**Myotrophic action of androgens**

The myotrophic effects of androgens on muscle strength and mass are the main reason for their popularity among androgen users. Androgens also increase lean body mass, decrease fat mass, enhance performance, sustain intensive training periods, and can improve
appearance. The effects on lean body mass were shown by treating young men with a gonadotropin-releasing hormone (GnRH) analogue that suppressed endogenous T production. These men showed decreased rates of whole body protein synthesis, muscle strength and fat oxidation, together with an increased fat mass. When T was replaced through supplementation, there was a restoration of muscle size and strength with a concomitant reduction in fat mass. Androgens including T increase muscle fiber hypertrophy in human skeletal muscle by enhancing protein synthesis. This occurs via the activation of satellite cells and the promotion of myonuclear accretion when existing myonuclei become unable to sustain further enhancement of protein synthesis. The use of androgen therapy during aging is primarily to promote muscle strength by improving or maintaining muscle mass [13084].

Endogenous androgens

Cholesterol is the starting substance for formation of glucocorticoids, mineralocorticoids and sex steroids. Precursors of androgens are formed in the adrenals and biotransformed in the endocrine target organs, the gonads and the prostate gland. Dihydrotestosterone (DHT) is formed from testosterone in the prostate and is a more potent androgen than testosterone itself. Some of the testosterone precursors have weak androgenic effects such as dehydroepiandrosterone (DHEA) and androstenedione. They may be present in dietary products and abused per se. Most of the end products are eliminated via the kidneys after conjugation with glucuronic acid or sulphate groups by enzymes in the uridine glucuronosyl transferase (UGT) and sulphate transferase (SULT) super families, respectively. The UGT enzymes have different selectivities for the androgens and androgen metabolites. The specificity, the activity and the genetic variation of the various UGT enzymes is of particular interest in the conjugation and excretion of testosterone and epitestosterone as these steroids and their conjugates are quantified in the urine in conventional testosterone doping tests. Genetic variation in other steroid metabolizing enzymes may also influence the disposition of testosterone and other AAS [12192].

Androgen disposition and genetic variation

Genetic variation in genes involved in the synthesis, breakdown and elimination of androgens may affect the bioavailability of administrated AAS, and hence affect the degree of anabolic and toxic effects of doping. Moreover, genetic variation in the androgen receptor (AR) may modulate the pharmacodynamic effects of AAS. As a corollary, several of these polymorphisms may change the serum concentrations of AAS and excretion of AAS metabolites in the urine. Therefore, genetic variations are an important source of confounders in doping tests. Several functional polymorphisms in these genes known to alter the systemic load and excretion rate of androgens are discussed below in relation to doping and doping control [12192].

The metabolism of testosterone is revisited. Four previously unreported metabolites were detected in urine after hydrolysis with KOH using a liquid chromatography-tandem mass spectrometry method and precursor ion scan mode. The metabolites were characterized by a product ion scan obtained with accurate mass measurements. Androsta-4,6-dien-3,17-dione, androsta-1,4-dien-3,17-dione, 17-hydroxy-androsta-4,6-dien-3-one and 15-androsten-3,17-dione were proposed as feasible structures for these metabolites on the basis of the mass spectrometry data. The proposed structures were confirmed by analysis of synthetic reference compounds. Only 15-androsten-3,17-dione could not be confirmed, owing to the lack of a commercially available standard. That all four compounds are testosterone metabolites was confirmed by the qualitative analysis of several urine samples collected
before and after administration of testosterone undecanoate. The metabolite androsta-1,4-dien-3,17-dione has a structure analogous to that of the exogenous anabolic steroid boldenone. Specific transitions for boldenone and its metabolite 17β-hydroxy-5β-androst-1-en-3-one were also monitored. Both compounds were also detected after KOH treatment, suggesting that this metabolic pathway is involved in the endogenous detection of boldenone previously reported by several authors [10325].

**Metabolism – phase I enzymes**

Aldo-keto reductases (AKR1C) are divided into three families of which the AKR1C members AKR1C1, AKR1C2, AKR1C3, AKR1C4 and AKR1D1 have been shown to play an important role in steroid metabolism. Genetic variations in AKR1C genes may regulate the local concentration of steroid hormones. AKR1C3 is involved in the formation and inactivation of testosterone. Cytochromes P450 (CYPs) CYPs are the most important phase I enzymes in drug metabolism, accounting for the metabolism of more than 60 percent of all drugs. Several of the members of the CYP superfamily are also important catalysts in the metabolic network of steroids, e.g. CYP3A4 catalyzing 4-hydroxylation of testosterone. There is a large variation in the CYP3A4 activity between individuals, partly explained by polymorphisms in the CYP3A4 gene and partly by induction or inhibition by exogenous compounds and drugs. Several polymorphisms in this gene have been associated with altered serum concentrations of testosterone and oestrogens [12192].

Many anabolic steroids on the market are available as esters in order to achieve a retarded release. These pro-drugs need to be hydrolyzed prior to activation. It has been shown that PDE7B is involved in the hydrolysis of testosterone enanthate and nandrolone decanoate with implications for the serum concentrations and bioavailability of testosterone enanthate. It is conceivable that higher serum concentrations of testosterone convey an advantage in terms of strong androgenic influence on the organ receptors, thus being likely to improve the physical achievements [12192].

**Metabolism – phase II enzymes**

Uridine glucuronosyl transferases (UGTs) UGT enzymes are considered to be the main enzymes for inactivation and quantitative metabolic elimination of steroid hormones. UGT2B17 has been shown to be the main enzyme in testosterone glucuronidation activity in vitro and in vivo. It has been possible to demonstrate a large variation in the gene deletion both within, and between ethnic populations with important consequences for the interpretation of the T : E test. There is a large variation in urinary testosterone concentrations in UGT2B17 carriers, and this SNP may contribute to the variation in excretion within each UGT2B17 deletion polymorphism mode. UGT2B7 has also been identified as the main phase II enzyme involved in epitestosterone glucuronidation. Whether other SNPs in the UGT2B7 (or in other epitestosterone metabolizing enzyme genes) could explain the inter-individual variation in epitestosterone excretion needs to be investigated. Sulphotransferases (SULT) Even though glucuronides are the main conjugated metabolites of androgens, many steroids are also sulphated to different extent. For some steroids such as DHEA, sulphation is the major metabolic phase II pathway. As a matter of fact DHEA and DHEA sulphate are the most abundant androgen precursors in the circulation [12192].

Lately genetic polymorphisms in many transporter proteins have been shown to affect the outcome of drug treatment. The superfAMILY of organic anion transporting polypeptides (OATP), encoded by solute carrier organic anion transporter (SLCO) genes, mediates the uptake of various endogenous compounds including hormones. SLCO1B3 is involved in the uptake of several hormones including testosterone and SLCO2B1 mediates the transport of steroid conjugates, such as DHEA sulphate. Both these SLCOB genes are polymorphically expressed and have been shown to be functional, i.e. there is a correlation with the capacity
to transport hormones [12192].

**Steroid hormone binding globulin (SHBG)**

Steroid hormone binding globulin (SHBG) Testosterone and DHT bind with high affinity to SHBG, thereby regulating the concentration of bioactive testosterone. Several genetic polymorphisms have been characterized in the human SHBG gene. SHBG polymorphisms are associated with serum concentration of DHT and testosterone. Since genetic variation in the SHBG gene affects both the binding (and thereby bioavailability) and the urinary excretion of androgens, polymorphisms in SHBG may modulate both the effects of steroid doping and the analysis of illicit AAS [12192].

**Hydroxysteroid dehydrogenase**

The 3β-hydroxysteroid dehydrogenase/delta(5)-delta(4) isomerase (3β-HSD) and 1alpha-hydroxylase/17,20-lyase cytochrome P450 (P450c17) enzymes are important in determining the balance of the synthesis of different steroids such as progesterone (P4), glucocorticoids, androgens, and estrogens. How this is achieved is not a simple matter because each of the two enzymes utilizes more than one substrate and some substrates are shared in common between the two enzymes. The two synthetic pathways, delta(4) and delta(5), are interlinked such that it is difficult to predict how the synthesis of each steroid changes when any of the enzyme activities is varied. In addition, the P450c17 enzyme exhibits different substrate specificities among species, particularly with respect to the 17,20-lyase activity. The mathematical model developed in this study simulates the network of reactions catalyzed by 3β-HSD and P450c17 that characterizes steroid synthesis in human, non-human primate, ovine, and bovine species. In these species, P450c17 has negligible 17,20-lyase activity with the delta(4)-steroid 17alpha-hydroxy-progesterone (17OH-P4); therefore androstenedione (delta4) is synthesized efficiently only from dehydroepiandrosterone (DHEA) through the delta(5) pathway. The model helps to understand the interplay between fluxes through the delta(4) and delta(5) pathways in this network, and how this determines the response of steroid synthesis to the variation in 3beta-HSD activity or in the supply of the precursor substrate, pregnenolone (P5). The model simulations show that A4 synthesis can change paradoxically when 3beta-HSD activity is varied. A decrease in 3beta-HSD activity to a certain point can increase A4 synthesis by favouring metabolism through the delta(5) pathway, though further decrease in 3beta-HSD activity beyond that point eventually limits A4 synthesis. The model also showed that due to the competitive inhibition of the enzymes' activities by substrates and products, increasing the rate of P5 supply above a certain point can suppress the synthesis of A4, DHEA, and 17OH-P4, and consequently drive more P5 towards P4 synthesis [11568].

**Modulation of follistatin and myostatin propeptide**

The purpose of one pilot study was to investigate the impact of training, anabolic steroids and endogenous hormones on myostatin-interacting proteins in order to identify manipulations of myostatin signalling. To identify whether analysis of the myostatin interacting proteins follistatin and myostatin propeptide is suitable to detect the abuse of anabolic steroids, their serum concentrations were monitored in untrained males, bodybuilders using anabolic steroids and natural bodybuilders. In addition, we analysed follistatin and myostatin propeptide serum proteins in females during menstrual cycle. Our results showed increased follistatin concentrations in response to anabolic steroids. Furthermore, variations of sex steroid levels during the menstrual cycle had no impact on the expression of follistatin and myostatin propeptide. In addition, we identified gender differences
in the basal expression of the investigated proteins. In general, follistatin and myostatin propeptide concentrations were relatively stable within the same individual both in males and females. In conclusion, the current findings provide an insight into gender differences in myostatin-interacting proteins and their regulation in response to anabolic steroids and endogenous hormones. Therefore our data provide new aspects for the development of doping prevention strategies [13162].

**Chromosomal damage**

The aim of one study was to evaluate DNA damage (micronucleus) and cellular death (pyknosis, karyolysis and karyorrhexis) in exfoliated buccal mucosa cells from anabolic steroid users after 2 months of exposure. Two experimental groups consisting of 15 adult males who practise weight lifting and are anabolic steroid users or 15 adult males who practise weight lifting, but are non-anabolic steroid users, were recruited. In addition, 20 sedentary males, who do not practise any physical activity regularly, were matched by age with experimental groups. No significant statistical differences were noticed in individuals who practise physical activity only. On the other hand, an increase of micronucleated cells (MNCs) in anabolic steroid (decadurabulin and winstrol) users was observed. Regarding cytotoxic parameters, the same observation has occurred, that is, significant statistical differences were noticed in the group exposed to anabolic steroids when compared with other controls, as depicted by high frequencies of pyknosis, karyolysis and karyorrhexis. Taken together, the results suggest that genomic instability and cytotoxicity are induced by anabolic steroid administration in oral mucosa cells as assessed by the micronucleus test [10446].

**Androgen receptors**

The androgen receptor (AR) is expressed in many tissues and is activated through binding of testosterone and DHT. Activation of AR interacts with a broad variety of physiological processes. Therefore it is likely that genetic variation in AR may correlate with both anabolic effects and adverse side effects of androgen abuse. It has been speculated that these polymorphisms may affect the sensitivity to adverse psychic reactions such as aggression. Several polymorphisms have been described in the AR affecting the receptor affinity. In particular, a trinucleotide repeat (CAG) has been extensively studied and associated with disease risks, total fat free mass in men and acne, a common side effect of AAS abuse. In addition to their binding to AR, the testosterone metabolite 3alpha-androstanediol can bind to GABAA receptors, known to have an important role in the mediation of aggression [12192].

Nearly every cell in the human body has receptors for steroids, so that every organ system is susceptible to the effects of these molecules. Giving physiologic amounts of testosterone has no net effect on plasma levels because feedback inhibition shuts down endogenous production [07031].

The androgen receptor (AR) is a member of the steroid and nuclear receptor superfamily, and is a soluble protein that functions as an intracellular transcriptional factor. Structurally, AR contains three major functional domains, N-terminal domain (NTD), DNA-binding domain (DBD), and ligand binding domain (LBD). AR ligands regulate receptor function through binding to the LBD, which initiates sequential conformational changes of the receptor. Upon agonist binding, the receptor then undergoes dissociation from the chaperones, dimerization, phosphorylation, translocation to the nucleus, and binding to the androgen response element. Recruitment of other transcription coregulators and transcriptional machinery further ensures the transactivation of the AR-regulated gene expression upon agonist activation. AR
is mainly expressed in androgen target tissues, such as the prostate, skeletal muscle, liver, and central nervous system (CNS), with the highest expression level observed in the prostate, adrenal gland, and epididymis as determined by real-time PCR. AR can be activated by the binding of endogenous androgens, including testosterone and DHT. Physiologically, functional AR is responsible for male sexual differentiation in utero and for male pubertal changes. In adult males, androgen is mainly responsible for maintaining libido, spermatogenesis, muscle mass and strength, bone mineral density, and erythropoiesis. The actions of androgen in reproductive tissues, including prostate, seminal vesicle, testis, and accessory structures, are known as androgenic effects, while the nitrogen-retaining effects of androgen in muscle and bone are known as anabolic effects. Gonadal production of testosterone is under the feedback regulation of circulating testosterone through the hypothalamo-pituitary-gonadal axis [06049].

Androgen receptor (AR) is a member of the nuclear receptor (NR) superfamily of ligand-dependent transactivation factors. Androgens such as testosterone and 5alpha-dihydrotestosterone (DHT) act as agonists of AR. AR mediates various biological effects such as the development of male reproductive tissues, sexual development, and spermatogenesis. Since androgen declining with age contributes to age-related bone and muscle loss and increase in fat mass, the anabolic effect of androgen is attractive for the maintenance of health. Antagonists of AR (flutamide, bicalutamide, and nilutamide) are in use. AR is structurally characterized by an amino-terminal trans-activation domain (NTD/activation function 1, AF1), a DNA binding domain (DBD), and a ligand binding domain (LBD) including a carboxy-terminal transactivation domain (activation function 2, AF2). In the absence of a ligand, AR is localized in the cytoplasm, where it forms complexes with chaperones. Upon ligand binding, AR translocates into the nucleus. Following nuclear translocation, AR binds to androgen responsive elements (ARE) in the promoter regions of its target genes as a homodimer. Generally, the transcriptional activity of nuclear receptors is modulated by their interaction with cofactors such as coactivators and corepressors. The type of ligand that binds to the receptor determines which type of cofactor is chosen. In the case of agonists, AR interacts with coactivators dominantly over corepressors, and vice versa in the case of antagonists. Unlike other nuclear receptors, AR AF2 demonstrates weak transcriptional activity. However, ligand-dependent interaction between NTD and LBD/AF2 (which is termed as the N/C interaction) endows AR with synergistic transactivation potential) Thus, the N/C interaction is important for the ligand-dependent transactivation potential of AR [11062].

Androgens primarily exert their effects by binding to and activating a specific receptor, AR, which is expressed in most cells. The AR is a 110 kDa protein to which the natural androgens, T and DHT, bind with high affinity. In its inactivated state, AR is bound in the cytoplasm to heat shock proteins. When androgens bind to the AR via the ligand binding domain (LBD) of the AR, a conformational change is induced that promotes the dissociation of the heat shock proteins and AR subunits dimerize to form a homodimer. The dimerized AR complex translocates to the nucleus where it binds to androgen response elements (ARE) in the regulatory regions of androgen target genes. Binding of AR to the ARE occurs via the DNA binding domain (DBD). Regulatory cofactors interact with AR to promote DNA binding. Transcriptional activation by AR also involves cofactors that modify the chromatin structure and histone complexes in the DNA surrounding the ARE. This is important because the ARE can be located distantly from the transcription start site of androgen-regulated genes, therefore, cofactors that alter DNA shape and flexibility are needed for AR to augment transcription [13084].

**Androgen receptor polymorphism**
Muscle mass and strength, as well as aerobic fitness (VO$_{2\text{max}}$) are related to health and mortality. Muscle mass and strength is determined by environmental factors, principally endocrine, nutritional and mechanical loading, and by the genetic background. Gene polymorphisms, like those encoding for the insulin-like growth-factor-1 (IGF-1), type I collagen (COL1A1), ciliary neurotrophic factor (CNTF), interleukin-6 (IL-6), the vitamin D receptor (VDR), IGF-2, resistin (RETN) and androgen receptor (AR), have an influence on either muscle mass or strength. The AR gene is located to the X chromosome (q11.2–q12), and contains eight exons. The exon 1 contains a polyglutamine tract encoded by CAG repeats and a polyglycine tract (GGN) encoded by (GGT)$_3$(GGG(GGT)$_2$)GGC. Polymorphic tracts are close to the region encoding the transactivation-1 domain of the AR protein. The CAG and GGN polymorphisms of the androgen receptor (AR) gene are associated with the fat-free mass phenotype in healthy elders. However, it remains to be established if the AR polymorphism influences muscle mass and fitness in young adults. The polyglycine repeat length of AR ranges from 10 to 30. Short GGN repeats are associated with increased AR protein content in cell cultures that may in turn enhance the response to androgen stimulation. It remains unknown if a short GGN repeat number is associated to increased muscle mass or strength in humans. The exon-1 of the androgen receptor gene thus contains two repeat length polymorphisms which modify either the amount of AR protein inside the cell (GGN$_n$, polyglycine) or its transcriptional activity (CAG$_n$, polyglutamine). Shorter CAG and/or GGN repeats provide stronger androgen signalling and vice versa. To test the hypothesis that CAG and GGN repeat AR polymorphisms affect muscle mass and various variables of muscular strength and fitness traits, the length of CAG and GGN repeats was determined by PCR and fragment analysis and confirmed by DNA sequencing of selected samples in 282 men. Individuals were grouped as CAG short (CAG$_S$) if harbouring repeat lengths of ≤21 and CAG long (CAG$_L$) if CAG >21. GGN was considered short (GGN$_S$) or long (GGN$_L$) if GGN ≤23 or >23, respectively. No significant differences in lean body mass or fitness were observed between the CAG$_S$ and CAG$_L$ groups, or between GGN$_S$ and GGN$_L$ groups, but a trend for a correlation was found for the GGN repeat and lean mass of the extremities. In summary, the length of CAG and GGN repeat of the AR gene do not appear to influence lean mass or fitness in young men. Additional studies are required to test if men harbouring the combination CAG$_S$ and GGN$_S$ have more jumping capacity.

**Antagonists of the androgen receptor**

While the androgens of testicular origin (representing about 50% of total androgens in men over 50 years) can be completely eliminated by surgical or medical castration with GnRH (gonadotropin-releasing hormone) agonists or antagonists, the antiandrogens currently available as blockers of androgen binding to the androgen receptor (AR), namely bicalutamide (BICA), flutamide (FLU) and nilutamide have too weak affinity to completely neutralize the other 50 percent of androgens made locally from dehydroepiandrosterone (DHEA) in the prostate cancer tissue by the mechanisms of intracrinology. Series of steroid derivatives having pure and potent antagonistic activity on the human and rodent AR were synthesized. Assays of AR binding and activity in carcinoma mouse Shionogi and human LNCaP cells as well as in vivo bioavailability measurements and in vivo prostate weight assays in the rat were used. The chosen lead steroidal compound, namely EM-5854, has a 3.7-fold higher affinity than BICA for the human AR while EM-5855, an important metabolite
of EM-5854, has a 94-fold higher affinity for the human AR compared to BICA. EM-5854 and EM-5855 are 14 times more potent than BICA in inhibiting androgen (R1881)-stimulated prostatic specific antigen (PSA) secretion in human prostatic carcinoma LNCaP cells in vitro. MDV3100 has a potency comparable to bicalutamide in these assays. Depending upon the oral formulation, EM-5854 is 5- to 10-times more potent than BICA to inhibit dihydrotosterone (DHT)-stimulated ventral prostatic weight in vivo in the rat while MDV3100 has lower activity than BICA in this in vivo model. These data are supported by respective 40-fold and 105-fold higher potencies of EM-5854 and EM-5855 compared to BICA to inhibit cell proliferation in the androgen-sensitive Shionogi carcinoma cell model. Although the preclinical results data need evaluation in clinical trials in men, combination of the data obtained in vitro in human LNCaP cells as indicator of potency in the human prostate and the data on metabolism evaluated in vivo on ventral prostate weight in the rat, could suggest the possibility of a 70- to 140-fold higher potency of EM-5854 compared to bicalutamide (Casodex) for the treatment of prostate cancer in men [12115].

Physiological and clinical effects and side effects

The physiological direct effects of testosterone and AASs (AR-mediated) are well known. They include increases in renal erythropoiesis, lipolysis, protein synthesis, sebaceous secretion, hair growth and libido. However, the indirect effects should also be considered. These include antiglucocorticoid effects, which are mediated by testosterone occupation of cortisol receptors (which have a remarkable affinity with testosterone) and create an anti-catabolic effect. An increase in muscular activity is certainly the leading result of AAS use. It constitutes a complex phenomenon involving hypertrophy of skeletal muscle fibres that contain muscle cells and undifferentiated satellite cells. The latter become myoblasts that are incorporated into skeletal muscle cells, increasing the number of nuclei, and also the amount of cytoplasm, actin and myosin, making them larger and more potent. Notably, this phenomenon does not increase the number of fibres, only their size. Side effects of AASs are also well known. Their incidence is unclear, as the denominator of AAS use is not clear. Acne, alopecia and LUTS attributable to prostate enlargement are usually related to the strong androgenic 5DHT-effect. Erectile dysfunction and libido loss may also occur, especially after discontinuation, when endogenous testosterone levels are usually low. A sustained increase in testosterone levels during “cycles” leads to higher aromatization rates of testosterone, which accounts for the gynaecomastia typically found in steroid users. Hepatic effects are most often related to oral alkylated agents. They include the uncommon hepatic peliosis, cholestatic jaundice and hepatic neoplasms, such as focal nodular hyperplasia, which are all closely related to dose and duration of usage. Hepatocellular carcinoma and Wilm’s tumour are serious and rare side effects that are always related to long-term and heavy use. Interestingly, there are no reports linking AASs to prostate cancer or significant increases in PSA levels. The most severe consequences of long-term AAS use are associated with the cardiovascular system. Hypertension, arrhythmia, erythrocytosis and ventricular dysfunctions have been reported. Mortality risk among chronic users is estimated to be 4.6 times higher than among non-users. Cases of renal failure secondary to rhabdomyolysis and diffuse membranoproliferative glomerulonephritis in heavy users have been reported. Aggressive behaviour, depression, mood swings, altered libido, euphoria and even psychosis are some of the psychiatric patterns related to AAS. Overpharmacy may increase the risk of violent criminality. Withdrawal syndrome and dependency were also described, and the likelihood of psychiatric effects is greater where there is previous psychiatric history, or alcohol or drug abuse [11028].

The adverse endocrine effects of AASs are best understood if one first looks at the native effects of testosterone. Testosterone is responsible for the in utero masculinization of internal
genitalia, postnatal skeletal muscle development, and the development of male secondary sexual characteristics. In addition, testosterone is converted in peripheral tissues by 5-alpha-reductase to dihydrotestosterone (DHT), which contributes to fetal development of external genitalia, prostate, and seminal vesicles. DHT acts in the cell nucleus of target tissues, such as skin, male accessory glands, and the prostate, exerting predominantly androgenic, but also anabolic, effects. Testosterone is converted by the enzyme aromatase to estradiol and estrone, which are involved in the sexual differentiation of the brain, bone mass accretion, and fusion of the epiphyses at the conclusion of puberty, in addition to feminizing effects. Under normal physiologic circumstances, aromatase has a limited role; however, with high-dose AAS use, this role increases, and, therefore, so does the level of estrogens. An antiestrogen effect may be present as well with supraphysiologic doses of AASs. Excess AASs lead to a down-regulation of androgen receptors and AASs then compete with estrogens for the estrogen receptor. The net outcome of these two different pathways is difficult to predict. With this information, it is easier to understand the adverse outcomes of AAS use because many of the effects are amplifications of physiologic effects. Testosterone acts at the androgen receptor to increase protein synthesis; it also has effects through conversion to DHT and estrogens. At normal physiologic levels of testosterone, androgen receptors are saturated, and it is believed that some of the effects of AASs may be through one or more different mechanisms. Research has shown an antagonist effect at the glucocorticoid receptor at supraphysiologic levels that leads to an antianabolic effect.

Glucocorticoids influence glucose synthesis and protein catabolism. The stimulation of glucocorticoid receptors by glucocorticoids leads to increased protein breakdown in muscle. High-dose AASs may displace glucocorticoids from the glucocorticoid receptor and inhibit muscle protein breakdown that leads to an overall anabolic or muscle-building effect. By competing with glucocorticoids for the glucocorticoid receptor, AASs block the depressed protein synthesis that usually occurs during stressful training. AASs also exert some effect on the growth hormone (GH)-insulin-like growth factor (IGF)-1 axis. There seems to be an androgen-induced stimulation of GH secretion and a direct stimulation of hepatic IGF-1 synthesis. IGF-I stimulates skeletal muscle formation, and GH exhibits anabolic effects. AASs act on osteoblasts to stimulate proliferation and differentiation that may inhibit bone breakdown. There also may be some degree of “placebo effect” that allows AAS users to train harder and increase muscle mass as a result of the increased aggression, euphoria, and decreased fatigue and recovery time that many AAS users report [07058].

**Anticorticoid effects**
The supraphysiologic doses of AAS used by athletes reduce the catabolic effects of endogenous cortisol, which is increased during training, by competitive inhibition of cortisol binding to the glucocorticoid receptor. This helps to preserve muscle mass by inhibiting glucocorticoid stimulation of muscle glycogen breakdown and gluconeogenesis. AAS also increase 2, 3-diphosphoglycerate concentration which decreases hemoglobin-oxygen affinity, facilitating release of oxygen to the tissues [07002].

**Commonest AASs in use worldwide, according to main effect [11028]**

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Brand name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Testosterone-like effect</strong></td>
<td></td>
</tr>
<tr>
<td>Testosterone esters: cypionate</td>
<td>Deposteron®, Testex Leo®</td>
</tr>
<tr>
<td>Testosterone esters: undecanoate</td>
<td>Nebido®, Androxon®</td>
</tr>
<tr>
<td>Testosterone esters: blends</td>
<td>Durateston®, Testoviron®, Sustanon®, Omnadren®</td>
</tr>
<tr>
<td>Methyltestosterone</td>
<td>Methyltestosterone®, Metandren®</td>
</tr>
</tbody>
</table>
Anabolic androgenic steroids, a class of steroid hormones related to testosterone, are natural ligands of androgen receptor (AR), a member of the nuclear receptor superfamily of ligand-activated transcription factors. AR binds specific DNA elements, known as androgen-response elements. Testosterone, the main male sexual hormone, binds AR directly and indirectly, through conversion into dihydrotestosterone (DHT), its more active metabolite. The effects of androgens can differ depending on the target cells and/or tissues. To gain insight into transcription activation mechanisms of AR, it was investigated AR protein signaling in human peripheral blood lymphocytes treated with supraphysiological doses of DHT. It was performed a comparative proteomic analysis and was identified about 30 differentially expressed proteins. At least five species contained a consensus androgen-response

<table>
<thead>
<tr>
<th>Steroid Name</th>
<th>Trade Names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methandrostenedolone</td>
<td>Dianabol®, Anabol®, Naposim®</td>
</tr>
<tr>
<td>Chlorodehydromethyltestosterone</td>
<td>Turinabol®</td>
</tr>
<tr>
<td>Fluoxymesterone</td>
<td>Halotestin®</td>
</tr>
<tr>
<td>Boldenone</td>
<td>Equipoise®, Equilon®</td>
</tr>
</tbody>
</table>

**DHT-like effect**

<table>
<thead>
<tr>
<th>Steroid Name</th>
<th>Trade Names</th>
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</thead>
<tbody>
<tr>
<td>Stanozolol</td>
<td>Winstrol®, Stromba®</td>
</tr>
<tr>
<td>Oxandrolone</td>
<td>Anavar®</td>
</tr>
<tr>
<td>Oxymetholone</td>
<td>Anadrol®, Hemogenin®, Anapolon®</td>
</tr>
<tr>
<td>Mesterolone</td>
<td>Proviron®</td>
</tr>
<tr>
<td>Methenolone</td>
<td>Primobolan®</td>
</tr>
</tbody>
</table>

**Nandrolone-like effect**

<table>
<thead>
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<th>Steroid Name</th>
<th>Trade Names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nandrolone decanoate</td>
<td>Decadurabolin®</td>
</tr>
<tr>
<td>Nandrolone phenylpropionate</td>
<td>Durabolin®</td>
</tr>
<tr>
<td>Trenbolone</td>
<td>Finaplix®, Parabolan®</td>
</tr>
<tr>
<td>Nandrolone undecanoate</td>
<td>Dynabolon®</td>
</tr>
</tbody>
</table>

Commonly abused anabolic-androgenic steroids [07031]

**Oral preparations**

- Fluoxymesterone (Halotestin)
- Mesterolone (Proviron)
- Methandienone (Dianabol)
- Methyltestosterone (Virilon)
- Mibolerone (Cheque)
- Oxandrolone (Anavar, Oxandrin)
- Oxymetholone (Anadrol)
- Stanozolol (Winstrol)

**Intramuscular preparations**

- Boldenone undecylenate (Equipoise)
- Methenolone enanthate (Primobolan)
- Nandrolone decanoate (Deca Durabolin)
- Nandrolone phenylpropionate (Durabolin)
- Testosterone cypionate (Depotest)
- Testosterone enanthate (Andro-Estro)
- Testosterone propionate (Testex)
- Trenbolone acetate (Finajet)

**Molecular function**

Anabolic androgenic steroids, a class of steroid hormones related to testosterone, are natural ligands of androgen receptor (AR), a member of the nuclear receptor superfamily of ligand-activated transcription factors. AR binds specific DNA elements, known as androgen-response elements. Testosterone, the main male sexual hormone, binds AR directly and indirectly, through conversion into dihydrotestosterone (DHT), its more active metabolite. The effects of androgens can differ depending on the target cells and/or tissues. To gain insight into transcription activation mechanisms of AR, it was investigated AR protein signaling in human peripheral blood lymphocytes treated with supraphysiological doses of DHT. It was performed a comparative proteomic analysis and was identified about 30 differentially expressed proteins. At least five species contained a consensus androgen-response
elements sequence in the promoter region of related coding genes. The analysis also revealed that high doses of DHT activate the drug detoxification process, could stimulate an increase in cell motility and exert a prosurvival effect rather than an apoptotic one [10324]

**Physiological cellular effects of androgens**

Androgens promote anabolism in the musculoskeletal system while generally repressing adiposity, leading to lean body composition. Circulating androgens decline with age, contributing to frailty, osteoporosis, and obesity, however the mechanisms by which androgens modulate body composition are largely unknown. Here we demonstrate that aged castrated rats develop increased fat mass, reduced muscle mass and strength, and lower bone mass. Treatment with testosterone or 5alpha-dihydrotestosterone (DHT) reverses the effects on muscle and adipose tissues while only aromatizable T increased bone mass. During the first week, DHT transiently increased soleus muscle nuclear density and induced expression of insulin-like growth factor-1 (IGF-1) and its splice variant mechano growth factor (MGF) without early regulation of the myogenic factors MyoD, myogenin, MNF, or myostatin. A genome-wide microarray screen was also performed to identify potential pro-myogenic genes that respond to androgen receptor activation in vivo within 24 hours. Of 24,000 genes examined, 70 candidate genes were identified whose functions suggest initiation of remodeling and regeneration, including the type II muscle genes for myosin heavy chain II and parvalbumin and the chemokine MCP-1. Interestingly, Axin and Axin2, negative regulators of beta-catenin, were repressed, indicating modulation of the beta-catenin pathway. DHT increased total levels of beta-catenin protein, which accumulated in nuclei in vivo. Likewise, treatment of C2C12 myoblasts with both IGF-1Ea and MGF c-terminal peptide increased nuclear beta-catenin in vitro. Thus it was propose that androgenic anabolism involves downregulation of Axin, and induction of IGF-1, leading to nuclear accumulation of beta-catenin, a pro-myogenic, anti-adipogenic stem cell regulatory factor [09053].

**Episodical secretion**

Testosterone and cortisol respond to exercise stimuli and modulate adaptation. Episodic basal secretion of these hormones may modify the responsiveness of these hormones. It was attempted to identify episodic steroid secretion via frequent salivary sampling and investigate any interaction between ultradian rhythmicity and induced changes in testosterone. Salivary testosterone and cortisol concentrations of seven males (age 20-40 years) were measured every 10 min between 0800 and 1600 h on three consecutive days. On either the second or third day, three interventions designed to elicit a hormonal response were randomly assigned: sprint exercise (two 30-s maximal efforts on a cycle ergometer); boxing (two 30-s maximal punching efforts); and a violent video game (10 min of player vs. player combat). On the other days subjects were inactive. Testosterone data on non-intervention days suggested pulsatile secretion with a pulse interval of 47 ± 9 min (mean ± SD). The sprint intervention substantially affected hormones: it elicited a small transient elevation in testosterone (by a factor of 1.21; factor 90 % confidence limits x/ divided by 1.21) 10 min after exercise, and a moderate elevation in cortisol peaking 50 min post-exercise (factor 2.3; x/ divided by 2.6). The testosterone response correlated significantly with the change in testosterone concentration in the 10 min prior to the sprint and with a measure of randomness in testosterone fluctuations. Thus, the salivary testosterone response to exercise may be dependent on the underlying ultradian rhythm and aspects of its regulation. This interaction may have important implications for adaptation to exercise [10326].
Circadian rhythm

Diurnal variation of sports performance usually peaks in the late afternoon, coinciding with increased body temperature. This circadian pattern of performance may be explained by the effect of increased core temperature on peripheral mechanisms, as neural drive does not appear to exhibit nycthemeral variation. This typical diurnal regularity has been reported in a variety of physical activities spanning the energy systems, from Adenosine triphosphate-phosphocreatine (ATP-PC) to anaerobic and aerobic metabolism, and is evident across all muscle contractions (eccentric, isometric, concentric) in a large number of muscle groups. Increased nerve conduction velocity, joint suppleness, increased muscular blood flow, improvements of glycogenolysis and glycolysis, increased environmental temperature, and preferential meteorological conditions may all contribute to diurnal variation in physical performance. However, the diurnal variation in strength performance can be blunted by a repeated-morning resistance training protocol. Optimal adaptations to resistance training (muscle hypertrophy and strength increases) also seem to occur in the late afternoon, which is interesting, since cortisol and, particularly, testosterone (T) concentrations are higher in the morning. T has repeatedly been linked with resistance training adaptation, and higher concentrations appear preferential. This has been determined by suppression of endogenous production and exogenous supplementation. However, the cortisol (C)/T ratio may indicate the catabolic/anabolic environment of an organism due to their roles in protein degradation and protein synthesis, respectively. The morning elevated T level (seen as beneficial to achieve muscle hypertrophy) may be counteracted by the morning elevated C level and, therefore, protein degradation. Although T levels are higher in the morning, an increased resistance exercise-induced T response has been found in the late afternoon, suggesting greater responsiveness of the hypothalamo-pituitary-testicular axis then. Individual responsiveness has also been observed, with some participants experiencing greater hypertrophy and strength increases in response to strength protocols, whereas others respond preferentially to power, hypertrophy, or strength endurance protocols dependent on which protocol elicited the greatest T response. It appears that physical performance is dependent on a number of endogenous time-dependent factors, which may be masked or confounded by exogenous circadian factors. Strength performance without time-of-day-specific training seems to elicit the typical diurnal pattern, as does resistance training adaptations. The implications for this are athletes are advised to coincide training times with performance times, and individuals may experience greater hypertrophy and strength gains when resistance training protocols are designed dependent on individual T response [10054].

Testosterone and cortisol respond to exercise stimuli and modulate adaptation. Episodic basal secretion of these hormones may modify the responsiveness of these hormones. We sought to identify episodic steroid secretion via frequent salivary sampling and investigate any interaction between ultradian rhythmicity and induced changes in testosterone. Salivary testosterone and cortisol concentrations of seven males (age 20-40 years) were measured every 10 min between 0800 and 1600 h on three consecutive days. On either the second or third day, three interventions designed to elicit a hormonal response were randomly assigned: sprint exercise (two 30-s maximal efforts on a cycle ergometer); boxing (two 30-s maximal punching efforts); and a violent video game (10 min of player vs player combat). On the other days subjects were inactive. Testosterone data on non-intervention days suggested pulsatile secretion with a pulse interval of mean 47 ± 9 min. The sprint intervention substantially affected hormones: it elicited a small transient elevation in testosterone (by a factor of 1.21) 10 min after exercise, and a moderate elevation in cortisol peaking 50 min post-exercise (factor 2.3). The testosterone response correlated with the change in testosterone concentration in the 10 min prior to the sprint and with a measure of randomness in testosterone fluctuations. Thus, the salivary testosterone response to
exercise may be dependent on the underlying ultradian rhythm and aspects of its regulation. This interaction may have important implications for adaptation to exercise [10055].

Testosterone is the principal androgenic steroid produced by the testes. Testosterone is also a precursor to estrogen synthesis by the ovary in women. Steroids are hormones derived from cholesterol, and androgens promote the development and maintenance of male characteristics.. Many androgen actions in the body are mediated by binding to the androgen receptor, a nuclear receptor that modulates transcription of responsive genes. Whether of endogenous or exogenous origin in males and in females, excess testosterone creates an advantage in sports. While the anabolic effects of testosterone in hypogonadal males were well-accepted, early studies testing the effects of testosterone supplementation to eugonadal men were not well-controlled. More recent studies have shown that testosterone stimulates muscle mass and reduces body fat. Androgens likely also act on specific substrates in the brain to increase aggression and motivation for competition. Exogenous testosterone has been banned from Olympic competition since 1976, and was classified in the United States as a controlled substance by the Anabolic Steroid Control Act of 1990. Defining the upper limit for endogenous testosterone is complicated by the dynamic changes in testosterone across a number of temporal scales. On the shortest time-scale, testosterone production in the gonads follows the pulsatile release of luteinizing hormone. This introduces a level of unpredictability for estimating circulating androgen concentrations in any single biologic sample. Secondly, in both sexes, testosterone follows a diurnal rhythm with peak concentrations in the morning followed by progressive decline over the course of the day, rising again at night during sleep. In women, there is evidence that testosterone concentrations also vary as a function of the menstrual cycle, with peak testosterone concentrations in the peri-ovulatory window, and lower values in the early follicular and late luteal phases. On a somewhat longer time-scale, testosterone concentrations exhibit circannual variation and peak in the fall. Lastly, men's testosterone concentrations slowly decline over the lifespan while women face an abrupt decline in testosterone at menopause. Due to the dynamic regulation of endogenous testosterone production, including the acute effects of competition and exercise, testosterone concentrations may vary considerably within and among individuals. Accordingly, it has been difficult to establish a threshold separating endogenous testosterone from exogenous sources. Furthermore, disorders of sexual differentiation (DSD) can produce elevated concentrations of endogenous androgens, potentially creating a competitive advantage for female athletes with DSD. Due to variability in endogenous secretion, and similarities with exogenous testosterone, it has been challenging to establish allowable limits for testosterone in competition. Endogenous androgen production is dynamically regulated by both exercise and winning in competition. Furthermore, testosterone may promote athletic performance, not only through its long-term anabolic actions, but also through rapid effects on behavior [12100].

**Endogenous steroids**

The detection of misuse with naturally occurring steroids is a great challenge for doping control laboratories. Intake of natural anabolic steroids alters the steroid profile. Thus, screening for exogenous use of these steroids can be established by monitoring a range of endogenous steroids, which constitute the steroid profile, and evaluate their concentrations and ratios against reference ranges. Elevated values of the steroid profile constitute an atypical finding after which a confirmatory IRMS procedure is needed to unequivocally establish the exogenous origin of a natural steroid. However, the large inter-individual differences in urinary steroid concentrations and the recent availability of a whole range of natural steroids (e.g. dehydroepiandrosterone and androstenedione) which each exert a different effect on the monitored parameters in doping control complicate the interpretation of
the current steroid profile. The screening of an extended steroid profile can provide additional parameters to support the atypical findings and can give specific information upon the steroids which have been administered. The natural concentrations of 29 endogenous steroids and 11 ratios in a predominantly Caucasian population of athletes were determined. The upper reference values at 97.5 percent, 99 percent and 99.9 percent levels were assessed for male (n=2027) and female (n=1004) populations. Monitoring minor metabolites and evaluation of concentration ratios with respect to their natural abundances could improve the interpretation of the steroid profile in doping analysis [09052].

The detection of the administration of an androgen such as testosterone that could be present normally in human bodily fluids is based upon the methodical evaluation of key parameters of the urinary profile of steroids, precisely measured by GC/MS. Over the years, the markers of utilization were identified, the reference ranges of diagnostic metabolites and ratios were established in volunteers and in populations of athletes, and their stability in individual subjects were studied. The direct confirmation comes from the measurement of delta $^{13}$C values reflecting their synthetic origin, ruling out a potential physiological anomaly. Several factors may alter the individual GC/MS steroid profile besides the administration of a testosterone-related steroid, the nonexhaustive list ranging from the microbial degradation of the specimen, the utilization of inhibitors of 5alpha-reductase or other anabolic steroids, masking agents such as probenecid, to inebriating alcohol drinking. The limitation of the testing strategy comes from the potentially elevated rate of false negatives, since only the values exceeding those of the reference populations are picked up by the GC/MS screening analyses performed by the laboratories on blind samples, excluding individual particularities and subtle doping. Since the ranges of normal values are often described from samples collected in Western countries, extrapolating data to all athletes appears inefficient. Furthermore, with short half-life and topical formulations, the alterations of the steroid profile are less pronounced and disappear rapidly. GC/C/IRMS analyses are too delicate and fastidious to be considered for screening routine samples. An approach based upon the individual athlete's steroid profiling is necessary to pick up variations that would trigger further IRMS analysis and investigations [10090].

One review attempted to give a synopsis of the major aspects concerning the biochemistry of endogenous androgens, supplemented with several facets of physiology, particularly with respect to testosterone. Knowledge regarding the precursors and metabolism of endogenous testosterone is therefore fundamental to understanding many of the issues concerning doping with testosterone and its prohormones, including the detection of their administration. Further, adverse findings for nandrolone are frequent, but this steroid and 19-norandrosterone are also produced endogenously, an appealing hypothesis being that they are minor by-products of the aromatization of androgens. At sports tribunals pertaining to adverse analytical findings of natural androgen administration, experts often raise issues that concern some aspect of steroid biochemistry and physiology. Salient topics included within this review are the origins and interconversion of endogenous androgens, the biosynthesis of testosterone and epitestosterone, the mechanism of aromatization, the molecular biology of the androgen receptor, the hypothalamic-pituitary-testicular axis, disturbances to this axis by anabolic steroid administration, the transport (binding) of androgens in blood, and briefly the metabolism and excretion of androgens [10091].

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concentrations and the recent availability of a whole range of natural steroids (e.g., dehydroepiandrosterone and androstenedione) which each exert a different effect on the monitored parameters in doping control complicate the interpretation of the current steroid profile. The screening of an extended steroid profile can provide additional parameters to support the atypical findings and can give specific information upon the steroids which have been administered. The natural concentrations of 29 endogenous steroids and 11 ratios in a predominantly Caucasian population of athletes were determined. The upper reference values at 97.5 percent, 99 percent and 99.9 percent levels were assessed for male (n=2027) and female (n=1004) populations. Monitoring minor metabolites and evaluation of concentration ratios with respect to their natural abundances could improve the interpretation of the steroid profile in doping analysis [10092].

By means of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) urinary steroids obtained from a reference population of 56 subjects were analyzed for their $^{13}$C/$^{12}$C-ratios. The analytes encompassed androsterone (A), etiocholanolone (E), 11beta-hydroxyetiocholanolone (OHE), 11beta-hydroxyandrosterone (OHA), and 5beta-pregnane-3alpha,20alpha-diol (PD). A and E represent androgen metabolites (AM). PD, OHE, and OHA have sources independent from androgen metabolism. The delta(13)C-values of the latter compounds may be compared to those of AM in order to detect doping with synthetic androgens and thus may serve as endogenous reference compounds (ERC). In order to allow for classification of conspicuous samples, reference ranges and limits were calculated for delta$^{13}$C-values of selected steroids and differences hereof (Delta$^{13}$C-values). When A is compared to ERCs, Delta$^{13}$C-values larger than 3 per thousand are very unlikely. A set of additional parameters was surveyed by a questionnaire. Several factors turned out to exert significant influence on the delta$^{13}$C-values of urinary steroids. These encompass the identity of the steroid itself, gender, oral contraception, travels, and physical activity [07059].

**Effect of smoking**

Cigarette tobacco smoke is a potent environmental contaminant known to adversely affect health including fertility and pregnancy. To examine the associations between second-hand cigarette tobacco-smoke exposure, or active smoking and serum concentrations of steroid hormones using tandem mass spectrometry. Healthy women (18-45 years) from the general community in the Metropolitan Washington, DC were recruited at the follicular stage of their menstrual cycle. Participants were assigned to one of three study groups: active smokers (n=107), passive smokers (n=86), or non-smokers (n=100). Classifications were based on a combination of self-reporting and serum cotinine concentrations. Serum androgens, estrogens, progestins, androstenedione, aldosterone, cortisol, corticosterone, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), 11-deoxycorticisol and 25-hydroxy-vitamin D3 (25-OHvitD3) and cotinine were measured by isotope dilution tandem mass spectrometry (LC/MS/MS) (API-5000). Serum estrone, estradiol, and estriol concentrations were lower in active and passive smokers than in non-smokers. The three study groups differed significantly in serum concentrations of 16-OHE1, aldosterone and 25-OHVitD3, as well as in the ratios of many of the steroids. Pair-wise comparison of the groups demonstrated significant differences in hormone concentrations between smokers and non-smokers for aldosterone; passive smokers and non-smokers for aldosterone, progesterone and estriol. Moreover, for smokers and passive smokers, there were no significant differences in these hormone concentrations. It was concluded that smoke exposure was associated with lower than normal median steroid hormone concentrations. These processes may be instrumental in explaining some adverse effects of tobacco smoke on female health and fertility [11070].
Effects of dietary components on testosterone metabolism

The potential interference in testosterone metabolism through ingested substances has ramifications for:

- a range of pathologies such as prostate cancer
- medication contra-indications
- disruption to the endocrine system
- potential confounding effects on doping tests

Conjugation of anabolic steroids during phase II metabolism, mainly driven by UDP-glucuronosyltransferase (UGT) 2B7, 2B15, and 2B17, has been shown to be impaired in vitro by a range of compounds including xenobiotics and pharmaceuticals. Following early reports on the effects of a range of xenobiotics on UGT activity in vitro, the work was extended to reveal similar effects with common non-steroidal anti-inflammatory drugs. Notably, recent studies have evidenced inhibitory effects of the common foodstuffs green tea and red wine, along with their constituent flavonoids and catechins. This review amalgamates the existing evidence for the inhibitory effects of various pharmaceutical and dietary substances on the rate of UGT glucuronidation of testosterone; and evaluates the potential consequences for health linked to steroid levels, interaction with treatment drugs metabolized by the UGT enzyme and steroid abuse in sport [13209].

As a major route for excretion of exogenous and endogenous compounds, there is considerable interest in the roles of the UDP-glucuronosyltransferase (UGT) family, which has led to widespread investigations of their potential effects in health and disease. In particular, genetic and chemical modification of UGT activity relating to steroid metabolism has ramifications for a range of pathologies such as prostate cancer, medication contra-indications, disruption to the endocrine system, and potential confounding effects on doping tests in sport. Therefore, it is timely to review lifestyle factors that affect UGT activity. Variations in the activity of UGT isozymes occur as a result of gender and ethnic origins giving different levels of expression of UGT forms and altered ratios of testosterone/testosterone excreted in urine). In addition to genetic variations, from a steroid metabolism viewpoint, one current focus of investigation is on the regulation of specific UGT activity via induction or inhibition by exogenous compounds such as pharmaceuticals and dietary components. Several reports show induction of UGT activity by a range of compounds including phytochemicals and pharmaceuticals. Early studies reported the effects of drugs and dietary compounds on UGT activity in isolated microsomes or in rats without detailing the specific UGT isozymes involved. Liver microsomal glucuronidation of estradiol and estrone was inhibited by green and black teas, along with a constituent catechin [(-)-epigallocatechin gallate] and several flavonoids (kaempferol, quercetin, rutin, flavone, naringenin, hesperetin). Green tea polyphenols had a strong inhibitory effect of glucuronidation in vitro and showed a small increase in liver glucuronidation activity against estrone and estradiol was observed in vitro in rats with green tea as the sole fluid source. Consequent alterations in steroid metabolism have been debated to have a range of putative effects including varying responses to doping tests, inter-medication interactions, and susceptibility to developing cancer. From a treatment perspective, the roles of common compounds, including dietary components have been investigated as UGT inhibitors with a view to enhancing bioavailability of drugs. This approach to impairing metabolism and thus increasing the half-lives of drugs has been the subject of patent protection for a wide range of drugs (raloxifene, 2-methoxyestradiol, irinotecan, estradiol, labetalol, dilevalol, zidovudine,
and morphine) using numerous inhibitors from plant origin (epicatechin gallate, epigallocatechin gallate, octyl gallate, propyl gallate, quercetin, tannic acid, benzoin gum, capsaicin, dihydrocapsaicin, eugenol, gallocatechin gallate, geraniol, menthol, menthyl acetate, naringenin, allspice berry oil, N-vanillylnonanamide, clovebud oil, peppermint oil, silybinin, and silymarin) [13210].

Early reports demonstrated that a number of compounds interfere with the activity of UGT2B17 which is the major isozyme for clearance of anabolic steroids, having greater than double the activity of the next most active form UGT2A1. It has been reported that epitestosterone and two non-steroidal anti-inflammatory drugs (NSAID) act as competitive inhibitors against UGT2B17. Using human microsomes and recombinant enzymes they demonstrated that diclofenac and ibuprofen inhibited testosterone glucuronidation without having significant effects on epitestosterone glucuronidation. Similar inhibitory effects on testosterone glucuronidation were reported for both UGT2B15 and UGT2B17 isozymes in *in vitro* studies. The authors measured IC$_{50}$ values for diclofenac inhibition of testosterone glucuronidation by UGT2B15 and UGT2B17 of 25 microM and 65 microM respectively, at testosterone concentrations of 10 μM. The corresponding IC$_{50}$ values for ibuprofen were 121 μM and 1340 microM against UGT2B15 and UGT2B17 respectively. To date, no commensurate studies have been reported demonstrating an effect of pharmaceuticals on testosterone glucuronidation *in vivo*. A recent report showed only a slight modification but no significant effects of concomitant use of maximum recommended doses of ibuprofen or diclofenac with testosterone on the urinary ratios of testosterone/epitestosterone in individuals with either two, one, or no allele of the UGT2B17, and no effect when ibuprofen/diclofenac was administered prior to single dose of testosterone. Given the competitive nature of the inhibition, at least for diclofenac, the experiment was limited by restriction to maximum doses of the NSAID. Thus, doses of 50 mg × 3 per day of the single competitive inhibitor, although well reasoned, may not elicit an inhibitory effect given that ibuprofen can also elevate UGT enzyme activity *in vivo*. Although reports of *in vivo* studies are lacking to date, the potential effects of inhibiting major testosterone-metabolizing enzymes warrants further exploration, especially if common substances are considered where maximum dosage effects do not limit intake. From one standpoint, this effect could alter the results of a doping test which is based on the ratio of the glucuronidated testosterone and epitestosterone. Following these advances, researchers have recently explored the effects of dietary components on steroid metabolism [13210].

**Tea and cacao**

It was first reported the effects of dietary green and white teas on the activity of UGT2B17 toward testosterone glucuronidation. Using an high performance liquid chromatography (HPLC) assay, testosterone glucuronidation was monitored in the presence of tea extracts using human UGT2B17 supersomes. Under the conditions studied, green and white tea preparations inhibited the reaction by about 20 percent with a white tea powder inhibiting glucuronidation by 30 percent. HPLC analysis of the teas revealed key constituents such as epicatechin (EC) and epigallocatechin gallate (EGCG). Analysis via a Dixon plot revealed that EGCG was acting as a competitive inhibitor with an IC$_{50}$ value of 64 microM which equaled that found previously for diclofenac. At a concentration of 1 mM, EC inhibited testosterone (at 10 microM) glucuronidation by some 55 percent [13210].

Cacao also inhibits UGT2B17 but to a lesser extent (15 %). Under these conditions, at testosterone concentrations of 12 microg/mL, white and green tea preparations inhibited glucuronidation by 30 percent. Under the conditions studied, green and white tea preparations inhibited the reaction by about 20 percent with a white tea powder inhibiting glucuronidation by 30 percent. HPLC analysis of the teas revealed key constituents such as epicatechin (EC) and epigallocatechin gallate (EGCG). Analysis via a Dixon plot revealed that EGCG was acting as a competitive inhibitor with an IC$_{50}$ value of 64 microM which equaled that found previously for diclofenac. At a concentration of 1 mM, EC inhibited testosterone (at 10 microM) glucuronidation by some 55 percent [13210].
epigallocatechin gallate (78 %), and catechin gallate (90 %). Analysis of the tea and cacao samples by HPLC revealed catechins were present in these samples at lower levels in comparison to the tea samples. The cacao samples, whilst inhibiting testosterone glucuronidation, did so at a much lesser rate than the tea samples which could be linked with having lower levels of inhibiting catechin compounds at the same concentrations of the tea samples [13210].

Red wine
Red wine and its constituents were shown to inhibit testosterone glucuronidation by human UGT2B17 supersomes. Under the conditions studied, red wine inhibited glucuronidation by up to 70 percent over a 2-h period, with little effect arising from the alcohol content. Phenolic components were selected following HPLC analysis of the selected red wine and quercetin, caffeic acid, and gallic acid inhibited UGT2B17 testosterone glucuronidation by 72, 22, and 9 percent respectively, with concentrations of phenolic : testosterone of 100 : 250 microM. For the most active phenolic, reducing the quercetin concentration to 2 microM, maintained inhibition of 20 percent in spite of the 10-fold excess of testosterone [13201].

Testosterone plus an ornithine decarboxylase inhibitor

Because of its anabolic effects on muscle, testosterone is being explored as a function-promoting anabolic therapy for functional limitations associated with aging; however, concerns about testosterone's adverse effects on prostate have inspired efforts to develop strategies that selectively increase muscle mass while sparing the prostate. Testosterone's promyogenic effects are mediated through upregulation of follistatin. It was shown that the administration of recombinant follistatin (rFst) increased muscle mass in mice, but had no effect on prostate mass. Consistent with the results of rFst administration, follistatin transgenic mice with constitutively elevated follistatin levels displayed greater muscle mass than controls, but had similar prostate weights. To elucidate signaling pathways regulated differentially by testosterone and rFst in prostate and muscle, we performed microarray analysis of mRNAs from prostate and levator ani of castrated male mice treated with vehicle, testosterone, or rFst. Testosterone and rFst shared the regulation of many transcripts in levator ani; however, in prostate, 593 transcripts in several growth-promoting pathways were differentially expressed after testosterone treatment, while rFst showed a negligible effect with only 9 transcripts differentially expressed. Among pathways that were differentially responsive to testosterone in prostate, we identified ornithine decarboxylase (Odc1), an enzyme in polyamine biosynthesis, as a testosterone-responsive gene that is unresponsive to rFst. Accordingly, we administered testosterone with and without α-difluoromethylornithine (DFMO), an Odc1 inhibitor, to castrated mice. DFMO selectively blocked testosterone's effects on prostate, but did not affect testosterone's anabolic effects on muscle. Co-administration of testosterone and Odc1 inhibitor presents a novel therapeutic strategy for prostate-sparing anabolic therapy [13211].

Influence of alcohol on steroid metabolism

Besides the (mis)use of natural steroids, the impact of ethanol consumption on steroid profiles was subjected to further investigations. In a comprehensive study with 21 male and 15 female volunteers, alterations in steroid profile parameters were correlated with urinary ethanol-glucuronide and ethanol-sulfate concentrations, and threshold values of 48 microg/ml and 15.5 microg/ml for men and women, respectively, were suggested. When exceeded, an
influence on urinary steroid profiles due to ethanol-induced suppression of steroid biotransformation processes should be considered during data interpretation [13012].

Salivary hormones

Saliva contains cells and compounds, of local and non-local oral origin, namely inorganic, organic non-protein, protein/polypeptide, and lipid molecules. Moreover, some hormones, commonly assayed in plasma, such as steroids, are detectable in oral fluid and peptide/protein, and non-steroid hormones have been investigated. The sports practice environment and athletes’ availability, together with hormone molecule characteristics in saliva and physical exercise behavior effects, confirm this body fluid as an alternative to serum. One review focused on the relation between salivary steroids and psychophysiological stress and underlines how the measurement of salivary cortisol provides an approach of self-report psychological indicator and anxiety change in relation to exercise performance. The correlation between salivary and plasma steroid hormone (cortisol, testosterone, and dehydroepiandrosterone (DHEA)) levels, observed during exercise, has been considered, underlining how the type, duration, and intensity of the exercise influence the salivary steroid concentrations in the same way as serum-level variations. Training conditions have been considered in relation to the salivary hormonal response. One review focuses on studies related to salivary hormone measurements, mainly steroids, in physical exercise. Saliva use in physical disciplines, as a real alternative to serum, could be a future perspective [11068]

The combination of resistance and plyometric training, or complex training, may yield greater functional gains than either method alone. As steroid hormones respond to exercise stimuli and modulate the functional outcomes, it is possible that complex training creates an enhanced anabolic physiological milieu for adaptation. It was investigated acute responses of salivary testosterone and cortisol to complex exercise bouts. After a standardized warm-up, 16 semiprofessional rugby players performed 1 of 4 exercise bouts in a cross-over manner: power-power; power-strength; strength-power; or strength-strength. Each player completed each of the 4 bouts twice over a 4-week period in a balanced random order such that each player performed a total of 8 bouts. The power block consisted of 3 sets of 3 repetitions of jump squat exercise at 50 percent of 1-repetition maximum load. The strength block consisted of three sets of three repetitions of box squat exercise at a 3-repetition maximum load. There were 3-minute rest periods between sets and 4-minute rest periods between exercise blocks. Saliva was sampled before, during, and immediately after the exercise bout. The greatest overall hormonal responses were a small increase in testosterone (13 %; 90 % confidence limits ± 7 %) and a trivial increase in cortisol (27 %; ± 30 %) after the strength-power bout. A clear difference was observed between the strength-power and the power-power bouts immediately after exercise for testosterone (10 %; ± 8 %) and cortisol (29 %; ± 17 %). The preceding exercise block had little effect on subsequent strength and power performance. The hormonal response after the strength-power bout suggests that this exercise sequence provides an enhanced anabolic milieu for adaptation [11069].

Salivary testosterone

The aim of one study was to examine the acute response to plasma and salivary cortisol and testosterone to three training protocols. Ten trained endurance athletes participated in three experimental trials, such as interval training (INT), tempo run (TEMP) and bodyweight-only circuit training (CIR), on separate days. Blood and saliva samples were collected pre- and 0, 15, 30 and 60 min post-exercise. Peak post-exercise salivary cortisol was higher than pre-
exercise in all trials. After INT, salivary cortisol remained elevated above pre-exercise than 60 min post-exercise. Salivary testosterone also increased post-exercise in all trials. Plasma and salivary cortisol were correlated between individuals and within individuals. Plasma and salivary testosterone was also correlated between and within individuals.Peak cortisol and testosterone levels occurred simultaneously in plasma and saliva, but timing of post-exercise hormone peaks differed between trials and individuals. Further investigation is required to identify the mechanisms eliciting an increase in hormones in response to CIR. Furthermore, saliva is a valid alternative sampling technique for measurement of cortisol, although the complex, individual and situation dependent nature of the hormone response to acute exercise should be considered [13212].

Salivary testosterone (T) and cortisol (C) concentrations were monitored across a sports competition. Data were compared using two enzyme-immunoassay (EIA) methods and two sample preparations to determine their influence on hormone concentrations. A group of male athletes (n=19) provided a saliva sample the morning before and one day after (24h post) an international rugby union match. Following an extraction procedure, the samples were analysed for T and C concentrations using a commercial kit (CM(E)) and an in-house method (IH(E)). Raw samples (no extraction procedure) were also tested using the commercial kit (CM(R)). There were no significant changes in T and C levels from pre to post competition with each EIA method and sample preparation, but significant differences in T (IH(E)>CM(E)>CM(R)) and C (CM(R)>IH(E) and CM(E)) concentrations were seen when both samples were pooled. Bland-Altman analyses confirmed the presence of fixed and proportional bias. Strong and significant correlations were demonstrated between the IH(E) and CM(E) measures of salivary T and C. The T and C values from the raw and extracted samples were also strongly correlated. The measurement of salivary T and C concentrations across an international sports event was influenced by different EIA methods and sample preparations, but all measures were strongly correlated with some bias. Both T and C were unresponsive to the sports event, but within the group results large individual variation was seen [13213].

One study examined salivary cortisol and testosterone responses to two, different high-intensity, about 30-min cycles separated by 2 h rest before and after an 11-day intensified training period. Twelve recreationally active, healthy males completed the study. Saliva samples were collected before, immediately after and 30 min after both bouts with salivary cortisol and testosterone concentrations assessed. Compared with pre-training blunted exercise-induced salivary cortisol, testosterone and cortisol/testosterone responses to both bouts post-training were observed. Comparing pre- with post-training the absolute exercise-induced salivary cortisol, testosterone and cortisol/testosterone decreased from 11.1 to 3.1 and 7.0 to 4.4 nmol/L (cortisol), from 407 to 258 and from 473 to 274 pmol/L (testosterone) and from 12 to 4 and 7 to 5 (cortisol/testosterone) for the first and second bouts, respectively. No differences in the pre- and post-training rating of perceived exertion (RPE) and heart rate (HR) responses during the cycles or times to fatigue were found. Fatigue and Burnout scores were higher post- compared with pre-training. These high-intensity exercise bouts can detect altered hormonal responses following intensified training. This test could assess an athlete’s current hormonal status, reductions in salivary cortisol and testosterone responses suggestive of increased fatigue [13214].

In soccer
One study investigated the contribution of salivary testosterone concentration, years from peak height velocity (YPHV) and height by body mass interaction on jumping performance (counter movement jump; CMJ) and aerobic fitness (Yo-Yo intermittent endurance test, level 1) in young elite soccer players. Forty-five participants (age: 13 years; body mass: 49 kg, height: 156 cm) belonging to a top level Brazilian soccer club were evaluated at four time
points across a single semester. None of the assessed players had reached PHV. The data from the four evaluations were averaged and multiple linear regression analysis conducted. For CMJ, the model explained 43 percent of the variance; salivary testosterone concentration was the primary contributor and the YPHV contributed 10 percent of the variance. The model explained 29 percent of the variance in Yo-Yo. The salivary testosterone was the primary and single significant contributor. A significant difference was noted between high and low testosterone groups divided a posteriori to CMJ performance. These results suggest an important role for hormonal status in interpreting physical performance in preadolescent soccer players [13215].

Effects on cognitive functions

The illicit use of anabolic androgenic steroids (AAS) has gained popularity among adolescents in the last decade. However, although it is known that exposure to AAS impairs cognition in adult animal models, the cognitive effects during adolescence remain undetermined. An inhibitory avoidance task (IAT) was used to assess the effect of AAS (17alpha-methyltestosterone; 17alpha-meT, 7.5 mg/kg) in male and female periadolescent rats. A single injection of 17α-meT immediately before the footshock produced significant impairment of inhibitory avoidance learning in males but not females. Generalized anxiety, locomotion, and risk assessment behaviors (RAB) were not affected. The results show that exposure to a single pharmacological dose of 17alpha-meT during periadolescence exerts sex-specific cognitive effects without affecting anxiety. Thus, disruption of the hormonal milieu during this early developmental period might have negative impact on learning and memory [13114].

Effects of training

Performing strength exercise, whether acutely or in a training programme, leads to alterations at the hypothalamic-pituitary-testicular and hypothalamic-pituitary-adrenal axes. One way to evaluate these changes is by analysis of the excretion of steroid hormones in the urine. One study determined the variations in the urine profile of glucuroconjugated steroids after a single session of strength exercise and after a 4-week programme of strength training. The subjects were a group (n=20) of non-sportsman male university students who worked out 3 days a week, performing the exercises at 70-75 percent of one repetition maximum strength (1-RM). Four urine samples were collected per subject: (A) before and (B) after a standard session prior to initiating the training programme, and (C) before and (D) after the same standard session at the end of the study, and they were assayed by gas chromatography coupled to mass spectrometry. The concentrations of the different hormones were determined relatively to the urine creatinine level (ng steroid/mg creatinine) to correct for diuresis. After the exercise sessions, both before and after the training programme, there was a fall in the urine excretion of androgens and estrogens, but no statistically significant changes in the excretion of tetrahydrocortisol (THF) and tetrahydrocortisone (THE). The anabolic/catabolic hormones ratio also decreased after the acute session, although only androstenedione + dehydroepiandrosterone (DHEA)/THE + THF ratio had a significant decrease. After the training programme, there was a significant improvement in the strength of the muscle groups studied, and an increased urinary excretion of all the androgens with respect to the initial state of repose, with the difference being significant in the case of epitestosterone. The androsterone (A) + etiocholanolone (E)/THE + THF ratio increased significantly concerning the initial state. It was therefore concluded that subjects suffer variations of the urine profile with regard to the steroid
hormones before and after the acute strength sessions and after the training period. The alteration after the training programme seems to be due to the subjects' hypothalamic-hypophysis-testicular and hypothalamic-pituitary-adrenal axes adaptations, which enable them to increase physical strength [07060].

The purpose of one study was to examine the effects of an 8-week basic training with added strength training or endurance training on both the performance of a 3K-combat loaded run test and the acute neuromuscular and hormonal responses. All training groups significantly improved their run-test times: strength training by 12 percent, endurance training by 12 percent, and normal training by 10 percent. Significant acute decreases were observed in maximal isometric force of leg extensors in all subject groups following the run. Increases were observed in acute testosterone responses after the test in all groups both at pre- and post-training. However, endurance training and normal training demonstrated significantly lower acute post-training serum cortisol responses than strength training. In conclusion, the present results indicate that within a demanding basic training, the added training for endurance training and especially strength training may be compromised in their adaptation potential due to interference from the demands of basic training [10044].

Age-related skeletal muscle loss is thought to stem from suboptimal nutrition and resistance to anabolic stimuli. Impaired microcirculatory (nutritive) blood flow may contribute to anabolic resistance by reducing delivery of amino acids to skeletal muscle. In one study, it was employed contrast-enhanced ultrasound, microdialysis sampling of skeletal muscle interstitium, and stable isotope methodology, to assess hemodynamic and metabolic responses of older individuals to endurance type (walking) exercise during controlled amino acid provision. It was hypothesized that older individuals would exhibit reduced microcirculatory blood flow, interstitial amino acid concentrations, and amino acid transport when compared with younger controls. It was reported that aging induces anabolic resistance following endurance exercise, manifested as reduced (by 40 %) efficiency of muscle protein synthesis. Despite lower (by 40-45 %) microcirculatory flow in the older than in the younger participants, circulating and interstitial amino acid concentrations and phenylalanine transport into skeletal muscle were all equal or higher in older individuals than in the young, comprehensively refuting our hypothesis that amino acid availability limits postexercise anabolism in older individuals. The data point to alternative mediators of age-related anabolic resistance and importantly suggest correction of these impairments may reduce requirements for, and increase the efficacy of, dietary protein in older individuals [10447].

The objectives of one investigation were to study the inflammatory and performance responses after an acute bout of intense plyometric exercise during a prolonged recovery period. Participants were randomly assigned to either an experimental group (P, n=12) that performed intense plyometric exercises or a control group (C, n=12) that rested. The delayed onset of muscle soreness (DOMS), knee range of motion (KROM), creatine kinase (CK) and lactate dehydrogenase (LDH) activities, white blood cell count, C reactive protein (CRP), uric acid (UA), cortisol, testosterone, IL-6, IL-1b strength (isometric and isokinetic), and counter-movement (CMJ) and static (SJ) jumping performance were measured at rest, immediately postexercise and at 24, 48, 72, 96, and 120 hours of recovery. Lactate was measured at rest and postexercise. Strength remained unchanged throughout recovery, but CMJ and SJ declined significantly by 8-20 percent. The experimental group induced a marked rise in DOMS, CK, and LDH (peaked 24-48 hours postexercise) and a KROM decline. An acute-phase inflammatory response consisting of leukocytosis (postexercise and at 24 hours), an IL-6, IL-1b, CRP, and cortisol elevation (during the first 24 hours of recovery) and a delayed increase of UA (peaked at 48 hours) and testosterone (peaked at 72 hours) was observed in P. The results of this investigation indicate that performing an acute bout of intense
plyometric exercise may induce a short-term muscle damage and marked but transient inflammatory responses. Jumping performance seems to deteriorate for as long as 72 hours postexercise, whereas strength appears to remain unchanged. The acute-phase inflammatory response after a plyometric exercise protocol appears to follow the same pattern as in other exercise models. These results clearly indicate the need of sufficient recovery between successive plyometric exercise training sessions [10045].

One study aimed to develop a quantitative and in vivo magnetic resonance imaging (MRI) approach to investigate the muscle growth effects of anabolic steroids. A protocol of MRI acquisition on a standard clinical 1.5 T scanner and quantitative image analysis was established and employed to measure the individual muscle and organ volumes in the intact and castrated guinea pigs undergoing a 16-week treatment protocol by two well-documented anabolic steroids, testosterone and nandrolone, via implanted silastic capsules. High correlations between the in vivo MRI and postmortem dissection measurements were observed for shoulder muscle complex, masseter, temporalis, neck muscle complex, prostate gland and seminal vesicles, and testis. Furthermore, the longitudinal MRI measurements yielded adequate sensitivity to detect the restoration of growth to or towards normal in castrated guinea pigs by replacing circulating steroid levels to physiological or slightly higher levels, as expected. These results demonstrated that quantitative MRI using a standard clinical scanner provides accurate and sensitive measurement of individual muscles and organs, and this in vivo MRI protocol in conjunction with the castrated guinea pig model constitutes an effective platform to investigate the longitudinal and cross-sectional growth effects of other potential anabolic steroids. The quantitative MRI protocol developed can also be readily adapted for human studies on most clinical MRI scanner to investigate the anabolic steroid growth effects, or monitor the changes in individual muscle and organ volume and geometry following injury, strength training, neuromuscular disorders, and pharmacological or surgical interventions [08151].

The purpose of one study was to examine the changes in neuromuscular, perceptual and hormonal measures following professional rugby league matches during different length between-match microcycles. Twelve professional rugby league players from the same team were assessed for changes in countermovement jump (CMJ) performance (flight time and relative power), perceptual responses (fatigue, well-being and muscle soreness) and salivary hormone (testosterone and cortisol) levels during 5, 7 and 9 d between-match training microcycles. All training was prescribed by the club coaches and was monitored using the session-RPE method. Lower mean daily training load was completed on the 5 d compared with the 7 and 9 d microcycles. CMJ flight time and relative power, perception of fatigue, overall well-being and muscle soreness were significantly reduced in the 48 h following the match in each microcycle. Most CMJ variables returned to near baseline values following 4 d in each microcycle. Countermovement jump relative power was lower in the 7 d microcycle in comparison with the 9 d microcycle. There was significantly increased fatigue at 48 h in the 7 and 9 d microcycles but had returned to baseline in the 5 d microcycle. Salivary testosterone and cortisol did not change in response to the match. Neuromuscular performance and perception of fatigue are reduced for at least 48 h following a rugby league match but can be recovered to baseline levels within 4 d. These findings show that with appropriate training, it is possible to recover neuromuscular and perceptual measures within 4 d after a rugby league match [10330].

The combination of resistance and plyometric training, or complex training, may yield greater functional gains than either method alone. As steroid hormones respond to exercise stimuli and modulate the functional outcomes, it is possible that complex training creates an enhanced anabolic physiological milieu for adaptation. It was investigated acute responses of salivary testosterone and cortisol to complex exercise bouts. After a standardized warm-up,
16 semiprofessional rugby players performed 1 of 4 exercise bouts in a cross-over manner: power-power; power-strength; strength-power; or strength-strength. Each player completed each of the 4 bouts twice over a 4-week period in a balanced random order such that each player performed a total of 8 bouts. The power block consisted of 3 sets of 3 repetitions of jump squat exercise at 50 percent of 1-repetition maximum load. The strength block consisted of three sets of three repetitions of box squat exercise at a 3-repetition maximum load. There were 3-minute rest periods between sets and 4-minute rest periods between exercise blocks. Saliva was sampled before, during, and immediately after the exercise bout. The greatest overall hormonal responses were a small increase in testosterone (13 %; 90 % confidence limits ± 7 %) and a trivial increase in cortisol (27 %; ± 30 %) after the strength-power bout. A clear difference was observed between the strength-power and the power-power bouts immediately after exercise for testosterone (10 %; ± 8%) and cortisol (29 %; +/− 17%). The preceding exercise block had little effect on subsequent strength and power performance. The hormonal response after the strength-power bout suggests that this exercise sequence provides an enhanced anabolic milieu for adaptation [10331].

Appropriate physical activity is one of the bases of healthy lifestyle. In fact, physical exercise and playing sport may be associated with both improvements and injury to both general and reproductive health. A biologically normal testosterone secretion appears fundamental in males to guarantee both a physiological exercise adaptation and safe sport participation. The reproductive system is highly sensitive to the effects of exercise-related stress and the reproductive hormones may both increase and decrease after different acute or chronic exercises. Exercise and sport participation may positively or negatively influence andrological health status depending on the type, intensity and duration of performed physical activity and on individual health status. In addition, prohibited substances administration (e.g. androgenic-anabolic steroids, and so forth) in competitive and non-competitive athletes represents the main cause of iatrogenic andrological diseases. Preventing and treating andrological problems in active healthy and unhealthy individuals is as important as promoting a correct lifestyle. Physicians need to be educated on the relationships between the male reproductive system and sport participation and on the great role of the pre-participation physical examination in the prevention of andrological diseases [12121].

**Morphology versus performance after use of anabolic steroids**

It was hypothesized that treatment with testosterone (T) and recombinant human growth hormone (rhGH) would increase lean mass (LM) and muscle strength proportionally and in a linear manner over 16 weeks. This was a multicenter, randomized, controlled, double-masked investigation of T and rhGH supplementation in older (71 ± 4 years) community-dwelling men. Participants received transdermal T at either 5 or 10 g/day as well as rhGH at 0, 3.0 or 5.0 μg/kg/day for 16 weeks. Body composition was determined by dual-energy X-ray absorptiometry (DEXA) and muscle performance by composite one-repetition maximum (1-RM) strength and strength per unit of lean mass (muscle quality, MQ) for five major muscle groups (upper and lower body) at baseline, week 8 and 17. The average change in total LM at study week 8 compared with baseline was 1.50 ± 1.54 kg in the T only group and 2.64 ± 1.7 in the T + rhGH group and at week 17 was 1 in the T only group and 2.14 ± 1.96 kg in the T + rhGH group. 1-RM strength improved modestly in both groups combined (12.0 ± 23.9 %) at week 8 but at week 17 these changes were twofold greater. MQ did not significantly change from baseline to week 8 but increased for the entire cohort, T only, and T + rhGH groups by week 17. Despite sizeable increases in LM measurements at week 8, tests of muscle performance did not show substantive improvements at this time point [12195].

**Effects on training in males**
The acute response of free salivary testosterone and cortisol concentrations to four resistance exercise (RE) protocols in 23 elite men rugby players was investigated. It was hypothesized that hormonal responses would differ among individuals after four distinct RE protocols: four sets of 10 repetitions (reps) at 70 percent of 1 repetition maximum (1RM) with 2 minutes' rest between sets (4 x 10-70 %); three sets of five reps at 85 percent 1RM with 3 minutes' rest (3 x 5-85 %); five sets of 15 reps at 55 percent 1RM with 1 minute's rest (5 x 15-55 %); and three sets of five reps at 40 percent 1RM with 3 minutes' rest (3 x 5-40 %). Each athlete completed each of the four resistance exercise protocols in a random order on separate days. Testosterone and cortisol concentrations were measured before exercise, immediately after exercise, and 30 minutes post exercise. Each protocol consisted of four exercises: bench press, leg press, seated row, and squats. Pooled testosterone data did not change as a result of resistance exercise, whereas cortisol declined significantly. Individual athletes differed in their testosterone response to each of the protocols, a difference that was masked when examining the pooled group data. When individual data were retrospectively tabulated according to the protocol in which each athlete showed the highest testosterone response, a significant protocol-dependent testosterone increase for all individuals was revealed. Therefore, resistance exercise induced significant individual, protocol-dependent hormonal changes lasting up to 30 minutes after exercise. These individual responses may have important ramifications for modulating adaptation to resistance exercise and could explain the variability often observed in studies of hormonal response to resistance exercise [08152].

In aging men
Worldwide many aging males practice sports. A high prevalence of late-onset male hypogonadism has been observed in general population. Sport-participation influences the neuroendocrine system and may decrease serum testosterone. One preliminary study was designed to estimate the prevalence and the symptoms of undiagnosed testosterone deficiency in aging athletes. This observational survey was performed in 183 caucasian male athletes >50 years, in the setting of pre-participation screening. Pituitary-gonadal hormones and symptoms of hypogonadism were investigated. Serum total testosterone (TT), sex hormone binding globulin, luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin (PRL), free-T4, and thyroid stimulation hormone (TSH) were assayed, and free testosterone, bioactive testosterone, and the LH/TT ratio were calculated. The International Index of Erectile Dysfunction (IIEF-15) and the Center for Epidemiological Studies Depression Scale (CES-D) were administered. Hypogonadal athletes were compared with eugonadal athletes as controls. Prevalence and clinical symptoms of severe (TT < 8 nmol/L) or mild (TT between 8 and 12 nmol/L) testosterone deficiency were investigated. The mean sample age was 62 ± 8 years (range 50-75). Severe or mild testosterone deficiency was observed in 12 percent and 18 percent respectively, of overall athletes, with the highest prevalence in athletes >70 years (28 % and 25 %, respectively). Serum total testosterone did not correlate with age, training duration, or questionnaire scores. No differences were observed for nonspecific symptoms of hypogonadism, IIEF-15 and CES-D scores between eugonadal and severe hypogonadal athletes. It was concluded that independently of its etiology, a significant percentage of aging athletes had undiagnosed testosterone deficiency. In a relevant number of these cases, testosterone deficiency was not overtly symptomatic. Our results suggest that sport-participation per se can influence the symptoms of hypogonadism [10334].

Effects on training in females
The purpose of one investigation was to study the effects of an 11-week training period
performed by female weightlifters. Two weeks before this investigation, baseline measures for total testosterone, cortisol, and testosterone:cortisol ratio were collected. The 11-week training program consisted of the core exercises (i.e. clean, clean and jerk, and snatch) and other supplemental exercises (i.e. clean pull, snatch pull, squat, and front squat). Hormonal, isometric, and dynamic middle thigh pull force-time curve characteristics were assessed biweekly throughout the duration of the investigation, whereas volume load and training intensity were assessed weekly throughout the investigation. The testosterone:cortisol ratio of the baseline was significantly different from the ratio of weeks 1 and 9. When the week-to-week values were compared, week 1 was significantly different from week 3. A very strong correlation was found between the percentage change of the testosterone:cortisol ratio and volume load from weeks 1 to 11. Moderate to very strong correlations were noted between the percentage change in volume load and isometric peak force, peak force during the 30 percent isometric peak force trial, and peak force during the 100-kg trial during the 11 weeks of training. The primary finding of this study was that alterations in training volume load can result in concomitant changes in the anabolic-to-catabolic balance, as indicated by the testosterone:cortisol ratio, and the ability to generate maximal forces [08153].

Although androgens are produced in small amounts in women, androgens have direct and significant effects on many aspects of female physiology. Moreover, androgens are precursors to estrogens, which are the predominant female sex hormones. The measurement of androgens in blood is important in the diagnosis of both gonadal and adrenal functional disturbances, as well as monitoring subsequent treatments. The accuracy of such measurements is crucial in sports medicine and doping control. Therefore, the concentration of androgens in female subjects is frequently measured. Analysing such compounds with accuracy is especially difficult, costly and time consuming. Therefore, laboratories widely use direct radioimmunoassay kits, which are often insensitive and inaccurate. It is especially complicated to determine the level of androgens in women, as the concentration is much lower compared to the concentration found in males. Additionally, the amount of androgens in fluids tends to decrease with aging. Analyses of hormone concentrations are influenced by a myriad of factors. The factors influencing the outcome of these tests can be divided into in vivo preanalytical factors (e.g. aging, chronobiological rhythms, diet, menstrual cycle, physical exercise, etc.), in vitro preanalytical factors (e.g. specimen collection, equipment, transport, storage, etc.) and as mentioned before, analytical factors. To improve the value of these tests, the strongly influencing factors must be controlled. This can be accomplished using standardised assays and specimen collection procedures. In general, sufficient attention is not given to the preanalytical (biological) factors, especially in the measurement of androgens in females. Biological factors (non-pathological factors) that may influence the outcome of these tests in female subjects have so far received little attention [08154].

The aim of one study was to examine the effect of supra-maximal exercise on circulating concentrations of salivary testosterone, salivary cortisol, and salivary immunoglobulin A in female adolescents. Nineteen apparently healthy females aged 15-16 years participated in this study. All participants completed 6 × 8 s sprints, interspersed with 30 s recovery intervals on a cycle ergometer. Salivary testosterone, cortisol, and immunoglobulin A samples were taken before and 5 min after exercise. Experimental procedures continued over two mornings, at least 3 h after a light breakfast. Participants refrained from performing any strenuous physical activity for at least 24 h prior to the exercise test. None of the participants were engaged in a structured training programme. The group mean for peak power output was 562 ± 113 W. Female adolescents recruited for this study showed no changes in salivary testosterone, cortisol or immunoglobulin A following repeated bouts of supra-maximal cycling. To date, there has been a paucity of information concerning adolescents' hormonal and mucosal immune function responses to supra-maximal exercise. The data
provide further guidance with regard to physical activities and sports prescription for female adolescents. Further research, on a larger sample of females, is required to elucidate the physiological significance of these findings [10335].

In virtually all sports, participants "warm-up" prior to formal competition. Women athletes from a highly ranked varsity college volleyball team and, in a second study, a highly ranked varsity college tennis team gave saliva samples before warm-up, at mid-warm-up (volleyball) or after warm-up (tennis), and immediately after intercollegiate competition. For volleyball and tennis, warm-up was associated with a substantial elevation in saliva levels of testosterone which was carried over through the period of actual competition. Cortisol levels were relatively unchanged during warm-up, but typically rose during competition. Thus, as women prepare for athletic competition by warming up, testosterone levels rise in apparent anticipation of the coming contest and then remain high through the period of play. In volleyball and tennis, after-practice testosterone level was significantly higher than before-practice level, and practice session increases in testosterone (but not cortisol) were positively correlated with increases in testosterone during intercollegiate competition. When practice and competitive play share as yet undetermined key elements, individual differences in this endocrine response to "competition" appear stable across practice and intercollegiate competition [10336].

**Effects on stress**

After dominance-related encounters, testosterone levels increase in winners and decrease in losers. In humans, many exceptions have been described. It is possible that the complicated patterns in humans result from the methods limitations – measurement of hormone concentrations in simulated competitive events or sport instead in real-life situations. It was studied changes in hormonal levels and self-estimated attractivity in real situations, namely in students after written exams. It was observed that the testosterone and cortisol increased or decreased in relation to the number of wrong answers on the exam. The number of wrong answers was a better predictor of the hormonal changes (increase of both testosterone and cortisol in successful, decrease in unsuccessful students) than the self-estimated number of wrong answers or a subjectively opinionated impression from the exam. On the contrary, the concentration of hormones before the exam and self-estimated attractivity were better predictors of the subjective impression from the exam than the number of wrong answers. The results suggest that the students' subconsciousness, which directly influences the concentration of hormones, is able to objectively estimate results of an exam better than their consciousness [10337].

**Effects on muscles and tendons**

Combined androgenic-anabolic steroids (AAS) and overloading affects tendon collagen metabolism and ultrastructure and is often associated with a higher risk of injury. The aim of this prospective study was to investigate whether such effects would be reflected in the patellar tendon properties of individuals with a history of long-term resistance training and AAS abuse (RTS group), compared with trained (RT) and untrained (CTRL) nonsteroids users. Tendon cross-sectional area (CSA), stiffness, Young's modulus, and toe limit strain were measured in vivo, from synchronized ultrasonography and dynamometry data. The patellar tendon of RT and RTS subjects was much stiffer and larger than in the CTRL group. However, stiffness and modulus were higher in the RTS group (26 % and 30 %, respectively) than in the RT group. Conversely, tendon CSA was 15% (P < 0.05) larger in the RT group than in RTS, although differences disappeared when this variable was normalized to quadriceps maximal isometric torque. Yet maximal tendon stress was higher in RTS than in
RT (15 %), without any statistical difference in maximal strain and toe limit strain between groups. The present lack of difference in toe limit strain does not substantiate the hypothesis of changes in collagen crimp pattern associated with AAS abuse. However, these findings indicate that tendon adaptations from years of heavy resistance training are different in AAS users, suggesting differences in collagen remodeling. Some of these adaptations (e.g. higher stress) could be linked to a higher risk of tendon injury [13116].

Skeletal muscle regeneration efficiency declines with age for both men and women. This decline impacts on functional capabilities in the elderly and limits their ability to engage in regular physical activity and to maintain independence. Aging is associated with a decline in sex hormone production. Therefore, elucidating the effects of sex hormone substitution on skeletal muscle homeostasis and regeneration after injury or disuse is highly relevant for the aging population, where sarcopenia affects more than 30 percent of individuals over 60 years of age. While the anabolic effects of androgens are well known, the effects of estrogens on skeletal muscle anabolism have only been uncovered in recent times. Hence, the purpose of this review is to provide a mechanistic insight into the regulation of skeletal muscle regenerative processes by both androgens and estrogens. Animal studies using estrogen receptor (ER) antagonists and receptor subtype selective agonists have revealed that estrogens act through both genomic and non-genomic pathways to reduce leukocyte invasion and increase satellite cell numbers in regenerating skeletal muscle tissue. Although animal studies have been more conclusive than human studies in establishing a role for sex hormones in the attenuation of muscle damage, data from a number of recent well controlled human studies is presented to support the notion that hormonal therapies and exercise induce added positive effects on functional measures and lean tissue mass. Based on the fact that aging human skeletal muscle retains the ability to adapt to exercise with enhanced satellite cell activation, combining sex hormone therapies with exercise may induce additive effects on satellite cell accretion. There is evidence to suggest that there is a “window of opportunity” after the onset of a hypogonadal state such as menopause, to initiate a hormonal therapy in order to achieve maximal benefits for skeletal muscle health. Novel receptor subtype selective ligands and selective estrogen and androgen receptor modulators (SERMs, SARMs) promise to reduce health risks associated with classical hormonal therapies, whilst maintaining the positive effects on muscle repair. Dietary supplements containing compounds with structural similarity to estrogens (phytoestrogens) are increasingly used as alternatives to classical hormone-replacement therapies (HRT), but the effects on skeletal muscle are currently largely unknown. Research has started to investigate the combined effects of exercise and alternative HRTs, such as soy isoflavones, on skeletal muscle regenerative processes to provide safer and more efficient therapies to promote muscle regeneration and maintenance of muscle mass and strength in the aging population [13117].

Androgen-dependent impairment of myogenesis in muscular atrophy
Spinal and bulbar muscular atrophy (SBMA) is an inherited neuromuscular disease caused by expansion of a polyglutamine (polyQ) tract in the androgen receptor (AR). SBMA is triggered by the interaction between polyQ-AR and its natural ligands, testosterone and dihydrotestosterone (DHT). SBMA is characterized by the loss of lower motor neurons and skeletal muscle fasciculations, weakness, and atrophy. To test the hypothesis that the interaction between polyQ-AR and androgens exerts cell-autonomous toxicity in skeletal muscle, we characterized the process of myogenesis and polyQ-AR expression in DHT-treated satellite cells obtained from SBMA patients and age-matched healthy control subjects. Treatment with androgens increased the size and number of myonuclei in myotubes from control subjects, but not from SBMA patients. Myotubes from SBMA patients had a reduced number of nuclei, suggesting impaired myotube fusion and altered contractile structures. The lack of anabolic effects of androgens on myotubes from SBMA patients was
not due to defects in myoblast proliferation, differentiation or apoptosis. DHT treatment of myotubes from SBMA patients increased nuclear accumulation of polyQ-AR and decreased the expression of interleukin-4 (IL-4) when compared to myotubes from control subjects. Following DHT treatment, exposure of myotubes from SBMA patients with IL-4 treatment rescued myonuclear number and size to control levels. This supports the hypothesis that androgens alter the fusion process in SBMA myogenesis. In conclusion, these results provide evidence of an androgen-dependent impairment of myogenesis in SBMA that could contribute to disease pathogenesis [13118].

**Long-term effects on muscles**

Previous strength training with or without the use of anabolic steroids facilitates subsequent re-acquisition of muscle mass even after long intervening periods of inactivity. Based on in vivo and ex vivo microscopy we here propose a cellular memory mechanism residing in the muscle cells. Female mice were treated with testosterone propionate for 14 days, inducing a 66% increase in the number of myonuclei and a 77 percent increase in fibre cross-sectional area. Three weeks after removing the drug, fibre size was decreased to the same level as in sham treated animals, but the number of nuclei remained elevated for at least 3 months (>10 % of the mouse lifespan). At this time, when the myonuclei-rich muscles were exposed to overload-exercise for 6 days, the fibre cross-sectional area increased by 31 percent while control muscles did not grow significantly. It was suggested that the lasting, elevated number of myonuclei constitutes a cellular memory facilitating subsequent muscle overload hypertrophy. The findings might have consequences for the exclusion time of doping offenders. Since the ability to generate new myonuclei is impaired in the elderly our data also invites speculation that it might be beneficial to perform strength training when young in order to benefit in senescence [13119].

**Inhibitory actions on tendons**

One study investigated the structural changes in the rat calcaneal tendon (CT), superficial flexor tendon (SFT), and deep flexor tendon (DFT) in response to jump exercises and anabolic androgenic steroids (AAS). Animals were divided into four groups: sedentary, trained, AAS-treated sedentary rats, and AAS-treated trained animals. Training increased the volume density (Vv%) of blood vessels in all regions of the CT and DFT, cell Vv% in the peritendinous sheath of the proximal and distal regions of the SFT and proximal region of DFT, and cell Vv% in the tendon proper of the proximal and distal regions of the SFT and DFT. The combination of AAS and load exercises showed little increased blood vessel Vv% at the proximal region of the CT, intermediate region of the SFT, and all regions of the DFT as opposed to an increase in adipose cell Vv% in the CT proximal region. The AAS reduced the levels of hydroxyproline in the proximal region of the DFT and in the distal region of the STF. In conclusion, exercise promoted benefits to the adaptation of the tendons to overload. These effects were absent when load exercise was combined with AAS. The abusive consumption of AAS contributes to tendon inertness and rigidity, and increases the potential risk of injury [13120].

**Influence on immunological function**

The in vitro effect of testosterone on human neutrophil function was investigated. Blood neutrophils from healthy male subjects were isolated and treated with 10 nM, 0.1 and 10 microM testosterone for 24 h. As compared with untreated cells, the testosterone treatment produced a significant decrease of superoxide production as indicated by the measurement of extra- and intracellular superoxide content. An increment in the production of nitric oxide was observed at 0.1 and 10 microM testosterone concentrations, whereas no effect was found for 10 nM. Intracellular calcium mobilization was significantly increased at 10 nM,
whereas it was reduced at 10 microM testosterone. There was an increase in phagocytic capacity at 10 nM and a decrease of microbicidal activity in neutrophils treated with testosterone at 10 microM. Glutathione reductase activity was increased by testosterone treatment, whereas no effect was observed in other antioxidant enzyme activities. An increase in the content of thiol groups was observed at all testosterone concentrations. Lipid peroxidation in neutrophils evaluated by levels of TBARS was decreased at 10 nM and 0.1 microM testosterone. These results indicate the antioxidant properties of testosterone in neutrophils as suggested by reduction of superoxide anion production, and lipid peroxidation, and by the increase in nitric oxide production, glutathione reductase activity and the content of thiol groups. Therefore, the plasma levels of testosterone are important regulators of neutrophil function and so of the inflammatory response [10338].

Influence on endurance

Anabolic androgenic steroids (AAS) are doping agents that are mostly used for improvement of strength and muscle hypertrophy. In some sports, athletes reported that the intake of AAS is associated with a better recovery, a higher training load capacity and therefore an increase in physical and mental performances. The purpose of one study was to evaluate, the effect of multiple doses of AAS on different physiological parameters that could indirectly relate the physical state of athletes during a hard endurance training program. In a double blind settings, three groups (n = 9, 8 and 8) were orally administered placebo, testosterone undecanoate or 19-norandrostenedione, 12 times during 1 month. Serum biomarkers (creatine kinase, ASAT and urea), serum hormone profiles (testosterone, cortisol and LH) and urinary catecholamines (noradrenalin, adrenalin and dopamine) were evaluated during the treatment. Running performance was assessed before and after the intervention phase by means of a standardized treadmill test. None of the measured biochemical variables showed significant impact of AAS on physical stress level. Data from exercise testing on submaximal and maximal level did not reveal any performance differences between the three groups or their response to the treatment. In the present study, no effect of multiple oral doses of AAS on endurance performance or bioserum recovery markers was found [06095].

The purpose of the study was to investigate the influence of a 14-week swimming training program on psychological, hormonal, and performance parameters of elite women swimmers. Ten Olympic and international-level elite women swimmers were evaluated 4 times along the experiment (i.e. in T1, T2, T3, and T4). On the first day at 8:00 am, before the blood collecting at rest for the determination of hormonal parameters, the athletes had their psychological parameters assessed by the profile of mood-state questionnaire. At 3:00 am, the swimmers had their anaerobic threshold assessed. On the second day at 3:00 am, the athletes had their alactic anaerobic performance measured. Vigor score and testosterone levels were significantly lower in T4 compared with T3. In addition, the rate between the peak blood lactate concentration and the median velocity obtained in the alactic anaerobic performance test significantly increased in T4 compared with T3. For practical applications, the swimming coaches should not use a tapering with the present characteristics to avoid unexpected results [10046].

The purpose of one study was to explore the mechanisms for increased exercise performance in conditions of competition. Endurance trained subjects (n=14) performed incremental treadmill running to exhaustion in control laboratory conditions (non-competition) and in conditions of simulated competition to assess performance (running duration). Heart rate and respiration gases were monitored continuously through each exercise condition. Blood lactate, cortisol, growth hormone and testosterone concentrations were also determined at pre- (rest) and postexercise in each condition. Results indicated competition exercise performance was significantly increased as was peak VO$_2$ response versus non-
competition. No significant differences were found in peak measurements of minute ventilation, respiratory exchange ratio, ventilation threshold, post-exercise lactate, heart rate, or the ventilation equivalent for O2 between the exercise conditions. In both conditions growth hormone and testosterone concentrations increased significantly in response to exercise, whereas cortisol responses post-exercise were significantly elevated in the competition but not in the control condition. These findings support that in competitive situations the affective state (motivation) experienced by athletes can enhance performance in exercise events, and lead to an increased peak oxygen uptake. The magnitude of the improvement is of a substantial nature and of a level seen with some training programs. Competitive conditions also augment the cortisol response to exercise, suggesting that enhanced sympatho-adrenal system activation occur in such situations which may be one of the key "driving forces" to performance improvement [10047].

Effect of hydration
Exercise intensity powerfully influences testosterone, cortisol, and testosterone/cortisol ratio (T/C) responses to endurance exercise. Hydration state may also modulate these hormones, and therefore may alter the anabolic/catabolic balance in response to endurance exercise and training. This study examined the effect of running intensity on testosterone, cortisol, and T/C when exercise was initiated in a hypohydrated state. Nine male collegiate runners (age 20 years) completed four 10-min treadmill runs differing in pre-exercise hydration status (euhydrated, or hypohydrated by 5 % of body mass) and exercise intensity (70 % or 85 % VO2max). Body mass, urine osmolality, and urine-specific gravity documented fluid balance; blood samples drawn pre-, immediately post-, and 20 min post-exercise were analyzed for testosterone, cortisol, and T/C. Except for heart rate measured during the 70 percent VO2max trials, heart rate, VO2, and plasma lactate were similar between euhydrated and hypohydrated conditions for a given intensity, suggesting hypohydration did not measurably increase the physiological stress of the exercise bouts. Furthermore, hydration state had no measurable effect on testosterone concentrations before, during, or after exercise at either intensity. Regardless of exercise intensity, cortisol concentrations were greater during hypohydration than euhydration pre-exercise and 20 min post-exercise. Additionally, T/C was significantly lower 20 min post-exercise at 70 percent VO2max when subjects were initially hypohydrated (T/C = 0.056) versus euhydrated (T/C = 0.072). These findings suggest that depending on exercise intensity, T/C may be altered by hydration state, therefore influencing the balance between anabolism and catabolism in response to running exercise performed at typical training intensities [06096].

Influence on strength
Performing strength exercise, whether acutely or in a training programme, leads to alterations at the hypothalamic-pituitary-testicular and hypothalamic-pituitary-adrenal axes. One way to evaluate these changes is by analysis of the excretion of steroid hormones in the urine. One study determined the variations in the urine profile of glucuroconjugated steroids after a single session of strength exercise and after a 4-week programme of strength training. The subjects were a group (n=20) of non-sportsman male university students who worked out 3 days a week (Monday, Wednesday, and Friday), performing the exercises at 70-75 percent of one repetition maximum strength (1-RM). Four urine samples were collected per subject: (A) before and (B) after a standard session prior to initiating the training programme, and (C) before and (D) after the same standard session at the end of the study, and they were assayed by gas chromatography coupled to mass spectrometry. The concentrations of the different hormones were determined relatively to the urine creatinine level (ng steroid/mg creatinine) to correct for diuresis. After the exercise sessions, both before and after the training programme, there was a fall in the urine excretion of androgens and estrogens, but
no statistically significant changes in the excretion of tetrahydrocortisol (THF) and tetrahydrocortisone (THE). The anabolic/catabolic hormones ratio also decreased after the acute session, although only androstenodione + dehydroepiandrosterone (DHEA)/THE + THF ratio had a significant decrease. After the training programme, there was a significant improvement in the strength of the muscle groups studied, and an increased urinary excretion of all the androgens with respect to the initial state of repose, with the difference being significant in the case of epistosterone (Epit). The androsterone (A) + etiocholanolone (E)/THE + THF ratio increased significantly concerning the initial state. It was therefore conclude that subjects suffer variations of the urine profile with regard to the steroid hormones before and after the acute strength sessions and after the training period. The alteration after the training programme seems to be due to the subjects’ hypothalamic-hypophysis-testicular and hypothalamic-pituitary-adrenal axes adaptations, which enable them to increase physical strength [06094].

Previous studies with different results have suggested that total and bioavailable testosterone levels are modified by physical exercise. Such changes may be related to modifications in cortisol levels and could be reflected in some urine androgens. To determine how weight lifting training may affect serum and urinary androgens, it was measured total serum testosterone (T), cortisol, sex hormone binding globulin (SHBG) and urinary testosterone, epistosterone, androsterone, and etiocholanolone, in a group of 19 elite weight lifters after 20 weeks of training. SHBG increased significantly (from 28 ± 10 to 35 ± 8 nM) whereas T/SHBG decreased significantly (from 1.1 ± 0.4 to 0.9 ± 0.3). Serum total testosterone and cortisol did not change significantly. In urine, androsterone and etiocholanolone decreased significantly, whereas testosterone and epistosterone remained unchanged. Changes in T/SHBG were related positively with changes in urinary androgens, and changes in SHBG were negatively related with changes in urinary androgens. These results suggest that intense physical activity may have an influence on the elimination of androgenic hormones due mainly to changes in their transporting protein SHBG [10048].

One study assessed an enzyme-immunoassay (EIA) kit for measuring the salivary testosterone (T) and cortisol (C) concentrations of weightlifters. Saliva samples (n=64) were collected from male and female weightlifters during normal training procedures and analysed for T and C using a commercial EIA kit and a criterion radioimmunoassay (RIA) method. Significant correlations were demonstrated between the EIA and RIA measurements of salivary T and C concentrations. Further examination by sample and gender revealed similar relationships. The EIA concentrations of salivary T and C were found to be slightly greater (10-13 %) than the RIA values. Similar discrepancies were noted when gender comparisons were made, although the relative information on T (males > females) and C (males=females) were consistent for both assay methods. In conclusion, a commercially available EIA kit provided valid measures of the salivary T and C concentrations of male and female weightlifters. Factors to consider when using an enzyme-immunoassay kit include the hormone(s) of interest, the magnitude of the correlations, as well as the descriptive information gained (e. g. absolute, relative) and its uses within sport [10049].

In weightlifters

One study examined the relationships between salivary testosterone (Sal-T) and cortisol (Sal-C) concentrations and training performance in Olympic weightlifters. Four male and four female Olympic weightlifters each provided saliva samples before and after four workouts during a four-week training period. Training involved the same three exercises; snatch, clean and jerk, and front squat with the one repetition maximum (1RM) calculated for each exercise during each workout. Significant training improvements in 1RM performance (4.0-5.2 %) were noted during the snatch and clean and jerk exercises, along with the Olympic total lift.
For male participants only, the pre-workout concentrations of Sal T were significantly correlated with the snatch and clean and jerk 1RM, and the Olympic total lift. It was concluded that short period of training improved the 1RM performance of Olympic weightlifters in two exercises (snatch and clean and jerk) and the Olympic total. For male participants, their Sal-T concentrations before each workout was also related to 1RM performance during these exercises, thereby highlighting one possible short-term causative mechanism [10332].

One study examined the acute hormonal responses to a single high power resistance exercise training session. Four weight trained men participated as subjects in two randomly ordered sessions. During the lifting session, serum samples were collected pre- and 5 min post-exercise, and later analyzed for testosterone (Tes), cortisol (Cort), their ratio (Tes/Cort), and lactate (HLa). The lifting protocol was 10 x 5 speed squats at 70 percent of system mass (1 RM ± BW) with 2 min inter-set rest intervals. Mean power and velocity were determined for each repetition using an external dynamometer. On the control day, the procedures and times (1600-1900 hrs) were identical except the subjects did not lift. Tes and Cort were analyzed via EIA. Mean power and velocity was 1377 ± 10 W and 0.79 ± 0.01 m/s respectively for all repetitions, and did not decrease over the 10 sets. Although not significant, post-exercise testosterone exhibited a very large effect size. No changes were observed for either cortisol or the Tes/Cort ratio. Lactate significantly increased post-exercise. The exercise protocol resulted in no significant changes in Tes, Cort or the Tes/Cort ratio. The acute increase for Tes is in agreement with previous reports that high power activities can elicit a Tes response. High power resistance exercise protocols such as the one used in the present study produce acute increases of Tes. These results indicate that high power resistance exercise can contribute to an anabolic hormonal response with this type of training, and may partially explain the muscle hypertrophy observed in athletes who routinely employ high power resistance exercise [1033].

**Influence of sprint**

The purpose of this study was to investigate the effects of a 6-month sprint training program on plasma androgens and catecholamine (CA) concentrations in response to a 6 s sprint in adolescent boys [training group (TG), n=6; control group (CG), n=6]. A 6 s-sprint test was performed on a cycle ergometer before and after training (Pre-T and Post-T, respectively). Plasma total testosterone (TT), bioavailable testosterone (BT), and CA concentrations were measured at rest, after a warm-up, immediately after a 6 s-sprint, and during the recovery (i.e. 5 and 20 min). After training period, plasma TT concentrations increased significantly at the end of the sprint and during the recovery in the TG. No effects for sampling times and period were observed in BT levels. Plasma TT concentrations after 5 min of recovery were positively correlated with the corresponding values of plasma lactate (La) concentrations and with post-6 s-sprint plasma adrenaline (A) concentrations.. These results suggest that sprint training increases plasma TT concentrations in response to sprint exercise in adolescent boys. Plasma A and plasma La concentrations increases in response to sprint exercise could be involved in this elevation of plasma TT concentrations [10050].

**Influence of bodybuilders’ fasting periods**

The purpose of one study was to investigate simultaneous effects of energy balance, caloric intake, and the hormonal anabolic-catabolic balance in bodybuilders prior to competition. Fourteen male bodybuilders took part in an 11-week energy-restricted period to reduce body fat. The subjects were divided into the energy-restricted group (ERG) (n=7), who were preparing for the competition, or the control group (CG) (n=7) who continued to train regularly.
and did not change their dietary or training pattern. Participants were tested at 11 weeks (T1), 5 weeks (T2), and 3 days (T3) before competition for diet, body composition, and fasting hormonal assessment. Body mass and body fat percentage of ERG were significantly (p < 0.05) decreased during the study period. In ERG, insulinlike growth factor-1 (IGF-1) and insulin decreased significantly during the 11-week weight-reduction period. Testosterone was decreased only from week 11 to week 5 (from 20 ± 6 to 18 ± 7 nmol/L). Changes in IGF-1 concentration were significantly related to changes in insulin, fat mass, lean body mass, and body mass. Changes in insulin concentrations were significantly related to changes in fat mass, and lean body mass. These data indicate that severe energy restriction to extremely low body energy reserves decreases significantly the concentrations of 3 anabolic pathways despite high protein intake. Monitoring of insulin and IGF-1 concentration is suggested to prevent losses in muscle mass in energy-restricted conditions [10051].

**Flywheel ergometer workouts**

The purpose of one study was to compare blood lactate and hormonal responses with flywheel ergometer (FERG) leg presses for preliminary assessment of workouts best suited for future in-flight resistance exercise. Comprised of 10 repetition sets, the workouts entailed 3 sets of concentric and eccentric (CE3) actions, or concentric-only actions done for 3 (CO3) or 6 (CO6) sets. Methods employed included assessment of blood lactate concentrations (BLa) before and 5 minutes postexercise. Venous blood was also collected before and at 1 and 30 minutes postexercise to assess growth hormone, testosterone, cortisol concentrations. Results showed blood lactate concentrations had a time effect. Growth hormone concentration showed gender x workout, gender x time, and workout x time interactions, whereas testosterone had a 3-way interaction. Testosterone-cortisol ratio yielded a gender x time interaction. It was concluded that, because CO6 and CE3 yielded similar anabolic hormonal data but the latter had a lower cortisol 30 minutes postexercise, CE3 served as the best workout. Although the FERG was originally designed for microgravity, the effort put forth by current subjects was like that for workouts aimed at greater athletic performance and conditioning. Practical applications suggest that eccentric actions should be used for FERG workouts geared toward muscle mass and strength improvement [10052].

**Watching a previous victory**

Previous research indicates that testosterone concentrations are highly responsive to human competitive interactions and that winners have elevated testosterone concentrations relative to losers. Also, there is some evidence that simply observing others compete can have a similar effect on the endocrine system. Here, in two studies, it was examined the extent to which elite male hockey players would demonstrate an increase in testosterone concentrations after watching themselves engaged in a previous successful competitive interaction. Results indicated that watching a previous victory produced a significant increase in testosterone concentrations (42-44 % increase), whereas watching a previous defeat or a neutral video did not produce a significant change in testosterone (17 % and 6 %, respectively). Given that natural fluctuations in testosterone have been shown to influence future competitive and aggressive behaviours, the current studies may have important practical implications for individuals involved in competitive sports [10053].

**Effects of magnesium supplementation**

One study was performed to assess how 4 weeks of magnesium supplementation and exercise affect the free and total plasma testosterone levels of sportsmen practicing tae
Kwon do and sedentary controls at rest and after exhaustion. The testosterone levels were determined at four different periods: resting before supplementation, exhaustion before supplementation, resting after supplementation, and exhaustion after supplementation in three study groups, which are as follows: Group 1 - sedentary controls supplemented with 10 mg magnesium per kilogram body weight. Group 2 - tae kwon do athletes practicing 90-120 min/day supplemented with 10 mg magnesium per kilogram body weight. Group 3 - tae kwon do athletes practicing 90-120 min/day receiving no magnesium supplements. The free plasma testosterone levels increased at exhaustion before and after supplementation compared to resting levels. Exercise also increased testosterone levels relative to sedentary subjects. Similar increases were observed for total testosterone. The results show that supplementation with magnesium increases free and total testosterone values in sedentary and in athletes. The increases are higher in those who exercise than in sedentary individuals [10056].

Effects of training on salivary levels

The aims of one study were to identify the time-course of change of salivary testosterone (sT), cortisol (sC) and IgA (SIgA), mood state and performance capacity during a 2-week taper in judo athletes, and to examine the diurnal variation in these salivary markers. Eleven male judo athletes completed 5 weeks of training: 1 week of normal training (NORM), 2 weeks of intensified training (INT) and 2 weeks of exponential tapering (TAPER). Once per week subjects completed vertical and horizontal countermovement jump tests, a grip strength test, a Special Judo fitness test (SJFT), a multistage aerobic fitness test (MSFT), a 3x300-m run test and anthropometric measurement. Subjects also completed questionnaires to assess mood state and muscle soreness. Two daily saliva samples (at 07:00 and 19:00) were collected at the end of each week during NORM and INT and every day during TAPER. Increased morning sT, decreased evening sC, lower muscle soreness and enhanced mood state were evident by the early phases of TAPER. A significant 7 percent improvement in 3x300-m performance time, a 7 percent improvement in the vertical jump and increased morning and evening SIgA secretion rate were observed during the middle-late phases of TAPER. The higher values of salivary variables were observed in the morning. The study indicates that salivary hormones display diurnal variation. Furthermore, changes in hormonal responses, mood state and muscle soreness precede enhancements in performance and mucosal immunity, suggesting that judo athletes taper for at least a week prior to competition [12122].

Effects of androgens on IGF-1

The mechanism whereby anabolic androgens are associated with hypertrophy of skeletal muscle is incompletely understood but may involve an interaction with locally generated insulin-like growth factor (IGF) 1. The present investigation utilized a cell culture model of human skeletal muscle-derived cell maturation to test the hypothesis that androgens increase differentiation of human muscle precursor cells in vitro and to assess effects of androgen with or without IGF-1 on IGF-1 messenger RNA (mRNA) expression in human muscle precursor cells. Differentiation of muscle-derived cells was induced under standard low-serum conditions. Cultures were then exposed to androgen (testosterone, T) at 50, 100, and 500 nM or IGF-1 (10-50 ng/mL). Immunocytochemistry and real-time polymerase chain reaction (RT-PCR) were used to assess effects of androgens and IGF-1 after 3- (early) or 7-d (late) muscle differentiation, respectively; RT-PCR was used to quantify the effects on androgen receptor expression. Under low-serum conditions, 3-d exposure to androgens or IGF-1 or both resulted in no significant increase in cellular myogenic commitment. After 7-d exposure, however, T and IGF-1 were both found to increase fusion index with no
observable synergistic effect. T also increased IGF-1 mRNA generation, whereas exogenous IGF-1 reduced IGF-1 mRNA transcription relative to control. The T effect was reversible after treatment with flutamide, an androgen receptor antagonist. Both T and IGF-1 increase myogenic commitment after 7-d exposure to a differentiation medium. With T causing a concomitant increase in IGF-1 mRNA underpinning IGF-1 as a central mediator in the cellular pathways associated with muscle hypertrophy, including those affected by androgens. The novel system described has the potential for elucidating the pattern of growth factor effects associated with androgens in skeletal muscle [12123].

Interactions with opioids

Over the past decades, research on doping agents, such as anabolic androgenic steroids (AAS), has revealed that these compounds are often used in combination with other drugs of abuse. It seems that misuse of AAS probably involves more than a desire to enhance appearance or sports performance and studies have revealed that steroids are commonly connected with alcohol, opioids, tobacco, and psychotropic drugs. It was observed that AAS may interact with the endogenous opioids, excitatory amino acids, and dopaminergic pathways involved in the brain reward system. Furthermore, studies provide evidence that AAS may induce an imbalance in these signal systems leading to an increased sensitivity toward opioid narcotics and central stimulants. In fact, studies performed in various clinics have shown that individuals taking AAS are likely to get addicted to opioids like heroin [12124].

Induction of nitric acid

Accumulating evidence indicates that abuse of anabolic androgenic steroids may cause cardiovascular adverse side-effects, including endothelial dysfunction. The aim of the present study was to investigate the effects of supra-physiological doses of testosterone on the endothelial production of nitric oxide (NO) and oxidative stress in vitro and in vivo. Testosterone enanthate was administrated as of a single 500 mg dose to healthy volunteers (n=27). Gene expression was studied in human vascular endothelial cells exposed to testosterone. The in vivo results show that the urinary NO level and the antioxidative capacity were significantly decreased two days after testosterone administration. In agreement, our in vitro studies show that testosterone inhibits the gene expression of endothelial NO synthase (eNOS) after 48 hours. When the antioxidant seleno-L-methionine was added, the down-regulation of mRNA specific eNOS was partly abrogated. The mRNA expression of antioxidizing enzyme genes was significantly inhibited after eight hours and recovered 48 hours after testosterone treatment of endothelial cells. These results show that a supraphysiological dose of testosterone decreases the expression of eNOS and consequently the formation of NO, which could partly be explained by oxidative stress. These results indicate that supraphysiological doses of testosterone may induce endothelial dysfunction, which is of interest in relation to the cardiovascular adverse side-effects observed in anabolic androgenic steroid abusers [13207].

Lack of influence of NSAID

When focusing on steroid glucuronides as diagnostic parameters in doping controls, the influence of dietary components on relevant enzymes (i.e. UDP-glucuronosyltransferases) involved in the conjugation reactions in vivo must be considered. A variety of reports demonstrating glucuronide inhibiting properties of pharmaceuticals (e.g. non-steroidal anti-inflammatory drugs, NSAIDs) and ingredients of green tea or red wine were published, the majority of which however was done in vitro. It was therefore investigated the influence of
NSAIDs (ibuprofen and diclofenac) on the renal elimination of TG and epiTG in a controlled, randomized cross-over study with 23 male volunteers with two (n=8), one (n=7), or no (n=8) allele of the UGT2B17 gene, thus representing the above mentioned ins/ins, ins/del, and del/del genotype, respectively. Both the baseline T/epiT ratios as well as the T/epiT values following an intramuscular injection of 500 mg of testosterone enanthate were not significantly influenced by repeated maximum daily doses of the NSAIDs, which suggests that the commonly employed steroid profile approach is not compromised by NSAID applications [13009].

Testosterone is one of the most commonly abused anabolic androgenic steroids (AAS) within doping in sports and for enhancement of physical performance. In 2011 anabolic agents represented the most frequently reported adverse analytical findings and atypical findings (59%) by accredited doping laboratories. Among these, elevated testosterone/epitestosterone ratios represented 60 percent of the findings, although only 10 percent of these were adverse analytical findings. The UDP Glucuronosyl Transferase (UGT) enzymes are important in the pharmacokinetics, and conjugation, of a variety of drugs including non-steroidal anti-inflammatory drugs (NSAIDs) as well as anabolic androgenic steroids (AAS). Testosterone glucuronidation capacity is strongly associated with a deletion polymorphism in the UGT2B17 gene. As the use of high doses of NSAIDs has been observed in athletes there is a risk for a drug-drug interaction that may influence the doping tests for AAS. In vitro studies show inhibitory potential on UGT2B7, 2B15, and 2B17 enzymes by NSAIDs. To discriminate exogenous testosterone from testosterone of endogenous origin the urinary ratio of testosterone glucuronide (TG) to epitestosterone glucuronide (EG) (T/E ratio) is used. Based on population studies a normal T/E ratio would be around 1.0 and a T/E ratio above six was initially considered suspicious of doping. However, additional knowledge showed that Asian individuals excreted low amounts of TG, and as a result low T/E ratios increasing the risk of false negative doping test results. Due to these findings the T/E ratio was lowered to 4.0 in 2004. Testosterone is inactivated, and excreted in urine, mainly as glucuronide conjugates, the formation of which is catalyzed by UDP-glucuronosyltransferases (UGTs). These enzymes play a key role in the homeostasis of a number of endogenous molecules including steroid hormones. The UGT super family is subdivided into UGT1A, UGT2A, and UGT2B families based on sequence identity. The human UGT2B genes are clustered on chromosome 4q13-21.1 and encode seven functional enzymes: UGT2B4, B7, B10, B11, B15, B17, and B28. In vivo, UGT2B17 has been identified as the main enzyme in testosterone glucuronidation where a gene deletion in UGT2B17 was associated with low, or negligible, excretion of testosterone in urine. All subjects devoid of UGT2B17 had a T/E ratio below 0.4. This polymorphism was considerably more common in a Korean Asian than in a Swedish Caucasian population, with 67 and 9 percent deletion/deletion (del/del) homozygotes, respectively. The UGT enzymes are important in the pharmacokinetics, and conjugation, of a variety of drugs including non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are a class of therapeutic agents used in the treatment of pain and inflammation and are commonly used by athletes. In fact, according to recent studies, inappropriate use of high doses and concomitant use of several different NSAIDs has been observed in athletes. Since steroids and NSAIDs are both inactivated by UGT enzymes there is a risk for a drug-drug interaction. In vitro studies show inhibitory potential on UGT2B7, 2B15 and 2B17 enzymes by NSAIDs. In the latter study both diclofenac and ibuprofen inhibited testosterone glucuronidation in liver microsomes, as well as recombinant UGT2B15 and UGT2B17 enzymes. However, epitestosterone glucuronidation activity in human liver microsomes was largely insensitive to ibuprofen and diclofenac. The aim of one study was to investigate if concomitant use of NSAIDs and a single dose of testosterone enanthate would affect the excretion rate of testosterone and epitestosterone glucuronide (TG and EG) as well as the T/E ratio, thereby affecting the outcome of the testosterone doping test. The study was designed as an open, randomized, cross-over study with subjects being their own control.
The 23 male healthy volunteers, with either two, one or no allele (ins/ins, ins/del, or del/del) of the UGT2B17 gene, received the maximum recommended dose of NSAID (Ibuprofen or Diclofenac) for 6 days. On day three, 500 mg of testosterone enanthate was administered. Spot urine samples were collected for 17 days. After a wash-out period of 4 months the volunteers received 500 mg testosterone enanthate only, with subsequent spot urine collection for 14 days. The glucuronides of testosterone and epitestosterone were quantified. NSAIDs did not affect the excretion of TG or EG before the administration of testosterone. The concomitant use of NSAIDs and testosterone slightly increased the TG excretion while the EG excretion was less suppressed compared to testosterone use only. The effects of the NSAIDs on the TG and EG excretion did not differ between the UGT2B17 genotype groups. In conclusion, the outcome of testosterone doping tests does not seem to be affected by the use of NSAIDs [13208].

Longitudinal steroid profiling

Another approach to detect testosterone use that is gaining widespread acceptance is longitudinal studies of urinary steroid concentrations. The concept is based on the observation that the T/E ratio for a single individual male typically varies by <30 percent, whereas between-individual variability is considerably larger. Individual T/E values from at least 3 test results are used to establish a baseline, and suspicious results that differ significantly from baseline are proof of synthetic testosterone use. Several statistical approaches have been used to detect outliers in longitudinal data. A Bayesian test using both population data and individual athlete test results appears to be superior to other statistical tests for detecting T/E ratio outliers. A Bayesian interpretation of T/E test results has been shown to produce 0 false-positive results for 43 true positives using a p-value of 0.1 percent. For the same data set, a population-based T/E cutoff of 4.0 resulted in 24 false positives and 34 true positives. Urine samples producing a test result significantly higher than baseline could then be submitted for GC/C/IRMS confirmation testing. This approach can be used to detect testosterone use in individuals with the UGT2B17 deletion polymorphism, possibly negating the need for genetic testing. Athlete-specific baseline T/E ratios, along with GC/C/IRMS testing on random and suspicious urine samples, were used at the Olympic Games 2008 to catch athletes that use synthetic testosterone and have low a T/E ratio. Longitudinal studies can also be applied to other urinary steroid metabolites to further enhance detection of synthetic steroid use [08016].

Long-time effects of anabolic steroids

To study the long-term impact of anabolic androgenic steroid (AAS) abuse on the cholesterol profile, and the potential to suppress endocrine activity in men working out at gym facilities a study of the relation between urinary biomarkers for testosterone and nandrolone abuse and the UGT2B17 genotype and time profile was performed. Subjects (n=56) were recruited through Anti-Doping Hot-Line. Serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), plasma levels of low density lipoprotein (LDL), high density lipoprotein (HDL) and urinary steroid profile were regularly measured for a period of up to one year after cessation of intramuscular AAS abuse. A sustained suppression of LH, and FSH was observed for several months. The nandrolone urinary biomarker 19-NA was detectable several months after the last nandrolone intake and was correlated to the levels of LH and FSH. Testosterone abuse on the other hand was detectable only for a few weeks, and some of the testosterone abusers did not test positive due to a genetic deletion polymorphism of the UGT2B17. Significantly increased levels of HDL and decreased levels of LDL were
observed for 6-months after cessation of AAS abuse. It was concluded that some individuals had a sustained suppression of LH and FSH for a period of 1 year whereas the cholesterol profile was normalized within 6 month. The long term consequences of these findings remain to be established [11570].

Effects of previous strength training can be long-lived, even after prolonged subsequent inactivity, and retraining is facilitated by a previous training episode. Traditionally, such "muscle memory" has been attributed to neural factors in the absence of any identified local memory mechanism in the muscle tissue. It was used in vivo imaging techniques to study live myonuclei belonging to distinct muscle fibers and observe that new myonuclei are added before any major increase in size during overload. The old and newly acquired nuclei are retained during severe atrophy caused by subsequent denervation lasting for a considerable period of the animal's lifespan. The myonuclei seem to be protected from the high apoptotic activity found in inactive muscle tissue. A hypertrophy episode leading to a lasting elevated number of myonuclei retarded disuse atrophy, and the nuclei could serve as a cell biological substrate for such memory. Because the ability to create myonuclei is impaired in the elderly, individuals may benefit from strength training at an early age, and because anabolic steroids facilitate more myonuclei, nuclear permanency may also have implications for exclusion periods after a doping offense [10306].

Anabolic androgenic steroids (AAS) use by adolescents is steadily increasing. Adolescence involves remodeling of steroid-sensitive neural circuits that mediate social behaviors, and previous studies using animal models document effects of AAS on male social behaviors. The present experiments tested whether AAS have persistent and more pronounced behavioral consequences when drug exposure occurs during adolescence as compared to exposure in adulthood. Male Syrian hamsters were injected daily for 14 days with either vehicle or an AAS cocktail containing testosterone cypionate (2 mg/kg), nandrolone decanoate (2 mg/kg), and boldenone undecylenate (1 mg/kg), either during adolescence (27-41 days of age) or adulthood (63-77 days of age). As adults, subjects were tested two or four weeks after the last injection for either sexual behavior with a receptive female or male-male agonistic behavior in a resident-intruder test. Compared with vehicle-treated males, AAS-treated males, regardless of age of treatment, displayed fewer long intromissions and a significant increase in latency to the first long intromission, indicative of reduced potential to reach sexual satiety. Increased aggression was observed in males exposed to AAS compared with males treated with vehicle, independently of age of AAS treatment. However, unlike hamsters exposed to AAS in adulthood, hamsters exposed to AAS during adolescence did not display any submissive or risk-assessment behaviors up to 4 weeks after discontinuation of AAS treatment. Thus, AAS have long-lasting effects on male sexual and agonistic behaviors, with AAS exposure during adolescence resulting in a more pronounced reduction in submissive behavior compared to AAS exposure in adulthood [09064].

Multi-parametric steroid profiling

Steroid profiling provides valuable information to detect doping with endogenous steroids. Apart from the traditionally monitored steroids, minor metabolites can play an important role to increase the specificity and efficiency of current detection methods. The applicability of several minor steroid metabolites was tested on administration studies with low doses of oral testosterone (T), T gel, dihydrotestosterone (DHT) gel and oral dehydroepiandrosterone (DHEA). The collected data for all monitored parameters were evaluated with the respective population based reference ranges. Besides the traditional markers T/E, T and DHT, minor metabolites 4-OH-Adion and 6α-OH-Adion were found as most sensitive metabolites to
detect oral T administration. The most sensitive metabolites for the detection of DHEA were identified as 16alpha-OH-DHEA and 7beta-OH-DHEA but longest detection up to three days (after oral administration of 50 mg) was obtained with non-specific 5beta-steroids and its ratios. Steroids applied as a gel had longer effects on the metabolism but were generally not detectable with universal decision criteria. It can be concluded that population based reference ranges show limited overall performance in detecting misuse of small doses of natural androgens. Although some minor metabolites provide additional information for the oral testosterone and DHEA formulations, the topical administered steroids could not be detected for all volunteers using universal reference limits. Application of other population based threshold limits did not lead to longer detection times [10307].

Steroid profiling is one of the most versatile and informative screening tools for the detection of steroid abuse in sports drug testing. Concentrations and ratios of various endogenously produced steroidal hormones, their precursors and metabolites including testosterone, epitestosterone, dihydrotestosterone (DHT), androsterone, etiocholanolone, dehydroepiandrosterone (DHEA), 5alpha-androstane-3alpha,17beta-diol, and 5beta-androstane-3alpha,17beta-diol as well as androstenedione, 6alpha-OH-androstenedione, 5beta-androstane-3alpha,17alpha-diol, 5alpha-androstane-3alpha,17alpha-diol, 3alpha,5-cyclo-5alpha-androstane-6beta-ol-17-one, 5alpha-androstenedione, and 5beta-androstenedione add up to a steroid profile that is highly sensitive to applications of endogenous as well as synthetic anabolic steroids, masking agents, and bacterial activity. Hence, the knowledge of factors that do influence the steroid profile pattern is a central aspect, and pharmaceutical (application of endogenous steroids and various pharmaceutical preparations), technical (hydrolysis, derivatization, matrix), and biological (bacterial activities, enzyme side activities) issues are reviewed [08184].

Prevalence of misuse

The prevalence of anabolic-androgenic steroids use has risen dramatically over the last two decades and filtered into all aspects of society. Support for AAS users has increased, but not by the medical profession, who will not accept that AAS use dependency is a psychiatric condition. Polypharmacy by self-prescription is prevalent in this sector. Most recently, AAS use has filtered through to "recreational street drug" users and is the largest growth of drugs in this subdivision. There is a degree of contentiousness in the scenario of anabolic-androgenic steroids drug use, both within and outside sport. AAS and associated doping agents are not illegal per se. Possession is not an offence, despite contravening sporting regulations and moral codes. Until AAS are classified in the same capacity as street drugs in the UK, where possession becomes a criminal offence, they will continue to attract those who want to win at any cost [08126].

Dependence

Anabolic-androgenic steroids (AAS) are widely used illicitly to gain muscle and lose body fat. Here it was reviewed the accumulating human and animal evidence showing that AAS may cause a distinct dependence syndrome, often associated with adverse psychiatric and medical effects. It was presented an illustrative case of AAS dependence, followed by a summary of the human and animal literature on this topic, based on publications known to us or obtained by searching the PubMed database. About 30 percent of AAS users appear to develop a dependence syndrome, characterized by chronic AAS use despite adverse effects on physical, psychosocial or occupational functioning. AAS dependence shares many features with classical drug dependence. For example, hamsters will self-administer AAS,
even to the point of death, and both humans and animals exhibit a well-documented AAS withdrawal syndrome, mediated by neuroendocrine and cortical neurotransmitter systems. AAS dependence may particularly involve opioidergic mechanisms. However, AAS differ from classical drugs in that they produce little immediate reward of acute intoxication, but instead a delayed effect of muscle gains. Thus standard diagnostic criteria for substance dependence, usually crafted for acutely intoxicating drugs, must be adapted slightly for cumulatively acting drugs such as AAS. It was concluded that AAS dependence is a valid diagnostic entity, and probably a growing public health problem. AAS dependence may share brain mechanisms with other forms of substance dependence, especially opioid dependence. Future studies are needed to characterize AAS dependence more clearly, identify risk factors for this syndrome and develop treatment strategies [09054].

Influence on reaction on pain

The purpose of one study was to investigate the effects of acute and chronic administration of anabolic-androgenic steroids (AAS) on nociception and morphine antinociception in acute pain models, as well as on chronic inflammatory nociception. In Experiment 1, adult, gonadally intact male rats were injected s.c. for 28 days with either 5mg/kg testosterone (T), dihydrotestosterone (DHT), stanozolol (STAN), or safflower oil vehicle (n=12-25/group). On day 28, rats in each group were tested on acute thermal and mechanical nociceptive assays, before and after morphine treatment. In Experiment 2, rats in each group (n=8-10/group) were injected with mineral oil or complete Freund's adjuvant (CFA) into one hindpaw after 28 days of AAS treatment, and then tested for thermal hyperalgesia, mechanical allodynia, inflammation and locomotor suppression intermittently for 28 days. Experiment 3 replicated nociceptive measurements in Experiments 1 and 2, but with a single AAS or vehicle injection occurring 3h prior to testing (n=10-12/group). While chronic AAS administration tended to decrease body weight gain and alter reproductive organ weights in the expected manner, it did not significantly alter acute nociception nor attenuate the development of various chronic pain indices after CFA administration. Morphine antinociceptive potency was significantly decreased by chronic DHT on the hotplate test only. Acute AAS administration also did not significantly alter acute or chronic nociception, or morphine antinociceptive potency. Comparisons between acute and chronic AAS administration suggest that steroid tolerance did not occur in rats treated with AAS chronically. Taken together, these data do not support the hypothesis that AAS exposure alters nociception or morphine antinociception in gonadally intact males [11071].

Overview of detection of AAS abuse

AAS misuse can be conventionally detected by steroid profiling including precursors and metabolites as well the urinary testosterone/epitestosterone (T/E) ratio, of which normal levels are below or up to 4.0. Any sample displaying levels above this threshold should be quantitatively analyzed for confirmation by tandem gas chromatography/mass spectroscopy (GC/MS). Guidelines for confirmatory analysis by GC/MS and LC/MS/MS have been released by WADA. High-performance liquid chromatography-tandem mass spectrometry (LC/MS/MS), also employed in recent years in forensic toxicology, has been established as a powerful and reliable tool for quantitative confirmatory analysis of drugs used for doping. Epitestosterone administration is prohibited because it lowers the urinary testosterone/epitestosterone ratio, a marker of testosterone administration. However, use of a gas chromatography–combustion–isotope ratio mass spectrometry method resulted in quantification of the delta C values for urinary epitestosterone as high in controls and lower in
the epitestosterone group. A two-step derivatization procedure has recently been introduced to enhance performance of electrospay ionization liquid chromatography-mass spectroscopy in detecting ASS, these being compounds that notably possess limited ionization efficiency. Nevertheless, the newly initiated approach based on high resolution/high accuracy MS and ion mobility has the capacity to analyze the gas phase dissociation behaviour of several new drugs. The thus enabled cartography of fragmentation routes of new compounds may permit a more rapid identification of metabolites and “tailor-made” analogues developed for doping purposes. The detection of doping is moving away from checking for quantified exposure to prohibited substance towards biologic assays detecting an effect of prohibited substances. Cell-based biological assays comprise the future generation of assays which should be implemented by anti-doping laboratories to detect presence of androgenic anabolic steroids and other human AR ligands as well as assess the biological activity when the structure of the compound is not known. Another method, the metabolomics-based approach that was introduced as a high-tech strategy to determine the anabolic steroid urine profile in livestock production, is an illicit use of natural steroids and is moreover hard to prove since the metabolites are unknown. Despite the present lack of compelling data, metabolomics, involving study of the fingerprints of ASS metabolites, is likely to be added to the arsenal of anti-doping methods and control programs [12011].

Ratio between testosterone and epitestosterone

In addition to the development of secondary sex characteristics, testosterone (T) has anabolic effects including increases in muscle size and strength and increases in lean body mass. In the case of exogenous administration of testosterone, the ratio of testosterone to its isomer, epitestosterone (E), is elevated. WADA has set a standard for T/E ratios of 4.0 as indicative of possible exogenous testosterone administration. Typically, a sample that screens for a T/E ratio above that threshold is then subjected to quantitative confirmation by GC/MS. This methodology, however, can limited due to sensitivity issues as well as a limited number of qualifying ions that can be used for unambiguous identification. It was therefore developed a confirmation method which uses liquid/liquid extraction, followed by room temperature Girard P derivatization, and analysis using LC/MS-ToF. Analysis time is decreased. Sensitivity is increased, resulting in limits of detection of 2 and 0.5 ng/ml for testosterone and epitestosterone, respectively. The number of diagnostic qualifier ions is also increased allowing more confident identification of the analytes. Finally, while this method has been developed on a QToF instrument, it should be easily transferable to any tandem LC/MS/MS system [08181].

In an effort to control androgen use for enhanced sport performance, WADA screens biological samples for the presence of androgens, metabolites, and/or masking agents. Professional athletes are tested both during, and before, competition. There are a number of routine screening tests that are used to detect exogenous androgen administration, including measuring the testosterone to epitestosterone (T/E) ratio by GC-MS. Epitestosterone is a co-secreted product of T and normally is present in urine at levels similar to testosterone. If exogenous T is administered this will elevate the T level, but not the epitestosterone level. This test is complicated by the fact that the values of testosterone and epitestosterone vary greatly between individuals, and therefore, any sample that meets any one of the following criteria will be sent off for further analysis using Isotope Ratio Mass Spectrometry (IRMS): T/E value greater than or equal to 4, concentration of T or E greater than 200 ng/mL, concentration of androsterone or etiocholanolone greater than 10,000 ng/mL, or concentration of DHEA greater than 100 ng/mL [13084].
If a sample is submitted for further evaluation by IRMS, the $^{13}\text{C}/^{12}\text{C}$ ratio of the androgen will be measured. This is because commercially produced androgens will have higher $^{13}\text{C}$ levels compared with endogenous androgens. Synthetic androgen use is screened by GC-MS. This is possible because each synthetic androgen has a distinctive chemical structure on GC-MS that is readily identifiable and can be matched to a catalogue kept by WADA. Even trace amounts of synthetic androgen intake are detectable months after the last administration with GC/MS able to detect concentrations in the pg/ml range. The GC-MS screening tests are very sensitive and specific for known androgens on the WADA list. However, these screening tests cannot provide a complete detection of all androgens because they are unable to detect designer androgens [13084].

**Specific laboratory techniques for anabolic steroids**

The list of prohibited substances of the World Anti-Doping Agency (WADA) classifies the administration of several steroids in sports as doping. Their analysis is generally performed using urine specimen as matrix. Lots of the steroids are extensively metabolised in the human body. Thus, knowledge of urinary excretion is extremely important for the sensitive detection of steroid misuse in doping control. The methods routinely used in steroid screening mainly focus on substances, that are excreted unconjugated or as glucuronides. Common procedures include deconjugation using a beta-glucuronidase enzyme. Following extraction and concentration the analytes are submitted to LC-MS(/MS) analysis and/or GC-MS(/MS) analyses. Besides the classical steroids, more and more products appear on the market for "dietary supplements" containing steroids that have never been marketed as approved drugs, mostly without proper labelling of the contents. To cover the whole range of potential products comprehensive screening tools have to be utilised in addition to the classical methods. Endogenous steroids, e.g. testosterone, represent a special group of compounds. As classical chemical methodology is incapable of discriminating synthetic hormones from the biosynthesised congeners, the method of steroid profiling is used for screening purpose. Additionally, based on isotope signatures a discrimination of synthetic and natural hormones can be achieved [10339].

A simple, rapid and sensitive method was developed for determining the presence of seven anabolic steroids (boldenone, nandrolone, testosterone, methyltestosterone, epiandrosterone, androsterone, and atnozolol) in human urine. Glucuronide-conjugates of these compounds were hydrolyzed with beta-glucuronidase. The anabolic steroids were analyzed by on-line in-tube solid-phase microextraction (SPME) coupled with liquid chromatography-mass spectrometry (LC-MS). The steroids were separated within 14 min by high performance liquid chromatography using a Chromolith RP-18e column and 5 mM ammonium formate/methanol (35/65, v/v) as a mobile phase at a flow rate of 1.0 mL/min. Electrospray ionization conditions in the positive ion mode were optimized for the MS detection of these compounds. The optimum in-tube SPME conditions were 20 draw/eject cycles with a sample size of 40 microL using a Supel-Q PLOT capillary column for the extraction. The extracted compounds could be desorbed readily from the capillary column by flow of the mobile phase, and no carryover was observed. Using the in-tube SPME LC-MS with SIM mode detection, good linearity of the calibration curve ($r>0.995$) was obtained in the concentration range of 0.5-20 ng/mL, except for stanozolol. The detection limits ($S/N=3$) of anabolic steroids were in the range 9-182 pg/mL and the proposed method showed 20-33-fold higher sensitivity than the direct injection method. The within-day and between-day precisions were below 4.0 and 7.3 percent (n=5), respectively. This method was applied successfully to the analysis of urine samples without the interference peaks. The recovery rates of anabolic steroids spiked into urine samples were above 85 percent. This method is
useful to analyze the urinary levels of these compounds in anti-doping tests [10340].

Triple quadrupole (QqQ), time of flight (TOF) and quadrupole-time of flight (QTOF) analysers have been compared for the detection of anabolic steroids in human urine. Ten anabolic steroids were selected as model compounds based on their ionization and the presence of endogenous interferences. Both qualitative and quantitative analyses were evaluated. QqQ allowed for the detection of all analytes at the minimum required performance limit (MRPL) established by the World Anti-Doping Agency (between 2 and 10 ng/mL in urine). TOF and QTOF approaches were not sensitive enough to detect some of the analytes (3’-hydroxy-stanozolol or the metabolites of boldenone and formebolone) at the established MRPL. Although a suitable accuracy was obtained, the precision was unsatisfactory (RSD typically higher than 20 %) for quantitative purposes irrespective of the analyser used. The methods were applied to 30 real samples declared positives either for the misuse of boldenone, stanozolol and/or methandienone. Most of the compounds were detected by every technique, however QqQ was necessary for the detection of some metabolites in a few samples. Finally, the possibility to detect non-target steroids has been explored by the use of TOF and QTOF. The use of this approach revealed that the presence of boldenone and its metabolite in one sample was due to the intake of androsta-1,4,6-triene-3,17-dione. Additionally, the intake of methandienone was confirmed by the post-target detection of a long-term metabolite [11073].

In routine screening, hormone residues of all known growth promoting agents are detected by immuno assays or chromatographical methods in combination with mass spectrometry. To overcome the detection by these routine screening methods new xenobiotic growth promoters and new ways of application were developed, e.g. the combination of different agents in hormone cocktails are employed. To enable an efficient tracing of misused anabolic substances it is necessary to develop new screening technologies for a broad range of illegal drugs including newly designed xenobiotic anabolic agents. The use of omic technologies like, transcriptomics, proteomics or metabolomics is a promising approach to discover the misuse of anabolic hormones by indirectly detecting their physiological action. With the help of biostatistical tools it is possible to extract the quested information from the data sets retrieved from the omic technologies [09065].
A simple, low cost system for the backflushing of capillary gas chromatography (GC) columns has been investigated and integrated into a method for the detection of anabolic steroids in equine urine. The modification to the method was simple to make and quick to setup and optimize. The use of backflushing technology was found to offer significant benefits in terms of sample throughput and improved system robustness [10067].

A molecularly imprinted polymer (MIP), templated with methyltestosterone, had been synthesized for the cleanup of hydrolyzed urine samples for subsequent testosterone (T) quantification by LC-MS/MS. A concentration of 2 ng/mL testosterone could be quantified after a single step extraction on the MIP. The limit of detection and quantification with the criteria of a signal-to-noise ratio of 3 and 5 were 0.3 and 2 ng/mL, respectively. These values meet the conditions set by the World Anti-Doping Agency for the minimum required performance limits for doping controls, between 2 and 10 ng/mL. Epitestosterone (E) was also separated on this polymer and could be detected at concentrations down to 0.3 ng/mL. The quantification of T and E gives access to the determination of the T/E ratio, essential in doping analysis. Hence, the polymers can offer a more specific extraction procedure, resulting in increased sensitivity with limits of detection 10 times lower than the ones achieved by the standard SPE C(18) sorbents employed in official testing laboratories [10068].

Phase-II metabolism has a major contribution to androgen metabolism, converting the highly non-polar compounds to a more easily excreted form prior to their excretion in urine. In the human body the main phase-II metabolic reactions are glucuronidation and sulphonation. These reactions are catalysed by enzymes, which are categorised into families and further subfamilies based on their function and similarities of their amino-acid sequences. Due to inter-individual variation of the metabolising enzymes and their activities, the metabolic patterns of prohibited substances should be estimated for efficient doping control. In addition to target analytes the phase-II reactions have an effect on the selection of sample preparation procedure, chromatographic technique and ionisation method of the analysis routine. For method development and identification purposes adequate reference material is required, and to replace the laborious in vivo excretion studies, in vitro methodologies have been implemented to produce intact phase-II metabolites of androgens [10069].

A simple, rapid and sensitive method was developed for determining the presence of seven anabolic steroids (boldenone, nandrolone, testosterone, methyltestosterone, epiandrosterone, androsterone, and atnozolol) in human urine. Glucuronide-conjugates of these compounds were hydrolyzed with beta-glucuronidase. The anabolic steroids were analyzed by on-line in-tube solid-phase microextraction (SPME) coupled with liquid chromatography-mass spectrometry (LC-MS). The steroids were separated within 14 min by high performance liquid chromatography using a Chromolith RP-18e column and 5 mM ammonium formate/methanol (35/65, v/v) as a mobile phase at a flow rate of 1.0 mL/min. Electrospray ionization conditions in the positive ion mode were optimized for the MS detection of these compounds. The optimum in-tube SPME conditions were 20 draw/eject cycles with a sample size of 40 microL using a Supel-Q PLOT capillary column for the extraction. The extracted compounds could be desorbed readily from the capillary column by flow of the mobile phase, and no carryover was observed. Using the in-tube SPME LC-MS with SIM mode detection, good linearity of the calibration curve (r>0.995) was obtained in the concentration range of 0.5-20 ng/mL, except for stanozolol. The detection limits (S/N=3) of anabolic steroids were in the range 9-182 pg/mL and the proposed method showed 20-33-fold higher sensitivity than the direct injection method. This method was applied successfully to the analysis of urine samples without the interference peaks. The recovery rates of anabolic steroids spiked into urine samples were above 85 percent. This method is useful to
analyze the urinary levels of these compounds in anti-doping tests [10070].

One work presented the validation study of the comprehensive two-dimensional gas chromatography (GC x GC)-time-of-flight mass spectrometry method performance in the analysis of the key World Anti-Doping Agency (WADA) anabolic agents in doping control. The relative abundance ratio, retention time, identification and other method performance criteria have been tested in the GC x GC format to confirm that they comply with those set by WADA. Furthermore, tens of other components were identified with an average similarity of >920 (on the 0-999 scale), including 10 other endogenous sterols, and full mass spectra of 5,000+ compounds were retained. The testosterone/epitestosterone ratio was obtained from the same run. A new dimension in doping analysis has been implemented by addressing separation improvement. Instead of increasing the method sensitivity, which is accompanied by making the detector increasingly "blind" to the matrix (as represented by selected ion monitoring mode, high-resolution mass spectrometry (MS) and tandem MS), the method capabilities have been improved by adding a new "separation" dimension while retaining full mass spectral scan information. Apart from the requirement for the mass spectral domain that a minimum of three diagnostic ions with relative abundance of 5% or higher in the MS spectra, all other WADA criteria are satisfied by GC x GC operation. The minimum of three diagnostic ions arises from the need to add some degree of specificity to the acquired mass spectrometry data; however, under the proposed full MS scan method, the high MS similarity to the reference compounds offers more than the required three diagnostic ions for an unambiguous identification [10071].

Doping control screening based on the enhanced resolution of comprehensive two-dimensional gas chromatography hyphenated to time of flight mass spectrometer was investigated. The identification of anabolic agents (clenbuterol, norandrosterone, epimetendiol, two methyltestosterone metabolites and 3'-hydroxystanozolol) contained in a spiked urine sample (2 ng/ml) has been demonstrated. Special emphasis was given to 3'-hydroxystanozolol, mainly considering the difficulty in its detection. In contrast to conventional GC-MS approaches that must use single-ion monitoring, the GCxGC-TOFMS method enabled the identification of that metabolite through the deconvolution of the full mass spectrum and also resolved the co-eluted peaks of 3'-hydroxystanozolol and an endogenous Components [08166].

Current threshold levels of steroids do not allow for the detection of all endogenous steroid misuse due to great interindividual variations in urinary steroid concentrations. A method has been developed and validated to screen for traditionally monitored endogenous steroids in doping control as well as specific hydroxylated/oxygenated metabolites in order to enhance the detection capabilities for the misuse of endogenous steroids [08167].

The urinary metabolism of the irreversible aromatase inhibitor androsta-1,4,6-triene-3,17-dione was investigated. It is mainly excreted unchanged and as its 17beta-hydroxy analogue. For confirmation, 17beta-hydroxyandrosta-1,4,6-trien-3-one was synthesized and characterized by nuclear magnetic resonance (NMR) in addition to the parent compound. In addition, several reduced metabolites were detected in the post-administration urines, namely 17beta-hydroxyandrosta-1,4-dien-3-one (boldenone), 17beta-hydroxy-5beta-androst-1-en-3-one (boldenone metabolite), 17beta-hydroxyandrosta-4,6-dien-3-one, and androsta-4,6-diene-3,17-dione. The identification was performed by comparison of the metabolites with reference material utilizing gas chromatography/mass spectrometry (GC/MS) of the underivatized compounds and GC/MS and GC/tandem mass spectrometry (MS/MS) of their trimethylsilyl (TMS) derivatives. Alterations in the steroid profile were also observed, most obviously in the androsterone/testosterone ratio. Even if not explicitly listed, androsta-1,4,6-triene-3,17-dione is classified as a prohibited substance in sports by the World Anti-Doping
Agency (WADA) due to its aromatase-inhibiting properties. In 2006 three samples from human routine sports doping control tested positive for metabolites of androsta-1,4,6-triene-3,17-dione. The samples were initially found suspicious for the boldenone metabolite 17beta-hydroxy-5beta-androst-1-en-3-one. Since metabolites of androst-4-ene-3,6,17-trione were also present in the urine samples, it is presumed that these findings were due to the administration of a product like “Novedex Xtreme”, which could be easily obtained from the sport supplement market [08168].

The detection of steroids originating from synthetic precursors in relation to their chemically identical natural analogues has proven to be a significant challenge for doping control laboratories accredited by WADA. Endogenous steroid abuse may be confirmed by utilising the atomic specificity of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) that enables the precise measurement of differences in stable isotope ratios that arise as a result of fractionation patterns inherent in the source of steroids. A comprehensive carbon isotope ratio (delta^{13}C) profiling study (n=1262) of urinary ketosteroids is reported that demonstrates the inter-individual variation that can be expected from factors such as diet, ethnicity, gender and age within and between different populations (13 countries). This delta^{13}C distribution is shown by principal component analysis (PCA) to provide a statistical comparison to delta^{13}C values observed following administration of testosterone enanthate [08169].

The product Orastan-A from Gaspari Nutrition was analyzed for its steroid content. According to the labeling, it is supposed to contain "5a-androstano[2,3-c]furazan-17b-tetrahydropyranol ether", also called furazadrol-THP ether. The GC-MS analyses of the liberated steroids (after extraction from the capsule matrix and cleavage of the THP ether, TMS-derivative andunderivatized) revealed mass spectra of two components, both inconsistent with the labeling. Thus, the steroids were characterized by different analytical techniques such as mass spectrometry, nuclear magnetic resonance spectroscopy and X-ray crystal structure analysis. They were identified as 17beta-hydroxyandrostano[3,2-c]isoxazole and -[2,3-d]isoxazole [08170].

The use of natural and synthetic anabolic steroids in animal fattening has been prohibited in many countries because of their potential toxic effect on human health. One paper describes a newly developed gas chromatography-ion trap-mass spectrometry (GC-IT-MS) method for the quantitative determination of various residual anabolic steroids in meat. Anabolic steroid was derivatized with N-methyl-N-trimethylsilyltrifluoroacetamide prior to GC-IT-MS analysis. MS^2 was employed for quantitative measurement. In addition, 2d-estradiol was used as an internal standard. Quantitative determination was based on the ratio of peak area of steroid derivative to peak area of internal standard derivative. Good linearity of each compound, 0.03-1.0 mug/ml, was determined. Solvent extraction was used to extract residual anabolic compounds in meat samples and a solid phase extraction procedure was utilized for sample cleanup and pre-concentration. The limits of detection of anabolic compounds approximately ranged from 0.1 to 0.4 mug/kg. The detection limit was comparable with or better than reported methods and was below the minimum required performance limits established by the European Community (EC). The application of this newly developed method was demonstrated by analyzing various beef, pork, chicken and several animal internal organ samples from local markets [08171].

Carbon isotope ratio (CIR) analysis of urinary steroids using gas chromatography-combustion isotope ratio mass spectrometry (GCC-IRMS) is a recognized test to detect illicit doping with synthetic testosterone. There are currently no universally used steroid isotopic standards (SIS). It was adapted a protocol to prepare isotopically uniform steroids for use as a calibrant in GCC-IRMS that can be analyzed under the same conditions as used for
steroids extracted from urine. Two separate SIS containing a mixture of steroids were created and coded CU/USADA 33-1 and CU/USADA 34-1, containing acetates and native steroids, respectively. CU/USADA 33-1 contains 5alpha-androst-3beta-ol acetate (5alphaA-AC), 5alpha-androstan-3alpha-ol-17-one acetate (androsterone acetate, A-AC), 5beta-androstan-3alpha-ol-11, 17-dione acetate (11-ketoetiocholanolone acetate, 11k-AC) and 5alpha-cholestane (Cne). CU/USADA 34-1 contains 5beta-androstan-3alpha-ol-17-one (etiocholanolone), 5alpha-androstan-3alpha-ol-17-one (androsterone), and 5beta-pregnane-3alpha, 20alpha-diol (5betaP). Each mixture was prepared and dispensed into a set of about 100 ampoules using a protocol carefully designed to minimize isotopic fractionation and contamination. A natural gas reference material, NIST RM 8559, traceable to the international standard Vienna PeeDee Belemnite (VPDB) was used to calibrate the SIS. Absolute delta^{13}C(VPDB) and DeltaDelta^{13}C(VPDB) values from randomly selected ampoules from both SIS indicate uniformity of steroid isotopic composition within measurement reproducibility. This procedure for creation of isotopic steroid mixtures results in consistent standards with isotope ratios traceable to the relevant international reference material [08172].

The applicability of comprehensive two-dimensional gas chromatography (GCxGC) for sterol analysis was investigated by separation and identification of endogenous sterols in standards, and spiked in human urine. The modulation temperature was optimized to achieve the best separation and signal enhancement. The separation pattern of trimethylsilyl derivatives of sterols was compared on two complementary column sets. Whilst the BPX5/BPX50 column set offers better overall separation, BPX50/BPX5 provides better peak shape and sensitivity. The average match quality for 19 analysed sterols on the BPX50/BPX5 column set was 950/1000 when searched against the in-house library; only four were identified against the NIST05 library, at a match threshold of 800. The study shows that GCxGC-TOFMS yields high specificity for steroids derived from urine, with detection limits appropriate for use in doping control [08173].

Exogenous testosterone is known to decrease the urinary excretion rate of epitestosterone glucuronide due to suppression of the secretion of luteinizing hormone [08271-08273]. In one study the epitestosterone glucuronide levels decreased after a testosterone injection. There were large interindividual differences, but six days after the testosterone administration 92 percent of all subjects had epitestosterone glucuronide levels below 30 percent of baseline leading to even higher increases of T/E ratios [08011].

By means of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) urinary steroids obtained from a reference population of 56 subjects were analyzed for their C13/C12-ratios. Androsterone and etiocholanolone represent androgen metabolites, whereas 11beta-hydroxyetiocholanolone, 11beta-hydroxyandrosterone, and 5beta-pregnane-3alpha,20alpha-diol have sources independent from androgen metabolism. The deltaC13-values of the latter compounds may be compared to those of androgen metabolites in order to detect doping with synthetic androgens and thus may serve as endogenous reference compounds (ERC). In order to allow for classification of conspicuous samples, reference ranges and limits were calculated for deltaC13-values of selected steroids and differences hereof (DeltaC13-values). When androsterone is compared to ERCs, deltaC13-values larger than 3 per thousand are very unlikely. A set of additional parameters was surveyed by a questionnaire. Several factors turned out to exert significant influence on the deltaC13-values of urin ary steroids. These encompass the identity of the steroid itself, sex, oral contraception, travels, and physical activity [08174].

The detection of new anabolic steroid metabolites and new designer steroids in urine is a challenge in doping analysis. An approach based on precursor ion scanning for the detection
of unknown anabolic steroids and metabolites is proposed. The study of the MS/MS spectra of selected anabolic steroids revealed different fragmentation pathways at low and medium collision energy depending on the steroid structure. However, after analysis at high collision energy three common ions at m/z 105, m/z 91, and m/z 77 were found for all studied anabolic steroids. These ions can be explained by the fragmentation of the steroid structure and corresponded to the methyl tropylium, tropylium, and phenyl ions, respectively. Because of the theoretical low specificity of these ions, the simultaneous presence of all of them was used as a starting point to consider a substance as a possible anabolic steroid. Hence, the developed approach is based on the simultaneous acquisition of the precursor ion scan of m/z 105, 91, and 77. The specificity of this approach has been checked by the injection of several doping agents including beta-agonists, corticosteroids, beta-blockers, and diuretics. In general, only compounds with a steroidal structure showed a signal at all three selected m/z values although some exceptions have been found. The applicability of the method was tested for three different scenarios: the detection of steroid metabolites, the detection of unknown steroids, and the analysis of prohormones. In metabolic studies, several recently reported fluoxymesterone metabolites were also found using this method. For detection of unknown steroids, some negative urine samples were spiked with the designer steroid THG and 33 other anabolic steroids and treated as blind samples. Finally, the applicability of the developed approach for the analysis of dietary supplements was checked by the analysis of a prohormone where several impurities and/or degradation products were found [08175].

In recent years products containing 6alpha-methylandrost-4-ene-3,17-dione have appeared on the sport supplement market. Scientific studies have proven aromatase inhibition and anabolic and mild androgenic properties; however, no preparation has been approved for medical use up to now. In sports 6alpha-methylandrost-4-ene-3,17-dione has to be classified as a prohibited substance according to the regulations of WADA. For the detection of its misuse the metabolism was studied following the administration of two preparations obtained from the Internet (Formadrol and Methyl-1-Pro). Several metabolites as well as the parent compounds were synthesized and the structures of 3alpha-hydroxy-6alpha-methyl-5beta-androstan-17-one, 6alpha-methylandrost-4-ene-3,17-dione, and 5beta-dihydromedroxy-progesterone were confirmed by nuclear magnetic resonance (NMR) spectroscopy. The main metabolite, 3alpha-hydroxy-6alpha-methyl-5beta-androstan-17-one, was found to be excreted as glucuronide and was still detectable in microg/mL amounts until urine collection was terminated (after 25 h). Additionally, samples from routine human sports doping control had already tested positive for the presence of metabolites of 6alpha-methylandrost-4-ene-3,17-dione. Screening analysis can be easily performed by the existing screening procedure for anabolic steroids using 3alpha-hydroxy-6alpha-methyl-5beta-androstan-17-one as target substance (limit of detection <10 ng/mL). Its discrimination from the closely eluting drostanolone metabolite, 3alpha-hydroxy-2alpha-methyl-5alpha-androstan-17-one, is possible as the mono-TMS derivative [08176].

Nine anabolic steroids (andosterone, nandrolone, estradiol, testosterone propionate, nandrolone-17 propionate, dydrogesterone, testosterone, epitestosterone, boldenone) and alpha-cholestanone as internal standard were studied by gas chromatography coupled with mass spectrometry (GC/MS). Anabolic steroids can be derivatised into one or two forms, mainly for andosterone into A-monoTMS and A-diTMS. The aim of one study was to research the optimization conditions of the derivatisation process (maximum yield of silylation reaction) of each anabolic steroid into only one form. The interaction "temperature-reaction time" is significant and has a positive effect on the improvement of the effectiveness of the derivatisation. Considering the large amount of information, often not convergent, a global desirability function was applied for multi-responses optimization. Thus, the optimized temperature and the reaction time of silylation were 85 degrees C and 24 min, respectively. Several GC/MS analytical parameters were also studied: linearity (regression coefficient
upper than 0.99 for each compound, sensibility (range of concentration 0.05-0.30µg/ml).

Confirmatory experiments were applied to check the predicted values and to validate the model. The confirmatory assay responses are relatively close to the responses predicted. It was observed satisfactory resolutions by GC/MS and a run lower than 12 min [08177].

An isocratic HPLC method for the determination with screening purposes of anabolic androgenic steroids (fluoxymesterone, boldenone, nortestosterone, metandrostenedione, norethindrone, methyltestosterone and bolasterone), used as growth promoting agents, in finishing pig feed samples has been developed and validated. The separation was achieved by using a reversed-phase Chromolith RP-18e column at controlled temperature, UV-detection at 245nm and epitestosterone as internal standard. The method development involved optimization of different aqueous-organic mobile phases using methanol or acetonitrile as organic modifiers, flow-rate and temperature. The optimized method was applied to the analysis of anabolic steroids in finishing pig feed samples. The extraction efficiencies, decision limits (CCalpha) and detection capabilities (CCbeta) for these compounds were in the range 83-96 percent, 27-37 and 32-47 per microg/kg range, respectively. The within-laboratory reproducibility at 1, 1.5 and 2 CCbeta concentration levels were smaller than 13, 10 and 8 percent, respectively. Finally, the proposed method was successfully applied to nine different kinds of animal feed [08178].

An approach based on precursor ion scanning for the detection of unknown anabolic steroids and metabolites was proposed. The study of the MS/MS spectra of selected anabolic steroids revealed different fragmentation pathways at low and medium collision energy depending on the steroid structure. However, after analysis at high collision energy three common ions at m/z 105, m/z 91, and m/z 77 were found for all studied anabolic steroids. These ions can be explained by the fragmentation of the steroid structure and corresponded to the methyl tropylium, tropylium, and phenyl ions, respectively. Because of the theoretical low specificity of these ions, the simultaneous presence of all of them was used as a starting point to consider a substance as a possible anabolic steroid. Hence, the developed approach is based on the simultaneous acquisition of the precursor ion scan of m/z 105, 91, and 77. The specificity of this approach has been checked by the injection of several doping agents including beta-agonists, corticosteroids, beta-blockers, and diuretics. In general, only compounds with a steroidal structure showed a signal at all three selected m/z values although some exceptions have been found. The applicability of the method was tested for three different scenarios: the detection of steroid metabolites, the detection of unknown steroids, and the analysis of prohormones. In metabolic studies, several recently reported fluoxymesterone metabolites were also found using this method. For detection of unknown steroids, some negative urine samples were spiked with the designer steroid THG and 33 other anabolic steroids and treated as blind samples. Finally, the applicability of the developed approach for the analysis of dietary supplements was checked by the analysis of a prohormone where several impurities and/or degradation products were found [08179].

A GC-MS method for the determination of anabolic steroids used as growth promoting agents using SIM in piglet feed samples has been developed and validated, using testosterone as internal standard. The formation of volatile steroid derivatives was carried out by derivatization with N-methyl-N-(trimethylsilyl)trifluoroacetamide. The optimum separation was achieved using a Zebron ZB-5 column under a gradient temperature elution, allowing the separation of steroids in 18 min. The extraction efficiencies, CCalpha and CCbeta for these compounds were in the ranges 78-98 percent, 10-21 and 18-35 µg/kg, respectively. The repeatability and the within-laboratory reproducibility at 1, 1.5, and 2 CCbeta concentration levels were smaller than 8.2, 7.5, and 5.8 percent and 12.2, 9.5, and 7.5 percent, respectively. Accuracy was in the 99-103 percent range. The robustness was evaluated using the Youden robustness test. The proposed method was applied to the
One paper presents the development, optimization and validation of a methodology to determine nine key steroid hormones (viz. pregnenolone, progesterone, dehydroepiandrosterone, androstenedione, testosterone, dihydrotestosterone, estrone, 17alpha-estradiol and 17beta-estradiol) expressed in the steroidogenesis in biological fluids. The analytical method allows for the determination of steroid hormones in blood plasma and serum down to 0.08-0.16 ng/mL for estrogens, 0.20-0.36 ng/mL for androgens and 0.36-0.43 ng/mL for progestagens. These limits of detection were obtainable using a two-step solid-phase clean-up for fractionation and elimination of interfering lipids (fatty acids, phospholipids, glycerides and sterols) from the steroid hormones. The accuracy of the method was 50-112 percent in the range 0.10 to 2.00 ng/mL [11075].

Trimethylsilylation of anabolic agents and their metabolites is frequently achieved by using the derivatization mixture N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA)/NH4I/2-mercaptetoethanol. Nevertheless, artifacts were formed when this mixture was employed in the monitoring of exemestane and its main metabolite 17β-hydroxyexemestane prior to GC-MS analysis. These artifacts were identified as the N-methyltrifluoroacetamide (MTFA) and trimethylsiloxyethylmercapto products of the respective trimethylsilyl (TMS) derivatives. Furthermore, artifact formation was evaluated taking the structure (1,4-diene-3-keto-6-exomethylene) of the compounds into account. Although these artifacts are relevant for investigations regarding the derivatization process and may be of interest in many fields, they are detrimental to cope with the requirements of the World Anti-Doping Agency (WADA) in terms of the limits of detection (LODs) required. To overcome this issue, a method using an alternative derivatization was proposed: formation of methyloxime-TMS derivatives through double derivatization using O-methylhydroxylamine/pyridine and MSTFA/TMS imidazole after enzymatic hydrolysis and liquid-liquid extraction. Samples from an excretion study after administration of exemestane to healthy volunteers were analyzed by the proposed method and detection of both exemestane and its main metabolite was possible. This method showed excellent results for both analytes meeting the LODs required for antiestrogenic agents (50 ng/mL) established by WADA. The method was validated for the main metabolite, it was robust and cost-effective for qualitative and quantitative purposes, with LOD and LOQ of 10 ng/mL and 25 ng/mL, respectively [10449].
orally ingested DHEA is preferentially converted into sulphated metabolites and that the renal clearance of sulphated steroids is slow, only the $^{13}$C/$^{12}$C ratio of EpiA demonstrated the potential to prolong the detection time for DHEA misuse [10450].

The use of two separate derivatization procedures with the formation of oxime (hydroxyl ammonium pretreatment) and picolinoyl (mixed anhydride method) derivate of anabolic steroids following HPLC-MS/MS analysis was proposed. The main product ions of obtained derivatives for 21 anabolic steroids were evaluated and fragmentation pathways were compared. The analysis of MS/MS spectra for underivatized steroids versus oxime or picolinoyl derivatives showed that in case of analytes containing conjugated double bonds in sterane core all of the observed MS/MS spectra contained abundant product ions of diagnostic value. The implementation of derivatization procedures to such compounds is useful for upgrading sensitivity or selectivity of the evaluated method. On the other hand, MS/MS spectra of underivatized and oxime analytes without conjugated double bonds in sterane core produce spectra with large amounts of low abundant product ions. Picolinoyl derivatives formation leads to highly specific spectra with product ions of diagnostic value coupled with sensitive and selective analysis at the same time. The intra- and inter-group comparison analysis revealed that fragmentation pathways for underivatized steroids and correspondent oxime derivatives are similar. The obtained oxime and picolinoyl derivatives provided 10-15 times higher ESI response in the HPLC-ESI-MS-selected reaction monitoring (SRM) when compared to those of underivatized molecules in positive HPLC-ESI-MS mode. Due to the laborious sample preparation it was suggested to use the performed strategy for confirmation analysis purposes, metabolic studies or while the identification of new steroids or steroid-like substances [10452].

A relatively selective, chemically and physically robust SPME fiber was developed in a simple way with testosterone-imprinted polymer, and then directly coupled with gas chromatography-mass spectrometry (GC-MS) for selective extraction and analysis of anabolic steroids. The factors influencing polymerization (i.e. cross-linker, polymerization solvent, polymerization time) were optimized in detail and the polymer was characterized by scanning electron microscope, infrared spectrometer and thermogravimetric analyzer. Furthermore, the extraction performance of the MIP-coated SPME fibers such as extraction ability and selectivity was evaluated. Moreover, the interaction mode between target analytes and fiber coating was deducted. Finally, the method for extraction and determination of androstenedione, stanolone, androstenedione and methyltestosterone by the homemade MIP-coated SPME fibers with GC-MS was obtained. It was applied to the simultaneous analysis of four anabolic steroids in the spiked human urine with the satisfactory recoveries [10451].

Testing for the illicit use of AAS consists of measurement of synthetic testosterone and determining the ratio of testosterone to epitestosterone in the urine. A normal ratio in a man rarely exceeds 1.3 and only 1 in 1000 men will have a ratio of 4.12 [07002].

Steroids with large conjugated or cross-conjugated electron systems such as trenbolone and gestrinone or boldenone, respectively, have demonstrated marginal gas chromatographic properties under commonly employed derivatization and analytical conditions. These problems have resulted in relatively high detection limits using GC-MS approaches. However, their particular structures provide them with considerable proton affinities and so they are well suited to LC-MS/MS approaches. Consequently, anabolic steroids as well as glucocorticosteroids that are difficult to assay using GC have been analyzed using LC-MS/MS methods that yield detection limits matching the minimum required performance limits (MRPL) as defined by WADA. Also, the designer steroid tetrahydrogestrinone (THG), which bears the same steroidal nucleus as gestrinone, has been determined using LC-
MS/MS ever since it was discovered by Catlin et al. The fact that THG was not detected for several years illustrates a drawback of the doping control screening protocols usually used, which are based on target analysis. Known drugs and/or metabolic products are determined via precursor/product ion pair measurements, which provide the utmost sensitivity but reduce the analytical result to a limited number of compounds. Drugs that have unknown molecular weights and dissociation pathways under conventional collision-induced dissociation (CID) conditions are provided with a cloak of invisibility and remain undetected. Hence, complementary analyses have been suggested that are based on precursor ion scanning of the product ions that characterize particular steroid structures or that utilize androgen bioassays in concert with high resolution MS (HRMS), allowing broader views of urinary steroids. These proposals provide a deeper insight into potentially misused anabolic androgenic steroids, but they can still not ensure the determination of surreptitiously altered steroids prepared solely for doping purposes, as primarily metabolic reactions may reduce or even impede the effectiveness of these assays [07050].

A qualitative liquid chromatography-tandem mass spectrometry method for the analysis of 22 sporting federation-banned anabolic agents (or their metabolite markers) and anti-estrogens in urine that are refractory to analysis by gas chromatography-mass spectrometry is presented. In addition, a quantitative method built around World Anti-Doping Agency (WADA) guidelines for the confirmatory analysis of 19-norandrosterone, the primary metabolite of nandrolone with a WADA-specified minimum required performance limit of 1 ng/mL, is included. Hydrolysis of glucuronide conjugates, liquid-liquid extraction, no clean-up derivatization with Girard's Reagent P, and analysis by quadrupole-time-of-flight mass spectrometry provide sensitivity and selectivity well beyond that required by the WADA [07079].

Characteristic of the preceding WADA prohibited lists, anabolic agents (in particular anabolic-androgenic steroids, AAS) are most frequently reported concerning adverse analytical findings in doping control samples. Despite the well-documented health risks attributed to the abuse of AAS and the reoccurring case reports of AAS-associated fatalities, the attraction of anabolic agents seems to be unconfined among cheating athletes. Consequently numerous studies have been conducted to improve anti-doping efforts concerning this prime category of substances monitored in sports drug testing programs. Enhanced/expanded screening methods, improved steroid profiling approaches, new/complementary confirmation assays based on either conventional mass spectrometric methodologies or isotope-ratio mass spectrometry (IRMS) [13012].

Androgenic anabolic steroids (AAS) are prohibited in sports due to their anabolic effects. Doping control laboratories usually face the screening of AAS misuse by target methods based on MS detection. Although these methods allow for the sensitive and specific detection of targeted compounds and metabolites, the rest remain undetectable. This fact opens a door for cheaters, since different AAS can be synthesized in order to evade doping control tests. This situation was evidenced in 2003 with the discovery of the designer steroid tetrahydrogestrinone. One decade after this discovery, the detection of unknown AAS still remains one of the main analytical challenges in the doping control field. Although important steps have been made in order to minimize this analytical problem and different analytical strategies have been proposed, there are still some drawbacks related to each approach [13163].

Initial testing procedures
Gas chromatography-(tandem) mass spectrometry, GC-MS/(MS) has been the primary tool for analytical approaches aiming at steroidal agents (with few exemptions) for decades. Nevertheless, small but relevant modifications to established assays have been applied to
tweak methods and gain a competitive edge, for example in terms of sensitivity, robustness, or specificity. Employing conventional sample preparation and chromatography strategies as well as established target analytes, the use of the triple-quadrupole mass analyzer enabled LODs for clenbuterol at 0.01 ng/ml and for the steroidal agents between 0.2 and 1 ng/ml on a routine basis. In order to strengthen and expand the detection capabilities of initial testing procedures particularly regarding the extension of detection windows, in-depth investigations revealing potential long-term metabolites of anabolic agents are of great importance [13012].

**Sweeping-MEKC sample concentration technique**

A reliable, convenient, and sensitive on-line sweeping-MEKC sample concentration technique has been applied for the simultaneous separation of six steroids including two pairs of epimer with 10mM phosphate buffer (pH 7.0) that contains 80 mM sodium dodecyl sulfate (SDS), 14 mM beta-cyclodextrin (beta-CD), and 4 percent (v/v) methanol. The column length was 105 cm (effective length, 90 cm). Samples were hydrostatically injected for 600 s. The separation was performed at ambient temperature under an applied voltage of 25kV. The external standard calibration curves of the six steroidal hormones proved good linearity within the concentration range 0.025-1.0 μg/mL. The limit of detections of the on-line sweeping-MEKC with the ultraviolet detector at 220nm for estrone, alpha-estradiol, beta-estradiol, androstenedione, epitestosterone, and testosterone were 10, 24, 28, 53, 73, and 11 ng/mL and were 240, 125, 93, 47, 32, and 200 times more sensitive than MEKC, respectively. The Saccharomyces cerevisiae mediated simultaneous stereoselective reduction of estrone and androstenedione exhibited a 100 percent stereoselectivity toward beta-estradiol and testosterone. The accuracy and precision achieved for the spiking experiments of the sweeping-MEKC were 95-98 beta and less than 3.8 percent (RSD), respectively [11573].

**Bayesian based screening**

In elite sports, indirect testing of testosterone abuse is mainly based on the testosterone over epitestosterone (T/E) ratio. Since this marker is characterized by a small ratio of intra- to inter-individual variation, it is surprising that current anti-doping strategy uses a screening test based on a population-based limit. From a database of more than 15,000 steroid profiles obtained from routine controls, the collection of steroids profiles of 11 elite athletes followed during 2 years, and a longitudinal study involving 17 amateur athletes, 8 of which were orally administrated testosterone undecanoate pills, we selected 12 case studies to represent the possible scenarios to which the anti-doping laboratories are confronted. Various detection strategies at the disposal of the laboratories are employed and discussed, including isotope ratio mass spectrometry (IRMS) analysis and a Bayesian interpretation of the T/E-time profile. The weak sensitivity versus specificity relation of a population-based limit for the T/E ratio is outlined. As a result, it was propose a Bayesian screening test whose T/E threshold progressively evolves from a population basis to a subject basis as the number of individual test results increases. It was found that this screening test heightens drastically the capacity to detect testosterone abuse, at no additional financial and administrative expenses for anti-doping authorities [07080].

It was developed a test that compares sequential measurements of a biomarker against previous readings performed on the same individual. A probability mass function expresses prior information on interindividual variations of intraindividual parameters. Then, the model progressively integrates new readings to more accurately quantify the characteristics of the individual. This Bayesian framework generalizes the two main approaches currently used in forensic toxicology for the detection of abnormal values of a biomarker. The specificity is independent of the number n of previous test results, with a model that gradually evolves
from population-derived limits when \( n = 0 \) to individual-based cutoff thresholds when \( n \) is large. We applied this model to detect abnormal values in an athlete's steroid profile characterized by the testosterone over epitestosterone (T/E) marker. A cross-validation procedure was used for the estimation of prior densities as well as model validation. The heightened sensitivity/specificity relation obtained on a large data set shows that longitudinal monitoring of an athlete's steroid profile may be used efficiently to detect the abuse of testosterone and its precursors in sports. Mild assumptions make the model interesting for other areas of forensic toxicology [07081].

**Purity certified reference materials**

The need for certified reference materials (CRM) of anabolic-androgenic steroids reference materials was emphasized by the Beijing 2008 Olympic game as a tool to improve comparability, ensuring accuracy and traceability of analytical results for competing athletes. The China National Institute of Metrology responded to the state request by providing seven anabolic-androgenic steroids (AAS) reference materials for Beijing Olympic anti-doping. It was described the production of the series of AAS CRMs, according to ISO Guides 34 and 35, which comprises the material processing, homogeneity and stability assessment, CRMs' characterization including moisture content, trace metal content. The AAS' purity values were assigned with collaborative study involved eight laboratories applying high resolution liquid chromatography-diode array detector (HPLC-DAD). Homogeneity of the AAS CRMs were determined by an in-house validated liquid chromatographic methodology. Potential degradation during storage was also investigated and a shelf-life based on this value was established. The certified values of CRMs were 99.8, 99.8, 99.6, 99.7, 98.8, 96.3, and 99.7 percent for methyltestosterone, testosterone propionate, nandrolone, nandrolone 17-propionate, boldenone, trenbolone acetate and testosterone respectively. It was concluded that the certified values for all the studied AAS reference materials are traceable to the international system of units (SI). The CRMs developed were applied by 32 laboratory including sports organizations and analytical laboratories during the 2008 Olympic game for anti-doping control [12150].

**Steroid profiling**

Steroid profile analyses represent an important resource of information concerning both the administration of natural (endogenous) steroids as well as those of xenobiotic origin. Steroid profiling has been utilized in sports drug testing for more than three decades and still much effort is invested in elaborating and improving this valuable tool, particularly to increase its screening efficiency and to allow for consideration of more recently clarified (genetically or pharmacologically induced) variations influencing the steroid profile interpretation [13009].

GC-MS/(MS)-based methods with electron ionization (EI) are still preferred over alternative options to produce steroid profile data; nevertheless, the utility of chemical ionization (CI) in combination with comprehensive 2-dimensional GC (GCxGC) and a fast-scanning quadrupole-MS was evaluated and found to be competitive with commonly used GC-MS benchtop systems concerning steroid quantification. The advantage of this approach was mentioned to be the superior GCxGC separation of analytes with full-scan EI-MS data recording, which supports the detection of presumably unknown anabolic agents. Here, the employed model steroids were measured mainly underivatized or acetylated, which is common to IRMS analyses but (yet) seldom to generic steroid screening assays [13012].

**Alkaline hydrolysis of steroid metabolites**
The alkaline hydrolysis (as opposed to commonly employed enzymatic deconjugation) of steroid metabolites has recently revealed additional analytes serving as potential markers for the abuse of natural steroids. The utility of these markers concerning the detection of orally administered testosterone undecanoate (120 mg) or dehydroepiandrosterone (DHEA) as well as transdermally applied dihydrotestosterone (DHT) or testosterone (T) was presented. Prolonged detection windows for testosterone undecanoate administration were recognized particularly when employing androsta-1,4-dien-3,17-dione (ADION) as one variable of the monitored steroid metabolite ratios. In cases of transdermal DHT and oral DHEA application, no advantage over established steroid profile ratios was observed; however, the detection of trandermal administered T was substantially improved when the ratio of ADION and androst-15-en-3,17-dione (15-AD) was monitored [13012].

Stacking method of repetitive large volume sample injection

In one research, a novel stacking capillary electrophoresis method, repetitive large volume sample injection and sweeping MEKC (rLVSI-sweeping MEKC) were developed to analyze the presence of three androgenic steroids considered as sport doping drugs, testosterone (T), epitestosterone (E) and epitestosterone glucuronide (EG) in urine. This method provides better sensitivity enhancement than the traditional large volume sample stacking-sweeping strategies due to sensitivity enhancement by repetitive injections. This multiple sampling method enhances sensitivity of monitoring of urine samples by UV detection (254 nm).

Firstly, the phosphate buffer was filled into an uncoated fused silica capillary and the samples were injected into the capillary at 10 psi for 20s, and then stacked at -10 kV for 1 min using phosphate buffer containing SDS. The above injecting and stacking steps were repeated five times. Finally, separation was performed at -20 kV, using phosphate buffer containing methanol, SDS and (2-hydroxypropyl)-beta-cyclodextrin. Method validation showed that calibration plots were linear over a range of 5-200 ng/mL for T, 20-200 ng/mL for E and 0.5-500 ng/mL for EG. The limits of detection were 1.0 ng/mL for T, 5.0 ng/mL for E and 200.0 pg/mL for EG. When evaluating precision and accuracy, values of RSD and RE in intra-day (n=3) and inter-day (n=5) analysis were found to be less than 10.0 percent.

Compared with the simple LVSS-sweeping, which is also a stacking strategy, this method further improves sensitivity up to 25 folds (about 2500 folds with MEKC without preconcentration). This method was applied to monitor 10 athletes' urine, and did not detect any analyte. The novel stacking method was feasible for monitoring of doping by sportsmen [12156].

The presence of microorganisms in urine samples, under favourable conditions of storage and transportation, may alter the concentration of steroid hormones, thus altering the correct evaluation of the urinary steroid profile in doping control analysis. According to the rules of the World Anti-Doping Agency (WADA technical document TD2004 EAAS), a testosterone deconjugation higher than 5 percent and the presence of 5alpha-androstane-3,17-dione and 5beta-androstane-3,17-dione in the deconjugated fraction, are reliable indicators of urine degradation. The determination of these markers would require an additional quantitative analysis since the steroids screening analysis, in anti-doping laboratories, is performed in the total (free+conjugated) fraction. The aim of this work is therefore to establish reliable threshold values for some representative compounds (namely 5alpha-androstane-3,17-dione and 5beta-androstane-3,17-dione) in the total fraction in order to predict directly at the screening stage the potential microbial degradation of the urine samples. Preliminary evidence on the most suitable degradation indexes has been obtained by measuring the urinary concentration of testosterone, epitestosterone, 5alpha-androstane-3,17-dione and 5beta-androstane-3,17-dione by gas chromatography-mass spectrometric every day for 15 days in the deconjugated, glucuronide and total fraction of 10 pools of urines from 60 healthy
subjects, stored under different pH and temperature conditions, and isolating the samples with one or more markers of degradation according to the WADA technical document TD2004EAAS. The threshold values for 5α-androstane-3,17-dione and 5beta-androstane-3,17-dione were therefore obtained correlating the testosterone deconjugation rate with the urinary concentrations of 5alpha-androstane-3,17-dione and 5beta-androstane-3,17-dione in the total fraction. The threshold values suggested as indexes of urine degradation in the total fraction were: 10 ng/mL for 5α-androstane-3,17-dione and 20 ng/mL for 5beta-androstane-3,17-dione. The validity of this approach was confirmed by the analysis of routine samples for more than five months (i.e. on a total of more than 4000 urine samples): samples with a concentration of total 5α-androstane-3,17-dione and 5β-androstane-3,17-dione higher than the threshold values showed a percentage of free testosterone higher than 5 of its total amount; whereas free testosterone in a percentage higher than 5 of its total amount was not detected in urines with a concentration of total 5alpha-androstane-3,17-dione and 5beta-androstane-3,17-dione lower than the threshold values [11072].

Minimum package of anabolic steroids in urine

One method comprises the screening of two groups of anabolic compounds, the stilbenes and several steroids. All compounds, inclusive their metabolites when possible, for which gas chromatography-mass spectrometry (GC-MS) currently is the preferred analytical technique, are included. Two different derivatives are prepared. One group, including the stilbenes, is detected as HFB derivative (Method 1), the second group is detected as TMS derivative (Method 2). The method is used to perform a qualitative and semi-quantitative analysis of a minimum package of anabolic steroids to be included in National Residue Control Plans based on Council Directive 96/23 and complies with the current Minimum Required Performance Limits. The method has been validated according to Commission Decision 2002/657/EC. The CCalpha and CCbeta values are based on the detection of the most abundant ion. Results of validation experiments are presented. The method is flexible and due to the non-specific sample clean-up more and new anabolic compounds can be easily added in order to new monitoring requirements [06098].

ELISA

A multianalyte enzyme-linked immunosorbent assay (ELISA) has been developed for the simultaneous detection of anabolic androgenic steroids (AAS) in human serum. The multiplexed method was developed according to a planar strategy in which the analytes are identified by their location in the microtiter plate. In the immunochemical procedure established here, human serum samples are mixed with a cocktail of antibodies and added to the distinct sections of a microplate biofunctionalized with different haptenized biomolecules. The cocktail of antibodies consists of a mixture of polyclonal antibodies raised against stanozolol (ST), boldenone (B), and tetrahydrogestrinone (THG). The whole immunochemical analytical procedure takes around 2 h including sample preparation, and many samples can be processed simultaneously to screen for the presence of the three AAS in a single run. Using this ELISA, ST, B, and THG can be detected and quantified individually. When used as a screening method, due to the cross-reactivity profiles of the immunoreagents used, the presence of up to 11 AAS can be detected simultaneously. The detectabilities achieved by this method in human serum are below the MRPLs (minimum required performance limits) proposed by WADA (World Anti-Doping Agency) and reference laboratories of the European Community [12151].

Liquid chromatography

Liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS)
method for simultaneous and direct detection of 12 glucuronide-conjugated anabolic androgenic steroid (AAS) metabolites in human urine was described. The compounds selected were the main metabolites detected in human urine after dosing of the most widely abused AAS in sports, e.g. methandienone, methenolone, methyltestosterone, nandrolone and testosterone, and certain deuterium-labeled analogs of these metabolites. Sample preparation and the LC-ESI-MS/MS method were optimized, validated, and the overall process was implemented and the results between seven laboratories were compared. All the metabolites were extracted simultaneously by solid-phase extraction (SPE) and analyzed by LC-ESI-MS/MS with positive ionization mode and multiple reaction monitoring (MRM). Recovery of the SPE for the anabolic androgenic steroid glucuronides was 89-100 percent and ten out of twelve compounds had detection limits in the range of 1-10 ng/ml in urine. The results for inter/intraday repeatability were satisfactory and the interlaboratory comparison with authentic urine samples demonstrated the ease of method transfer from one instrument setup to another. When equivalent triple quadrupole analyzers were employed the overall performance was independent from instrument manufacturer, electrospray ionisation (ESI) or atmospheric pressure chemical ionization (APCI) and liquid chromatoohraphic (LC) column, whereas major differences were encountered when changing from one analyzer type to another, especially in the analysis of those AAS glucuronides ionized mainly as adducts [08182].

Gas chromatography-triple quadrupole mass spectrometry

A rapid, sensitive and robust gas chromatography-triple quadrupole mass spectrometry method was developed for the determination of seven anabolic agents in human urine. The selection of analytes includes the main metabolites of all anabolics with higher sensitivity requirements. After optimizing the fragmentation conditions for each compound, a validation procedure for qualitative analysis was performed. The selectivity of the method showed that no interfering peaks were observed at the retention time of the compound. Adequate intermediate precision, below 14 percent, was observed for all of the compounds at the lower concentration tested. The concentrations assayed were in accordance with the performance limits required by the World Anti-Doping Agency (WADA). Unlike a previously published GC/QqQ method, detection of 17alpha-methyl-5beta-androstane-3alpha,17beta-diol (the main metabolites of methyltestosterone) at 2 ng/mL was accomplished under routine conditions. The qualitative method was applied to the analysis of 1367 samples in the span of 2 weeks, as part of the doping control of the XVI Pan American Games which took place in Mexico (14th-30th October, 2011). The high sensitivity was maintained during the analysis of all analytical batches, proving for the first time the excellent ruggedness of GC/QqQ methods [12152].

Liquid chromatography-tandem mass spectrometry method (LC-MS/MS)

The liquid chromatography-tandem mass spectrometry method (LC-MS/MS) was developed and validated to detect androgenic steroids: trenbolone, nortestosterone, boldenone, methylboldenone, testosterone, methyltestosterone, 17beta-1-testosterone and their metabolites in bovine urine. Sample preparation before LC-MS/MS analysis involved an enzymatic hydrolysis with glucuronidase AS-HP, isolation of free hormones from urine on C(18) SPE column and purification of the extract using liquid-liquid extraction with n-pentane and SPE NH(2) column. For the chromatographic separation of steroids, the Poroshell 120-EC C18 column (150 x 2.1 mm, 2.7 microm) was used. Mass spectrometric measurement was achieved using the API 4000 triple quadrupole instrument with a Turbolon-Spray source operating in positive electrospray ionization mode. The procedure was validated according to the Decision 2002/657/EC. Recovery ranged from 77 to 119 percent for all examined
compounds. The repeatability was below 20 percent and reproducibility did not exceed the 25 percent. The linearity was good for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients greater than 0.99. The application of an innovative Poroshell column allowed for very good chromatographic separation of steroids with a much shorter time of analysis [12153].

A fast and sensitive LC-MS/MS method for the quantitative analysis of seven steroid hormones in 150 microL of human serum was developed and validated. The following compounds were included: 17alpha-hydroxyprogrenolone, 17alpha-hydroxyprogesterone, androstenedione, dehydroepiandrosterone, testosterone, pregnenolone, and progesterone. Individual stable isotope-labeled analogues were used as internal standards. Sample preparation was performed by liquid-liquid extraction, followed by oxime derivatization to improve the ionization efficiency of the analytes. In contrast to the common derivatization-based methods, the reaction was incorporated into the sample preparation process and the only additional step due to the derivatization was a short heating of the autosampler vials before the sample injection. Chromatographic separation was achieved on a reversed-phase column using a methanol-water gradient. For the analyte detection, a triple quadrupole instrument with electrospray ionization was used. Total run time was 7.0 min and the lower limits of quantification were in the range of 0.03-0.34 nM (0.01-0.10 ng/mL), depending on the analyte. The method was validated using human serum samples from both sexes and applied for the serum steroid profiling of endometriosis patients [11572].

A specific and sensitive multi-method based on liquid chromatography-tandem mass spectrometry using atmospheric pressure chemical ionization (LC-APCI-MS/MS) has been developed for the determination of 20 anabolic steroids in muscle tissue (diethylstilbestrol, beta-estradiol, ethynylestradiol, alpha/beta-boldenone, alpha/beta-nortestosterone, methyltestosterone, beta-trenbolone, triamcinolone acetonide, dexamethasone, flumethasone, alpha/beta-zearalenol, alpha/beta-zearalanol, zearalenone, melengestrol acetate, megestrol acetate and medroxyprogesterone acetate). The procedure involved hydrolysis, extraction with tert-butyl methyl ether, defattening and final clean-up with solid phase extraction (SPE) on Oasis HLB and Amino cartridges. The analytes were analyzed by reversed-phase LC-MS/MS, in positive and negative multiple reaction monitoring (MRM) mode, acquiring two diagnostic product ions from each of the chosen precursor ions for the unambiguous confirmation of the hormones. The method was validated at the validation level of 0.5ng/g. The accuracy and precision of the method were satisfactory. The decision limits CC-alpha ranged from 0.03 to 0.14ng/g while the detection capabilities CC-beta ranged from 0.05 to 0.24ng/g. The developed method is sensitive and useful for detection, quantification and confirmation of these anabolic steroids in muscle tissue and can be used for residue control programs [09067].

Liquid chromatography/electrospray ion trap mass spectrometry
A fast and selective LC/MS/MS method for the screening of four anabolic steroids in human urine has been developed and validated. Liquid-liquid extraction with diethyl ether was applied after enzymatic hydrolysis. Analyses were performed on an ion trap mass spectrometer equipped with electrospray ionisation. MS/MS was applied for all compounds. The analytical run time was 11 min. The LOD for all compounds varied between 1 and 10 ng/mL. Left-over A samples, which were declared positive by GC/MS for the presence of 3'-hydroxystanozolol, were assessed using the described method [06099].

Ultra high performance liquid chromatography tandem mass spectrometry
The use of doping agents, once restricted to professional athletes, has nowadays become a problem of public health, since it also concerns young people and non-competing amateurs in different sports. The use is also diffused in social life for improving physical appearance
and enhancing performance and even dietary supplements assumed to improve performance often contain anabolic steroids. While decades ago the so-called "classical doping agents" (like stimulants and narcotics) were used, to-day anabolic steroids are more widely diffused. Anabolic steroids are synthetic substances prepared by introducing modifications in the molecular structure of testosterone, the main natural androgenic anabolic steroid that forms in testes interstitial cells. Over the years, always new doping substances are synthesized and, as a consequence, the list of prohibited compounds is continuously updated and new suitable analytical methods for their detection and determination in biological matrices are continuously required. In doping control analysis the knowledge of steroid metabolism pathway in human body is of primary importance and the analytical methods must permit the simultaneous detection and determination not only of the forbidden precursor agents but also of their metabolites. In addition, the potential presence and amount in the biological samples of species that can interfere in the analysis should be evaluated. Also the several anabolic steroids, specifically designed to circumvent doping control, put on the market have been incorporated in the list of the prohibited substances of the World Anti-Doping Agency (WADA). In WADA list steroids figure in three main classes, namely anabolic steroids, corticosteroids and substances with anti-estrogenic properties. It must be strongly reminded that assumption of doping agents not only leads to athletes the possible failing of doping tests but causes important health risk and WADA prohibited list establishes criteria to highlight the alteration of the natural steroid profile caused by exogenous administration. Doping control analyses are generally performed in urine, a matrix that provides a prolonged detection time window, and less often in blood, serum, plasma, hair, saliva, and nails. To identify the chemical structures of anabolic steroids the use of mass spectrometry detection is very advantageous. Gas chromatography-mass spectrometry (GC-MS) techniques allowed for the development of comprehensive screening methods. GC-MS methods are sensitive and robust but present the disadvantages of time-consuming sample pretreatment, that is often based on hydrolysis and derivatisation reactions. Liquid chromatography-mass spectrometry (LC-MS) methods have been successfully used to identify and determinate steroids in different matrices, as well as to study their metabolisms. Nowadays, automatic rapid ultra high performance liquid chromatography (UHPLC) tandem mass spectrometry has become the technique of choice for steroid analysis. Due to its generally higher speed, sensitivity, reproducibility and specificity with respect to HPLC, it can be used to simultaneously separate and determinate multi component steroid mixtures. The technique is of huge interest to separate conjugates anabolic androgenic steroids, as it allows efficiency enhancement due to the small particle (sub-2μm) column packing, which provides high peak capacity within analysis times even 5-10 fold shorter than conventional HPLC methods. Modern multiplex instruments can analyze thousands of samples per month so that, notwithstanding the generally high instrumental costs, the cost of the individual assay is affordable. In addition, the improved specificity and resolution offered by time-of-flight or quadrupole time-of-flight mass spectrometry allow their application in doping control analysis or in steroid profiling for accurate and sensitive full mass range acquisition. Aim of one review was to consider, compare and discuss the applications of the UHPLC/MS methods present in literature for the identification and determination of forbidden steroids and their metabolites in human biological matrices [13004].

**Ligand and structure-based virtual screening**

Parallel ligand- and structure-based virtual screenings of 269 steroids with anabolic activity evaluated in vivo were performed. The quantitative structure-activity relationship (QSAR) model expressed by selected descriptors as the octanol-water partition coefficient, the molar volume and the quantum mechanical calculated charge values on atoms C1, C2, C5, C9, C10, C14 and C17 of the steroid skeleton, expresses structural features of anabolic steroids (AS) contributing to the transport and steroid-receptor interaction. On the other hand,
computational simulations of a candidate ligand binding to a receptor study (a "docking" procedure) predict the association of these AS with the human androgen receptor (AR). Fourteen compounds were identified as lead; the most potent was the 7alpha-methylestr-4-en-3, 17-dione. It was concluded that a good anabolic activity requires hydrogen bonding interactions between both Arg752 and Gln711 residues in the cycles A with O3 atom of the steroid and either Asn705 and Thr877 residues in the cycles D of steroid with O17 atom [13171].

3-Oxo-steroidal agents

Focusing on 3-oxo-steroidal agents (methyltestosterone, nandrolone, boldenone, trenbolone, fluoxymesterone, mesterolone, and bolasterone), a MALDI-MS-based protocol was presented, reporting on the detection of the intact steroids as extracted from spiked urine at 2 ng/mL. Using a proprietary derivatization reagent with hydrazine-based chemistry, substantial sensitivity was accomplished enabling the above mentioned detection limits of the assay; however, as in the aforementioned study on CE and AAS, the target analytes (i.e. the intact drugs) were not an appropriate choice of compounds to provide proof-of-concept for a potential doping control method. Besides the fact that steroid analysis in sports drug testing necessitates an extraordinary comprehensive picture of xenobiotic and natural/endogenous steroids and their metabolites, which is not yet achievable by the MALDI-MS approach, the method would benefit from demonstrating the capability of the developed reagent to convert 17-oxo groups. Aiming at the facilitated data interpretation, the same group presented a software supporting the library-assisted identification of steroidal agents measured by means of MALDI-MS/(MS) [13009].

Stable isotope dilution liquid chromatography electrospray ionization

Prostate cancer is the most frequently diagnosed form of cancer in males in the United States. The disease is androgen driven and the use of orchietomy or chemical castration, known as androgen deprivation therapy (ADT) has been employed for the treatment of advanced prostate cancer for over 70 years. Agents such as GnRH agonists and non-steroidal androgen receptor antagonists are routinely used in the clinic, but eventually relapse occurs due to the emergence of castration-resistant prostate cancer. With the appreciation that androgen signaling still persists in these patients and the development of new therapies such as abiraterone and enzalutamide that further suppresses androgen synthesis or signaling, there is a renewed need for sensitive and specific methods to quantify androgen precursor and metabolite levels to assess drug efficacy. It was described the development, validation and application of a stable isotope dilution liquid chromatography electrospray ionization selected reaction monitoring mass spectrometry (SID-LC/ESI/SRM/MS) method for quantification of serum keto-androgens and their sulfate and glucuronide conjugates using Girard-T oxime derivatives. The method is robust down to 0.2-4 pg on column, depending on the androgen metabolite quantified, and can also quantify dehydroepiandrosterone sulfate (DHEA-S) in only 1 microL of serum. The clinical utility of this method was demonstrated by analyzing serum androgens from patients enrolled in a clinical trial assessing combinations of pharmacological agents to maximally suppress gonadal and adrenal androgens (Targeted Androgen Pathway Suppression, TAPS clinical trial). The method was validated by correlating the results obtained with a hydroxylamine derivatization procedure coupled with tandem mass spectrometry using selected reaction monitoring that was conducted in an independent laboratory [13173].

Microflow tile technology and LC-MS/MS
A novel microfluidic chromatography device coupled with tandem mass spectrometry (LC-MS/MS) was utilized for the multiplex analysis of 5 steroids (testosterone, dihydrotestosterone, progesterone, cortisol, cortisone) in human serum. The use of microfluidics allowed for reduction of the chromatographic flow rate to 3 microl/min with overall method run times comparable to standard flow LC-MS/MS methods reported in the literature, corresponding to a 150 fold decrease in solvent consumption. Furthermore, a simple sample preparation protocol was employed requiring injection of only 0.5 microl of sample, corresponding to a 100-400 fold increase in on-column sensitivity as compared to published standard flow assays. The measured LOQ for both testosterone and progesterone was 0.4 ng/mL, representing an improvement over reported literature values obtained by standard flow methods employing comparable sample preparation and large injection volumes. The LOQs for cortisol (1.9 ng/mL), cortisone (0.3ng/mL), and dihydrotestosterone (1.4 ng/mL) were all within a biologically relevant range. A comparison of clinical serum samples was performed for the analysis of testosterone using this microfluidic LC-MS/MS assay and the Beckman Access II automated antibody-based measurement system. The immunoassay results were systematically higher due to matrix interference which was easily resolved with the increased chromatographic resolution obtained in the microflow LC-MS/MS assay [13174].

**Effects of sample storage condition steroid hormones in saliva**

Measurement of steroid hormones in saliva is increasingly common in elite sport settings. However, this environment may enforce handling and storage practices that introduce error in measurement of hormone concentrations. It was assessed the influence of storage temperature and duration on reproducibility of salivary steroid levels. Nine healthy adults provided morning and afternoon saliva samples on two separate occasions. Each sample was divided into identical saliva aliquots which were stored long-term (i.e. 28 and 84 days) at -80°C or -20°C (testing day 1), and short-term (i.e. 1, 3, 7 and 14 days) at 4°C or 20°C (testing day 2). Samples were analyzed for cortisol, testosterone and estradiol using ELISA. In non-freezer conditions, there was a decrease from baseline to 7 days in testosterone (-26 ± 15 %) and estradiol (-58 ± 17 %) but not cortisol concentrations. This decrease was larger in samples stored at room temperature than in the refrigerator. There were small but significant changes in measured concentrations of all hormones after 28 and/or 84 days of storage in freezer conditions, but these were generally within 12 percent of baseline concentrations, and may be partly explained by inter-assay variability. Whole saliva samples to be analyzed for cortisol, testosterone and estradiol should be frozen at -20°C or below within 24 h of collection, and analyzed within 28 days. Storage of samples for measurement of testosterone and estradiol at temperatures above -20°C can introduce large error variance to measured concentrations [13175].

**IRMS**

Nowadays, the importance of IRMS in sports drug testing is substantial and, as recently concluded, its relevance is continuously growing and new instrumental options allow for enhanced detection assays. In doping controls, IRMS is currently applied mainly to the analysis of natural/endogenous anabolic-androgenic steroids and its metabolites; however, additional candidates such as cortisone or 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) have been subject of recent research projects and are conceivable future target analytes. IRMS represents a comparably complex and challenging analytical methodology that necessitates thorough consideration of information indicating the need for an IRMS-based analysis of a doping control sample and factors influencing the analytical result as well as its interpretation [13009].
Gas chromatography coupled to IRMS

The administration of anabolic steroids is one of the most important issues in doping control and is detectable through a change in the carbon isotopic composition of testosterone and/or its metabolites. Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), however, remains a very laborious and expensive technique and substantial amounts of urine are needed to meet the sensitivity requirements of the IRMS. This can be problematic because only a limited amount of urine is available for anti-doping analysis on a broad spectrum of substances. In this work we introduce a new type of injection that increases the sensitivity of GC-C-IRMS by a factor of 13 and reduces the limit of detection, simply by using solvent vent injections instead of splitless injection. This drastically reduces the amount of urine required. On top of that, by only changing the injection technique, the detection parameters of the IRMS are not affected and there is no loss in linearity [12154].

The application of a comprehensive gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS)-based method for stable carbon isotopes of endogenous urinary steroids was presented. The key element in sample preparation is the consecutive cleanup with high-performance liquid chromatography (HPLC) of underivatized and acetylated steroids, which allows the isolation of ten analytes (11beta-hydroxyandrosterone, 5alpha-androst-16-en-3beta-ol, pregnanediol, androsterone, etiocholanolone, testosterone, epitestosterone, 5alpha-androstane-3alpha,17beta-diol, 5beta-androstane-3alpha,17beta-diol and dehydroepiandrosterone) from a single urine specimen. These steroids are of particular importance to doping controls as they enable the sensitive and retrospective detection of steroid abuse by athletes. Depending on the biological background, the determination limit for all steroids ranges from 5 to 10 ng/mL for a 10 mL specimen. The method is validated by means of linear mixing models for each steroid, which covers repeatability and reproducibility. Specificity was further demonstrated by gas chromatography/mass spectrometry (GC/MS) for each analyte, and no influence of the sample preparation or the quantity of analyte on carbon isotope ratios was observed. In order to determine naturally occurring $^{13}$C/$^{12}$C ratios of all implemented steroids, a reference population (n=61) subjects was measured to enable the calculation of reference limits for all relevant steroidal delta values [08183].

An alternative calibration procedure for the gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) measurements of the World Antidoping Agency (WADA) accredited laboratories is presented. To alleviate the need for externally calibrated CO$_2$ gas for GC-C-IRMS analysis of urinary steroid metabolites, calibration using an external standard mixture solution of steroids with certified isotopic composition was investigated. The reference steroids of the calibration mixture and routine samples underwent identical instrumental processes. The calibration standards bracketed the entire range of the relevant delta$^{13}$C values for the endogenous and exogenous steroids as well as their chromatographic retention times. The certified delta$^{13}$C values of the reference calibrators were plotted in relation to measured m/z $^{13}$CO$_2$/$^{12}$CO$_2$ (i.e. R(45/44)) mass spectrometric signals of each calibrator. delta$^{13}$C values of the sample steroids were calculated from the least squares fit through the calibration curve. The effect of the external calibration on delta$^{13}$C values, using the same calibration standards and set of urine samples but different brands of GC-C-IRMS instruments, was assessed by an interlaboratory study in the WADA Accredited Laboratories of Sydney, Australia and Athens, Greece. Relative correspondence between the laboratories for determination of androsterone, etiocholanolone, 5beta-androstane-3alpha,17beta-diacetate, and pregnanediacetate means were SD(delta$^{13}$C) 0.012, 0.058, -0.034, and -0.040 percent, respectively. These data demonstrate that accurate intralaboratory external
calibration with certified steroids provided by United States Antidoping Agency (USADA) and without external CO$_2$ calibration is feasible and directly applicable to the WADA Accredited Laboratories for the harmonization of the GC-C-IRMS measurements [11076].

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**Gas chromatography-combustion-isotope ratio mass spectrometry**

The administration of anabolic steroids is one of the most important issues in doping control and is detectable through a change in the carbon isotopic composition of testosterone and/or its metabolites. Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), however, remains a very laborious and expensive technique and substantial amounts of urine are needed to meet the sensitivity requirements of the IRMS. This can be problematic because only a limited amount of urine is available for anti-doping analysis on a broad spectrum of substances. In this work we introduce a new type of injection that increases the sensitivity of GC-C-IRMS by a factor of 13 and reduces the limit of detection, simply by using solvent vent injections instead of splitless injection. This drastically reduces the amount of urine required. On top of that, by only changing the injection technique, the detection parameters of the IRMS are not affected and there is no loss in linearity [13170].

The analysis of urinary metabolites of testosterone-related steroids through the measurement of their carbon isotopic signature ($\delta^{13}C$) by gas chromatography combustion/mass spectrometry (GC/C/IRMS) is a confirmation method employed in doping control analyses. Stringent analytical conditions are essential to an accurate and precise analysis as well as the proper selection of the metabolites, which forms the basis of the refined method presented in one paper. In a simplified approach, following enzymatic hydrolysis and extraction from a relatively low volume of urine sample, a one-step high-performance liquid chromatography (HPLC) purification was developed for seven diagnostic urinary metabolites (TS) including testosterone itself, dehydroepiandrosterone, 5alpha- and 5beta-androstenediol, epitestosterone, androsterone, etiocholanolone and two endogenous reference compounds (ERC), 5beta-pregnanediol and 5alpha-androst-16-en-3beta-ol. These steroids were pooled in three fractions and analyzed as such. With regards to the GC/C/IRMS analysis, a multi-level isotopic calibration using the 'identical treatment' principle
was created. The proposed isotopic calibration yielded results for purified reference steroids with a precision ≤0.15 and accuracy of ≤0.030 percent (between-assay, n=26). Compared to other common endogenous reference compounds, those selected in this study had delta^{13}C values close to the target metabolites which, along with the proposed isotopic calibration, produced narrow reference intervals within ±0.3 percent for most diagnostic TS-ERC pairs, in compliance with the requirements of the World Anti-Doping Agency. These carefully controlled analytical conditions are compatible with routine operations, affording accurate and precise results for the more diagnostically relevant metabolites such as testosterone itself and the 5alpha- and 5beta-androstanediols. The values of the TS-ERC pairs measured in reference populations are described and the results from the routine testing of several hundreds of athletes' samples are discussed. Robust, this technique permitted the detection of adverse findings that would have been missed had these low level metabolites not been analyzed [13172].

**Gas chromatographic/time-of-flight mass-spectrometric**

The method of high sensitive gas chromatographic/time-of-flight mass-spectrometric (GC/TOF-MS) analysis of steroids was developed. Low-resolution TOF-MS instrument (with fast spectral acquisition rate) was used. This method is based on the formation of the silyl derivatives of steroids; exchange of the reagent mixture (pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)) for tert-butylmethylether; offline large sample volume injection of this solution based on sorption concentration of the respective derivatives from the vapour-gas mixture flow formed from the solution and inert gas flows; and entire analytes solvent-free concentrate transfer into the injector of the gas chromatograph. Detection limits for 100 microL sample solution volume were 0.5-2 pg/µL (depending on the component). Application of TOF-MS model 'TruTOFcoupled with gas chromatograph and ChromaTOF software (Leco, St Joseph, MO, USA) allowed extraction of the full mass spectra and resolving coeluted peaks. Due to use of the proposed method (10 microL sample aliquot) and GC/TOF-MS, two times more steroid-like compounds were registered in the urine extract in comparison with the injection of 1 microL of the same sample solution [11074].

**Carbon isotope ratio (CIR)**

Carbon isotope ratio (CIR) analysis has been routinely and successfully used in sports drug testing for many years to uncover the misuse of endogenous steroids. One limitation of the method is the availability of steroid preparations exhibiting CIRs equal to endogenous steroids. To overcome this problem, hydrogen isotope ratios (HIR) of endogenous urinary steroids were investigated as a potential complement; results obtained from a reference population of 67 individuals are presented herein. An established sample preparation method was modified and improved to enable separate measurements of each analyte of interest where possible. From the fraction of glucuronidated steroids; pregnanediol, 16-androstenol, 11-ketoetiocholanolone, androsterone (A), etiocholanolone (E), dehydroepiandrosterone (D), 5alpha- and 5beta-androstanediol, testosterone and epitestosterone were included. In addition, sulfate conjugates of A, E, D, epiaandrosterone and 17alpa- and 17beta-androstanediol were considered and analyzed after acidic solvolysis. The obtained results enabled the calculation of the first reference-population-based thresholds for HIR of urinary steroids that can readily be applied to routine doping control samples. Proof-of-concept was accomplished by investigating urine specimens collected after a single oral application of testosterone-undecanoate. The HIR of most testosterone metabolites were found to be significantly influenced by the exogenous steroid beyond the established threshold values. Additionally, one regular doping control sample with an extraordinary testosterone/
epitestosterone ratio of 100 without suspicious CIR was subjected to the complementary methodology of HIR analysis. The HIR data eventually provided evidence for the exogenous origin of urinary testosterone metabolites. Despite further investigations on HIR being advisable to corroborate the presented reference-population-based thresholds, the developed method proved to be a new tool supporting modern sports drug testing procedures [12155].

Isotope ratio mass spectrometry (IRMS) testing is performed to determine if an atypical steroid profile is due to administration of an endogenous steroid. Androsterone (Andro) and etiocholanolone (Etio), and/or the androstanediols (5alpha- and 5beta-androstane-3alpha,17beta-diol) are typically analyzed by IRMS to determine the $^{13}$C/$^{12}$C ratio. The ratios of these target compounds are compared to the $^{13}$C/$^{12}$C ratio of an endogenous reference compound (ERC) such as 5beta-pregnane-3alpha,20alpha-diol (Pdiol). Concentrations of Andro and Etio are high so $^{13}$C/$^{12}$C ratios can easily be measured in most urine samples. Despite the potentially improved sensitivity of the androstanediols for detecting the use of some testosterone formulations, additional processing steps are often required that increase labour costs and turnaround times. Since this can be problematic when performing large numbers of IRMS measurements, we established thresholds for Andro and Etio that can be used to determine the need for additional androstanediol testing. Using these criteria, 105 out of 2639 urine samples exceeded the Andro and/or Etio thresholds, with 52 of these samples being positive based on Andro and Etio IRMS testing alone. The remaining 53 urine samples had androstanediol IRMS testing performed and 3 samples were positive based on the androstanediol results. A similar strategy was used to establish a threshold for Pdiol to identify athletes with relatively $^{13}$C-depleted values so that an alternative ERC can be used to confirm or establish a true endogenous reference value. Adoption of a similar strategy by other laboratories can significantly reduce IRMS sample processing and analysis times, thereby increasing testing capacity [13095].

Carbon isotope ratio combined with hydrogen isotope ratio

Carbon isotope ratio (CIR) analysis has been routinely and successfully applied to doping control analysis for many years to uncover the misuse of endogenous steroids such as testosterone. Over the years, several challenges and limitations of this approach became apparent, e.g., the influence of inadequate chromatographic separation on CIR values or the emergence of steroid preparations comprising identical CIRs as endogenous steroids. While the latter has been addressed recently by the implementation of hydrogen isotope ratio (HIR), an improved sample preparation for CIR avoiding co-eluting compounds is presented herein together with newly established reference values of those endogenous steroids being relevant for doping controls. From the fraction of glucuronidated steroids 5beta-pregnane-3alpha,20alpha-diol, 5alpha-androst-16-en-3alpha-ol, 3alpha-Hydroxy-5beta-androstane-11,17-dione, 3alpha-hydroxy-5alpha-androstan-17-one (ANDRO), 3alpha-hydroxy-5beta-androstan-17-one (ETIO), 3beta-hydroxy-androst-5-en-17-one (DHEA), 5alpha- and 5beta-androstane-3alpha,17beta-diol (5aDIOL and 5bDIOL), 17beta-hydroxy-androst-4-en-3-one and 17alpha-hydroxy-androst-4-en-3-one were included. In addition, sulfate conjugates of ANDRO, ETIO, DHEA, 3beta-hydroxy-5alpha-androstan-17-one plus 17alpha- and androst-5-ene-3beta,17beta-diol were considered and analyzed after acidic solvolysis. The results obtained for the reference population encompassing 67 males and females confirmed earlier findings regarding factors influencing endogenous CIR. Variations in sample preparation influenced CIR measurements especially for 5alphiDIOL and 5betaDIOL, the most valuable steroidal analytes for the detection of testosterone misuse. Earlier investigations on the HIR of the same reference population enabled the evaluation of combined measurements of CIR and HIR and its usefulness regarding both steroid metabolism studies and doping control analysis. The combination of both stable isotopes would allow for lower reference limits
providing the same statistical power and certainty to distinguish between the endogenous or exogenous origin of a urinary steroid [13165].

**Capillary electrophoresis**

One report described a rapid, simple, and highly selective approach to perform testosterone competitive immunoassay by capillary electrophoresis (CE) and LIF detection. The method involves using synthesized fluorescence-labeled testosterone as a tracer to compete with testosterone. Two polyclonal antibodies arised and their respective tracers have been optimized and a system is used for the quantification of testosterone by CE-based immunoassay. The method is developed with a wide working range of 3.70-2000 ng/mL and a limit of detection at 1.11 ng/mL. Tests for normal and positive urine samples show that this method has the potential to be applied in testosterone doping control [08185].

**Prediction of metabolic pattern of new derivatives of AAS**

The aim of one work was to develop a flexible in vitro synthesis procedure, which can be applied in order to study and predict the metabolic patterns of new derivatives of anabolic androgenic steroids with respect to most prominent target compounds for doping control purposes. Microsomal and S9 fraction of human liver preparations were used as a source of metabolising enzymes and the co-substrates of the synthesis mixture were selected to favour phase-I metabolic reactions and glucuronidation as phase-II conjugation reactions. Model compounds within the study were 4,9,11-trien-3-one steroids, structural derivatives of gestrinone and trenbolone, which both are included in the list of prohibited compounds in sports by the World Anti-Doping Agency (WADA). The correlation between in vitro metabolism of human microsomes and in vivo excretion studies in human was compared with gestrinone and subsequently, the applicability of the in vitro model for prediction of AAS metabolic pathways for new doping agents was evaluated. All the AAS examined within this study were successfully metabolised using the developed in vitro model, hydroxylation, reduction and glucuronide conjugation being the most prominent reaction pathways. Hydroxylated and glucuronide-conjugated metabolites of in vivo experiment with gestrinone were the same metabolites formed in the enzyme-driven process, thus showing good in vitro-in vivo correlation. Liquid chromatographic-mass spectrometric and tandem mass spectrometric methods were developed, relying on the positive polarity of electrospray ionisation, which also allowed the direct detection of intact glucuronide-conjugated AAS metabolites. Due to charge delocalisation and high proton affinity, the developed method was proven effective in the analysis of anabolic steroids metabolites bearing extensive conjugated double bond systems in their structures [08187].

**Mass spectometry**

The metabolism of two anabolic steroids – oxymetholone and desoxymethyltestosterone – was reinvestigated to identify new targets potentially valuable for the antidoping analysis. Excretion urine samples from the laboratory reference collection were used in this study. Following fractionation of the urinary extract by means of high performance liquid chromatography (HPLC), each fraction was subjected to gas chromatography-mass spectrometry (GC-MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis after trimethylsilylation. About 20 metabolites were found for desoxymethyltestosterone and more than 40 for oxymetholone, with many of them being isomeric compounds. In addition to the well-known reduced and hydroxylated metabolites, 18-nor-17,17-dimethyl and 18-nor-17-hydroxymethyl-17-methyl steroids were also identified. Having evaluated all the metabolites in terms of how long they could be detected, it was suggested
that 18-nor-2epsilon,17beta-hydroxymethyl-17alpha-methyl-5alpha-androst-13-en-3alpha-ol is an important marker of oxymetholone abuse. In case of desoxymethyltestosterone, better detectability could be achieved if 18-nor-17,17-dimethyl-5alpha-androst-13-en-2epsilon, 3alpha-diol is monitored. These novel metabolites could be detected using GC-MS/MS at least for 14 days after administration of these anabolic steroids compared to 5-7 days for previously reported metabolites [12157].

Mass spectrometric identification of anabolic androgenic steroids challenges standard doping-control methods. To reveal a doping offence the presence of prohibited anabolic androgenic steroids at trace levels in the picogram-per-millilitre range must be confirmed as reliable. Human urine samples containing epitrenbolone, metandienone metabolite (17beta-hydroxymethyl-17alpha-methyl-18-norandrost-1,4,13-trien-3-one), stanozolol, 16beta-hydroxystanozolol and 4beta-hydroxystanozolol were analysed using LC-FAIMS-MS/MS. These substances are prohibited in sport according to World Anti-Doping Agency (WADA) regulations. Glucuronides were hydrolysed and prepared by liquid-liquid extraction. Excellent recovery and precision were obtained for all compounds. Linear calibration results for epitrenbolone and metandienone metabolite were obtained and concentration information could be determined in the ranges of reliable response between 750-1200 and 100-600 pg/mL, respectively. Limits of detection were estimated at 25 pg/mL (stanozolol), 50 pg/mL (metandienone metabolite, 16beta-hydroxystanozolol), 100 pg/mL (4beta-hydroxystanozolol) and 500 pg/mL (epitrenbolone). The assay was applied to doping-control samples. For all analytes, LC-FAIMS-MS/MS resulted in excellent interference removal, which effectively extends the post-dose detection time [09068].

Regularly new anabolic steroids appear on the black market. In most cases these substances are marketed on websites or are confiscated during inspections. 1,(5alpha)-Androstene-17beta-ol-3-one, also known as 1-testosterone, is one of these substances presented to body-builders as a nutritional supplement or a pro-hormone. 1-Testosterone closely resembles the natural hormone testosterone except for a 1,2-double bound instead of a 4,5-double bound. 1-Androstene-3beta,17beta-diol is transformed into 1-testosterone after oral administration. 1-Testosterone, 1-androstene-3beta,17beta-diol and some other related “new” anabolic steroids were studied with gas chromatography coupled to mass spectrometry (GC-MS) and Liquid chromatography coupled to tandem mass spectrometry (LC-MS2) methods. Similarities in spectra to known analytes, which may lead to pitfalls in the interpretation of the derivatised analytes, are discussed [06100].

Electrospray ionization tandem mass spectrometry

Anabolic steroids are structurally similar compounds, and their product-ion spectra obtained by tandem mass spectrometry under electrospray ionization conditions are quite difficult to interpret because of poly-ring structures and lack of a charge-retaining center in their chemical structures. In the present study, the fragmentation of nine anabolic steroids of interest to the racing industry was investigated by using triple quadrupole mass spectrometer, Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer, and a linear ion trap instrument. With the aid of an expert system software (Mass Frontier version 3.0), accurate mass measurements, and multiple stage tandem mass spectrometric (MS(n)) experiments, fragmentation pathways were elucidated for boldenone, methandrostenolone, tetrahydrogestrinone (THG), trenbolone, normethandrolone and mibolerone. Small differences in the chemical structures of the steroids, such as an additional double-bond or a methyl group, result in significantly different fragmentation pathways. The fragmentation pathways proposed in this paper allow interpretation of major product ions of other anabolic steroids reported by other researchers in a recent publication. The proposed fragmentation
pathways are helpful for characterization of new steroids. The approach used in this study for elucidation of the fragmentation pathways is helpful in interpretation of complicated product-ion spectra of other compounds, drugs and their metabolites [06101].

**GCxGC-TOFMS**

The application of comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GCxGC-TOFMS) for the analysis of six anabolic agents (AAs) in doping control is investigated in this work. A non-polar-polar column configuration with 0.2microm film thickness second dimension (2D) column was employed, offering much better spread of the components on 2D when compared to the alternative 0.1microm 2D column. The proposed method was tested on the "key" AA that the World Anti-Doping Agency (WADA) had listed at the low ng/mL levels ( clenbuterol, 19-norandrostosterone, epimethendiol, 17alpha-methyl-5alpha-androstane-3alpha,17beta-diol, 17alpha-methyl-5beta-androstane 3alpha,17beta-diol and 3'-OH-stanozolol). The compounds were spiked in a blank urine extract obtained by solid-phase extraction, hydrolysis and liquid-liquid extraction; prior to analysis they were converted to the corresponding trimethylsilyl (TMS) derivatives. The limit of detection (LOD) was below or equal to the minimum required performance limit (MRPL) of 2ng/mL defined by WADA, and the correlation coefficient was in the range from 0.995 to 0.999. The method allows choosing an ion from the full mass spectra which shows the least interference from the matrix and/or the best sensitivity; this can only be done if full scan mass spectral data are available. The advantage of GCxGC over classical one-dimensional GC (1D GC), in terms of separation efficiency and sensitivity, is demonstrated on a positive urine control sample at a concentration of 5ng/mL. The obtained similarity to the in-house created TOFMS spectra library at this level of concentration was in the range from 822 to 932 (on the scale from 0 to 999). Since full mass spectral information are recorded, the method allows the retro-search of non-target compounds or new “designer steroids”, which cannot be detected with established GC-MS methods that use (SIM) mode [09066].

**UHPLC-HRMS**

Aiming at the identification of new, complementary biomarkers for endogenous steroid abuse, the utility of a steroidomic approach using UHPLC-HRMS was assessed. In a controlled elimination study with orally administered testosterone undecanoate (80 mg), urine samples were subjected to a holistic steroid analysis followed by chemometric/statistical data evaluation. Here, numerous glucuronidated or sulfated steroids, the deconjugated analogs, of which mostly constitute the established steroid profile, were found to support the discrimination of the groups having received either placebo or testosterone undecanoate. The study demonstrated the principle of modern analytical approaches commonly referred to as “-omics” strategies and its potential application to issues of doping controls; in order to consider the whole (holistic) picture of such approaches, complementary analyses (e.g. by means of GC-HRMS) might be required to strengthen the outcome and value [13012].

**Single-stage-Orbitrap-MS**

A prominent trend which has been observed in recent years in the analysis of veterinary drugs and growth-promoting agents is the shift from target-oriented procedures, mainly based on liquid chromatography coupled to triple-quadrupole mass spectrometry (LC-QqQ-MS), towards accurate mass full scan MS (such as time of flight (ToF) and Fourier Transform (FT) Orbitrap MS). In this study the applicability of high resolution single-stage-Orbitrap-MS for confirmatory analysis of growth-promoting agents in meat was compared to that of a QqQ-MS. Validation according to CD 2002/657/EC demonstrated that steroid analysis based
on Orbitrap MS, operating at a resolution of 50,000 FWHM, is indeed capable to compete with QqQ-MS in terms of selectivity/specificity, while providing excellent linearity (for most compounds >0.99) but somewhat inferior sensitivity. Indeed, CCalphas reached from 0.04 to 0.88 microg/kg for the 34 anabolic steroids upon MS/MS detection, while upon Orbitrap MS detection a range of 0.07-2.50 microg/kg was observed. Using QqQ-MS adequate precision was obtained since relative standard deviations, associated with the repeatability and intralaboratory reproducibility, were below 20%. In the case of Orbitrap MS, for some compounds (i.e. some estrogens) this threshold was exceeded and thus poor precision was observed, which is possibly caused by the lack in sensitivity. Overall, it may be concluded that Orbitrap-MS offers an adequate performance in terms of linearity and precision but lacks in sensitivity for some of the compounds [13166].

**Full-capillary sample injection combined with a sweeping CE stacking method**

One study describes an on-line stacking CE approach by sweeping with whole capillary sample filling for analyzing five anabolic androgenic steroids in urine samples. The five anabolic steroids for detection were androstenedione, testosterone, epitestosterone, boldenone, and clostebol. Anabolic androgenic steroids are abused in sport doping because they can promote muscle growth. Therefore, a sensitive detection method is imperatively required for monitoring the urine samples of athletes. In this research, an interesting and reliable stacking capillary electrophoresis method was established for analysis of anabolic steroids in urine. After liquid-liquid extraction by n-hexane, the supernatant was dried and reconstituted with 30 mM phosphate buffer (pH 5.00) and loaded into the capillary by hydrodynamic injection (10 psi, 99.9 s). The stacking and separation were simultaneously accomplished at -20 kV in phosphate buffer (30 mM, pH 5.0) containing 100 mM sodium dodecyl sulfate and 40 % methanol. During the method validation, calibration curves were linear over a range of 50-1,000 ng/mL for the five analytes. In the evaluation of precision and accuracy for this method, the absolute values of the RSD and the RE in the intra-day (n=3) and inter-day (n=5) analyses were all less than 6.6 percent. The limit of detection for the five analytes was 30 ng/mL. Compared with simple MECK, this stacking method possessed a 108- to 175-fold increase in sensitivity. This simple and sensitive stacking method could be used as a powerful tool for monitoring the illegal use of doping [13167].

**Oxidizing adulterants’ effect on the steroid profile of human urine**

Steroid profiling is the most versatile and informative technique adapted by doping control laboratories for detection of steroid abuse. The absolute concentrations and ratios of endogenous steroids including testosterone, epitestosterone, androsterone, etiocholanolone, 5alpha-androstane-3alpha,17beta-diol and 5beta-androstane-3alpha,17beta-diol constitute the significant characteristics of a steroid profile. In the present study we report the influence of various oxidizing adulterants on the steroid profile of human urine. Gas chromatography-mass spectrometry analysis was carried out to develop the steroid profile of human male and female urine. Oxidants potassium nitrite, sodium hypochlorite, potassium permanganate, cerium ammonium nitrate, sodium metaperiodate, pyridinium chlorochromate, potassium dichromate and potassium perchlorate were reacted with urine at various concentrations and conditions and the effect of these oxidants on the steroid profile were analyzed. Most of the oxidizing chemicals led to significant changes in endogenous steroid profile parameters which were considered stable under normal conditions. These oxidizing chemicals can cause serious problems regarding the interpretation of steroid profiles and have the potential to act as masking agents that can complicate or prevent the detection of the steroid abuse [13168].

**Electrospray ionization tandem mass spectrometry (ESI-MS/MS)**
Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was used to investigate the effect of different substitutions introduced during metabolism on fragmentation patterns of four anabolic steroids including methyltestosterone, methandrostenolone, cis-androsterone and adrenosterone, along with their metabolites. Collision-induced dissociation (CID) analysis was performed to correlate the major product ions of 19 steroids with structural features. The analysis is done to portray metabolic alteration, such as incorporation or reduction of double bonds, hydroxylations, and/or oxidation of hydroxyl moieties to keto functional group on steroidal skeleton which leads to drastically changed product ion spectra from the respective classes of steroids, therefore, making them difficult to identify. The comparative ESI-MS/MS study also revealed some characteristic peaks to differentiate different steroidal metabolites and can be useful for the unambiguous identification of anabolic steroids in biological fluid. Moreover, LC-ESI-MS/MS analysis of fermented extract of methyltestosterone, obtained by Macrophomina phaseolina was also investigated [13169].

Glucuronocojugated metabolites

In humans, conjugation with glucuronic acid is the most important phase II metabolic reaction of steroidal compounds. Glucuronocojugated metabolites have been conventionally studied by using beta-glucuronidase enzymes to release the phase I metabolites. It is well-known that hydrolysis with beta-glucuronidase presents some limitations that may result in the underestimation of some conjugates. The aim of the present work was to develop and to evaluate liquid chromatography-tandem mass spectrometry (LC-MS/MS) scan methods for the open detection of steroid glucuronides in urine samples. The mass spectrometric behavior of thirteen representative steroid glucuronides, used as model compounds, was studied. Characteristic ionization and collision induced dissociation behaviors were observed depending on the steroid glucuronide structure. Neutral loss (NL of 176, 194, 211, and 229 Da) and precursor ion (PI of m/z 141, 159, and 177, in positive mode and m/z 75, 85, and 113, in negative mode) scan methods were evaluated. The NL scan method was chosen for the open detection of glucuronocojugated steroids due to its sensitivity and the structural information provided by this method. The application of the NL scan method to urine samples collected after testosterone (T) undecanoate administration revealed the presence of two T metabolites which remain conjugated as glucuronides after an enzymatic hydrolysis of the urine. 3alpha,6beta-Dihydroxy-5alpha-androstan-17-one (6beta-hydroxyandrostosterone) glucuronide and 3alpha,6beta-dihydroxy-5beta-androstan-17-one (6beta-hydroxyetiocholanolone) glucuronide were established as the structures for these metabolites, by comparing the structure of the steroids released after chemical hydrolysis with reference materials. An increase of 50-300-fold of these metabolites after oral administration of T undecanoate was observed, proving that their determination can be useful in the doping control field. Moreover, these results exemplify that significant information might be missed, unless direct methods for the determination of steroid glucuronides are employed [13164].

Relative retention times

A quantitative structure-retention relationship (QSRR) study has been performed to correlate relative retention times (RRTs) of trimethylsilylated (TMS) anabolic androgenic steroids (AAS) with their molecular characteristics, encoded by the respective descriptors, for the prediction of RRTs of novel molecules, using gas chromatography time-of-flight mass spectrometry (GC-TOF-MS). The elucidation of similarities and dissimilarities among the data structures was carried out using principal component analysis (PCA). Successful models were established using multiple linear regression (MLR) and partial least squares (PLS) techniques as a function of topological, three-dimensional (3D) and physicochemical descriptors. The models are useful for the estimation of RRTs of designer steroids for which
Variability in the $^{13}\text{C}/^{12}\text{C}$ ratios

The determination of the carbon isotope ratio in androgen metabolites has been previously shown to be a reliable, direct method to detect testosterone misuse in the context of antidoping testing. Here, the variability in the $^{13}\text{C}/^{12}\text{C}$ ratios in urinary steroids in a widely heterogeneous cohort of professional soccer players residing in different countries (Argentina, Italy, Japan, South Africa, Switzerland and Uganda) was examined. Carbon isotope ratios of selected androgens in urine specimens were determined using gas chromatography/combustion/isotope ratio mass spectrometry (GC-C-IRMS). Urinary steroids in Italian and Swiss populations were found to be enriched in $^{13}\text{C}$ relative to other groups, reflecting higher consumption of C3 plants in these two countries. Importantly, detection criteria based on the difference in the carbon isotope ratio of androsterone and pregnanediol for each population were found to be well below the established threshold value for positive cases. The results obtained with the tested diet groups highlight the importance of adapting the criteria if one wishes to increase the sensitivity of exogenous testosterone detection. In addition, confirmatory tests might be rendered more efficient by combining isotope ratio mass spectrometry with refined interpretation criteria for positivity and subject-based profiling of steroids [09070].

Principal components analysis

Principal components analysis was applied to anabolic androgenic steroids molecules referred in the WADA list of prohibited substances, resulting to their classification into six distinct groups related to structure features where metabolic alterations usually occur. The metabolites of the steroids participating to these six groups were treated using the Excel(c) classification filters showing that common metabolism routes are derived for each of the above principal components analysis classes, leading to the proposed metabolism schemes of the study. This rule-based approach is proposed for the prediction of the metabolism of unknown, chemically modified steroids, otherwise named as designer steroids. The metabolites of three known, in the literature, anabolic androgenic steroids are estimated using the proposed metabolism schemes, confirming that their use could be a useful tool for the prediction of metabolic pathways of unknown anabolic androgenic steroids [09071].

Androgen receptors assay

Anabolic androgenic steroids (AAS) share the activation of the androgen receptor (AR) as common mechanism of action. The mammalian androgen responsive reporter gene assay (AR CALUX bioassay), measuring compounds interacting with the AR can be used for the analysis of AAS without the necessity of knowing their chemical structure beforehand, whereas current chemical-analytical approaches may have difficulty in detecting compounds with unknown structures, such as designer steroids. One study demonstrated that AAS prohibited in sports and potential designer AAS can be detected with this AR reporter gene assay, but that also additional steroid activities of AAS could be found using additional mammalian bioassays for other types of steroid hormones. Mixtures of AAS were found to behave additively in the AR reporter gene assay showing that it is possible to use this method for complex mixtures as are found in doping control samples, including mixtures that are a result of multi drug use. To test if mammalian reporter gene assays could be used for the detection of AAS in urine samples, background steroidal activities were measured. AAS-spiked urine samples, mimicking doping positive samples, showed significantly higher androgenic activities than unspiked samples. GC-MS analysis of endogenous androgens and AR reporter gene assay analysis of urine samples showed how a combined chemical-
analytical and bioassay approach can be used to identify samples containing AAS. The results indicate that the AR reporter gene assay, in addition to chemical-analytical methods, can be a valuable tool for the analysis of AAS for doping control purposes [09072].

The utility of androgen receptor-based bioassays to probe for the presence of AAS and other non-steroidal anabolic agents in dietary products (with and without additional mass spectrometric measurement) has been demonstrated with various applications and reports in the past. One of the main advantages of this approach is the assay’s capability to indicate the presence of one or more substances able to bind to the androgen receptor, even if the structures and compositions of the substrates are unknown to the analyst. Moreover, the bioassay will provide information on the sum of androgen receptor activation. Hence, if two or more anabolic agents are present at low concentration, their detection is facilitated compared to methods that are designed to measure each analyte individually. Once suspicious bioassay results are obtained, products can be scrutinized for known as well as possibly unknown anabolic agents as recently shown in a study using a combined bioaffinity mass spectrometry methodology employing a competitive inhibition binding assay interfaced to a UHPLC-MS/MS system. In terms of doping controls, particularly the first-mentioned feature of measuring the combined androgen receptor binding of multiple analytes was evaluated as a potential means to tackle the issue of testosterone doping. The androgenic activity in urine as well as serum was measured prior to and after intramuscular testosterone enanthate administration, demonstrating that the readout of the bioassay was elevated independent from UGT2B17 ins/ins, ins/del, and del/del genotypes, suggesting that this approach might complement traditional steroid profile measurements [13009].

Although the specificity and unambiguous nature of mass spectrometry-based methods is undisputed, the search for complementary approaches, especially for initial test methods, is unbound. Here the utility of effect-based test methods such as those utilizing bioassays with androgen-receptors have been extensively reviewed. These assays can indicate the presence of agents stimulating the human androgen receptor without detailed knowledge of the substrate; however, proof of the misuse of anabolic agents remains to be provided, most likely by structural identification of the banned substance, for example, by mass spectrometry. In addition, immunological methodologies have been proposed to support the detection of AAS from human serum in a recent communication. By means of three different polyclonal antibodies (raised against boldenone, stanozolol, and tetrahydrogestrinone) and their respective cross reactivities, a total of 11 AAS is described to be detectable in less than 3 h. The authors highlight the sensitivity of the assay as being in agreement with WADAs MRPL; however, for serum samples no MRPL is given as to AAS concentrations and urine specimens have to be taken into account where metabolic processes and other potential [13012].

Receptor binding (competitive) assays
Receptor binding assays are based on the binding affinity of a ligand for its receptor. For this assay, purified receptor is immobilized on a column or suspended in a homogenate and to this, radiolabeled testosterone of known concentration is added. For the test, the suspect molecule is added to the radiolabeled testosterone, and it is measured whether the unknown molecule displaces the binding of the testosterone. If there is displacement then the unknown molecule has AR binding affinity. This type of assay only measures binding to AR and therefore is not able to differentiate between agonist and antagonist activity, or if any activity per se. Receptor binding assays require the use of radiolabelling, which presents a hazard if used as a routine screening test. These assays can be developed as high-throughput and are relatively easy to perform. To date, they have been used in a number of applications, including screening animal feed for growth hormones and screening for potential endocrine
disrupting chemicals (EDCs). They can also be used to investigate the potential potency of anabolic steroids by assessing the ability to bind to AR [13084].

**Androgen bioassays**

Androgen bioassays used for detection differ from the techniques described above because they mimic AR function and are not dependent on chemical structure. There are a number of different bioassays ranging from those based on whole animals to those based on mammalian or yeast cells. The Hershberger assay is an example of an *in vivo* androgen bioassay. The endpoint of this assay is a measured increase in the weight of androgen-dependent tissues. It is based on orchidectomised animals that produce little endogenous sex steroid hormones. These animals are treated with the test compound. If the test compound is androgenic, it will promote growth of androgen-dependent tissues. As this is an *in vivo* assay, metabolism of test molecules can also be tested by analyzing metabolites present in the blood stream and/or urine. As metabolism occurs upon treatment, this assay cannot be used to screen for activation or inactivation of androgens but it does allow the dissection of anabolic and androgenic effects of the test molecule or its metabolites. An assay based in animals is not feasible for routine sports doping screening in WADA laboratories. This has led to the development of *in vitro* cell-based androgen bioassays to screen for androgenic compounds. *In vitro* cell-based bioassays are widely used to detect androgenic molecules. They were first developed to test environmental pollutants (endocrine disrupting chemicals, EDCs) for their ability to alter normal hormonal function. Many substances including detergents (nonylphenol and other alkylphenols), plastics (bisphenol A), pesticides, insecticides, and even pharmaceutical wastes such as birth control tablets (ethinylestradiol) are now classified as EDCs. In vitro yeast androgen bioassays can be used in combination with other detection methods such as ultra high performance liquid chromatography combined with time-of-flight-tandem mass spectrometry (UHPLC/TOFMS) or liquid chromatography screening method. Moreover, bioassays can detect androgens in samples where LC-MS/MS could not, highlighting that bioassays have a valuable role in the fight against doping [13084].

**Cell-based androgen bioassays**

Cell proliferation assays can be used to measure the hormonal activity of a suspected agonist (or antagonist) in a sample because hormones, via their specific receptors, stimulate cell growth. In these assays, radioactive-labeled nucleotides are included in the culture media that become incorporated into DNA as cells proliferate. The radiolabel that incorporates into cells is a direct measurement of cell proliferation. These assays can measure both agonist and antagonist activity, as an agonist for the receptor of interest can stimulate cell growth, whereas an antagonist will block cell growth in the presence of an agonist. To date, this type of bioassay has not been extensively used to screen for androgens, however, it is the basis of the E-screen, which uses the human breast-cancer cell line (MCF-7) to screen for xenoestrogens. This assay is relatively simple to perform and is amenable to high-throughput readouts. However, results can be confounded by cell expression of other receptors (such as AR and glucocorticoid receptor) that induce non-specific cell proliferation. The assay relies on cell growth and, therefore, it can take days to produce results. Thus, it is not really feasible for use in sport doping laboratories [13084].

**Sensitivities of various androgen bioassays.**

The detection of prohormones or an understanding of potential metabolism of test extracts is central for the analysis of nutritional sport supplements. Mammalian cell lines may be limited in their metabolic capacity, compared to *in vivo* metabolism. To address this, a liver tissue metabolism step has been combined with the yeast AR/ARE/EGFP-based bioassay. In this two-step assay, test extracts are first incubated with a bovine liver S9 fraction, the extract
recovered, and then exposed to the yeast AR bioassay. As the liver tissue is whole, it is expected that this ex vivo approach will mimic the in vivo capacity for enzymatic conversions of steroids and therefore detect both prohormones and/or strong androgenic metabolites. In another example of introducing a pre-metabolism step prior to testing with a yeast AR bioassay to allow for prohormone or androgen metabolite detection, samples were pretreated with a *Helix pomatia* enzyme mix to activate inactive hormone conjugates including sulphates, glucuronides and glycosides. This example was in the setting of feed supplementation, rather than nutraceutical supplements, however, it is possible that a similar approach could be used to detect such conjugates if they were components of nutraceuticals [13084].

### Protein assays

The purpose of one study was to develop a rapid and sensitive method utilizing the state-of-the-art protein arrays technique to detect urinary anabolic steroids (ASs) in athletes. Three experiments were designed to investigate the feasibility of the protein arrays for ASs testing. Firstly, androgen receptor (AR) and estrogen receptor (ER) protein arrays were prepared on polysaccharide-coated slides to investigate whether they can bind to ASs (affinity tests). Secondly, in comparison to adrenergic receptor (the receptor of beta-blockers) and opioid receptor (the receptor of narcotic analgesics) arrays, AR and ER protein arrays were used to test whether they can determine the ASs positive urine sample specifically (specific binding tests). At last protein arrays were used to estimate qualitatively the ASs in positive urine samples (qualitative tests). From the results of the affinity tests the shape of the dose-dependence curve suggested a positive cooperative binding of ASs with the protein arrays. The AR and ER protein arrays showed affinities for fluorescence labelled testosterone and estradiol that were similar to those of literatures (0.65 vs 0.89 nM, 5.96 vs 10.3 nM, respectively). Based on the data, the sensitivity of testing can reach 0.1 nM that was much better than the World Anti-Doping Code (WADA) standard. Specific binding tests showed that the prohibited substance in positive urine samples belonged to the anabolic estrogenic inhibitor of ASs. From the results of the affinity tests the shape of the dose-dependence curve suggested a positive cooperative binding of ASs with the protein arrays. The total time of the test process for ASs in urine needed less than 1 h. In summary, the present study showed that the protein arrays method provided a highly sensitive and rapid alternative to screen urine samples for the detection of the misuse of ASs in athletes and was suitable for testing in both weekly training sessions as well as large-scale competition events [06102].

### Enzyme-immunoassay kit

One study assessed an enzyme-immunoassay (EIA) kit for measuring the salivary testosterone and cortisol concentrations of weightlifters. Saliva samples (*n*=64) were collected from male and female weightlifters during normal training procedures and analysed for testosterone and cortisol using a commercial EIA kit and a criterion radioimmunoassay (RIA) method. Significant correlations were demonstrated between the EIA and RIA measurements of salivary testosterone and cortisol concentrations. Further examination by sample and gender revealed similar relationships. The EIA concentrations of salivary testosterone and cortisol were found to be slightly greater (10-13 %) than the RIA values. Similar discrepancies were noted when gender comparisons were made, although the relative information on testosterone (males > females) and cortisol (males=females) were consistent for both assay methods. In conclusion, a commercially available EIA kit provided valid measures of the salivary testosterone and cortisol concentrations of male and female weightlifters. Factors to consider when using an EIA kit include the hormone(s) of interest,
the magnitude of the correlations, as well as the descriptive information gained (e.g. absolute, relative) and its uses within sport [10341].

**Triptorelin test**

In a case report a 34-year-old man had a single dose (100 mug) of triptorelin (triptorelin test). Within 1 month, the patient's serum testosterone was in the normal range, and he reported a return to normal energy and libido. The World Anti-Doping Code has proved to be a very powerful and effective tool in the harmonization of antidoping efforts worldwide, but it is insufficient to combat this illegal phenomenon. To tackle the serious side effects caused by doping we believe that it is necessary to increase monitoring and adopt severe sanctions, particularly with regard to Internet sites [10074].

**Serum inhibin B as a potential marker of testosterone doping**

The aim of one study was to explore effectors of the pituitary-testicular axis suitable as potential biochemical markers to screen for testosterone doping. A pilot study with male bodybuilding athletes with a self-reported history of testosterone doping (repeated intramuscular administration of testosterone preparations, last injection 8 weeks or less ago) were compared with an equal sized control group matched for sex, age, and body mass index. Fifteen healthy young men of white background were tested for inhibin B, testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH). Although the levels of testosterone, LH, and FSH did not differ between the 2 groups, the serum concentrations of inhibin B in individuals with a history of testosterone doping were exclusively at or below the lower limit of the normal range for adult men (100-400 pg/mL). Inhibin B was significantly lower in those men who used testosterone for weight lifting (76 ± 36 ng/L) than in controls (182 ± 35 ng/L). It was concluded that a low concentration of serum inhibin B may reflect the application of exogenous testosterone and appears to be a potential marker associated with anabolic androgenic steroid doping [10075].

**Two-dimensional gas chromatography**

This work presents the validation study of the comprehensive two-dimensional gas chromatography (GC x GC)-time-of-flight mass spectrometry method performance in the analysis of the key World Anti-Doping Agency anabolic agents in doping control. The relative abundance ratio, retention time, identification and other method performance criteria have been tested in the GC x GC format to confirm that they comply with those set by WADA. Furthermore, tens of other components were identified with an average similarity of >920 (on the 0-999 scale), including 10 other endogenous sterols, and full mass spectra of 5,000+ compounds were retained. The testosterone/epitestosterone ratio was obtained from the same run. A new dimension in doping analysis has been implemented by addressing separation improvement. Instead of increasing the method sensitivity, which is accompanied by making the detector increasingly "blind" to the matrix (as represented by selected ion monitoring mode, high-resolution mass spectrometry (MS) and tandem MS), the method capabilities have been improved by adding a new "separation" dimension while retaining full mass spectral scan information. Apart from the requirement for the mass spectral domain that a minimum of three diagnostic ions with relative abundance of 5 percent or higher in the MS spectra, all other WADA criteria are satisfied by GC x GC operation. The minimum of three diagnostic ions arises from the need to add some degree of specificity to the acquired mass spectrometry data; however, under the proposed full MS scan method, the high MS similarity to the reference compounds offers more than the required three diagnostic ions for an unambiguous identification. This should be viewed as an extension of the present criteria.
Effective detection of the abuse of androgenic-anabolic steroids in human and animal sports often requires knowledge of the drug's metabolism in order to target appropriate urinary metabolites. "Designer" steroids are problematic since it is difficult to obtain ethical approval for in vivo metabolism studies due to a lack of a toxicological profile. In one study, the in vitro metabolism of estra-4,9-diene-3,17-dione is reported for the first time. This is also the first study comparing the metabolism of a designer steroid in the three major species subject to sport's doping control; namely the equine, canine and human. In order to allow the retrospective analysis of sample testing data, the use of a high-resolution (HR) accurate-mass Thermo LTQ-Orbitrap LC-MS instrument was employed for metabolite identification of underivatised sample extracts. The full scan HR-LC-MS Orbitrap data was complimented by several further experiments targeted at elucidating more detailed structural information for the most abundant metabolites. These included; HR-LC-MS/MS of the underivatised metabolites, functional group selective chemical derivatisation followed by full scan HR-LC-MS, enzyme inhibition experiments and full scan electron ionization GC-MS analysis of methoxynamine-trimethylsilyl derivatives. The major metabolite detected in all species, and therefore the most suitable candidate for screening of estra-4,9-diene-3,17-dione abuse, was proposed to be an isomer of 17-hydroxy-estra-4,9-dien-3-one. Less significant metabolic pathways in all species included hydroxylation and reduction followed by hydroxylation. Reductive metabolism in the canine was less significant than in the other two species, while the equine was unique in producing a di-reduced metabolite (proposed to be an isomer of estra-4,9-diene-3,17-diol) and also relatively large quantities of d-ring hydroxy and hydroxy-reduced metabolites [10077].

Adequate detection of designer steroids in the urine of athletes is still a challenge in doping control analysis and requires knowledge of steroid metabolism. In one study we investigated whether uPA(+/+)-SCID mice carrying functional primary human hepatocytes in their liver would provide a suitable alternative small animal model for the investigation of human steroid metabolism in vivo. A quantitative method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed and validated for the urinary detection of 7 known methandienone metabolites. Application of this method to urine samples from humanized mice after methandienone administration allowed for comparison with data from in vivo human samples and with reported methandienone data from in vitro hepatocyte cultures. The LC-MS/MS method validation in mouse and human urine indicated good linearity, precision, and recovery. Using this method it was quantified 6 of 7 known human methandienone metabolites in the urine of chimeric mice, whereas in control nonchimeric mice we detected only 2 metabolites. These results correlated very well with methandienone metabolism in humans. In addition, it was detected 4 isomers of methandienone metabolites in both human and chimeric mouse urine. One of these isomers has never been reported before. The results of this proof-of-concept study indicate that the human liver-uPA(+/-)-SCID mouse appears to be a suitable small animal model for the investigation of human-type metabolism of anabolic steroids and possibly also for other types of drugs and medications [09073].

**Molecularly imprinted polymer filaments (MIPFs)**

An online system that can perform dynamic microextraction, on-coating derivatization and desorption, and subsequent GC-MS analysis with a large-volume injection was developed. A derivatization cell as the conjunction of the online system was developed for the online extraction and derivatization. To evaluate the feasibility of the online system,
methyltestosterone molecularly imprinted polymer filaments (MIPFs) were prepared for the selective online extraction of five androgenic steroids, namely, methyltestosterone, testosterone, epitestosterone, nandrolone, and metandienone. Under the optimized conditions, the detection limits of testosterone and epitestosterone were 0.09 and 0.12 μg/L, respectively, which were under the minimum required performance limits between 2 and 10 μg/L from the World Anti-Doping Agency. The detection limits of the other three androgenic steroids were varied from 0.04 to 0.18 microg/L. Finally, the MIPFs-GC-MS method was applied for the determination of androgenic steroids in urine, and satisfactory recovery (78-97 %) and reproducibility (3.2-8.9 %) were obtained. The proposed online coupling system offers an attractive alternative for hyphenation to GC instruments and could also be extended to other adsorptive materials [13176].

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**Urinary steroids**

One work presented a novel database search engine – MLibrary – designed to assist the user in the detection and identification of androgenic anabolic steroids (AAS) and its metabolites by matrix assisted laser desorption/ionization (MALDI) and mass spectrometry-based strategies. The detection of the AAS in the samples was accomplished by searching the mass spectrometric (MS) spectra against the library developed to identify possible positives and by comparison of the tandem mass spectrometric (MS/MS) spectra produced after fragmentation of the possible positives with a complete set of spectra that have previously been assigned to the software. The urinary screening for anabolic agents plays a major role in anti-doping laboratories as they represent the most abused drug class in sports. With the help of the MLibrary software application, the use of MALDI techniques for doping control is simplified and the time for evaluation and interpretation of the results is reduced. To do so, the search engine takes as input several MALDI-TOF-MS and MALDI-TOF-MS/MS spectra. It aids the researcher in an automatic mode by identifying possible positives in a single MS analysis and then confirming their presence in tandem MS analysis by comparing the experimental tandem mass spectrometric data with the database. Furthermore, the search engine can, potentially, be further expanded to other compounds in addition to AASs. The applicability of the MLibrary tool is shown through the analysis of spiked urine samples [13178].

**In saliva**

To validate the testosterone (T) and cortisol (C) concentration measures in saliva in
response to short high-intensity exercise 9 healthy males provided matching saliva and plasma samples before and after a 30-second Wingate cycle test. Saliva was assayed for T (Sal-T) and C (Sal-C) concentrations, and plasma for total T and total C, sex hormone-binding globulin, corticosteroid-binding globulin (CBG) and albumin concentrations. The plasma free and bioavailable hormones were calculated. The Sal-T and plasma T correlations were weak to moderate when examined between individuals (pooled data for all participants), but these relationships improved within individuals (data for each participant on average). The Sal-C and plasma C correlations were strong both between individuals and within individuals. The peak relative increases in Sal-T (35 ± 9 %) and Sal-C (63 ± 29 %) concentrations exceeded the plasma total and/or free hormones, but not the bioavailable hormones. Albumin and CBG also increased with exercise, along with blood lactate. It was concluded that the Sal-T and Sal-C concentration measures were validated in response to short high-intensity exercise, especially for individuals. The hormonal changes in saliva were also more sensitive to exercise (i.e. greater relative responses) than the plasma total and/or free hormones, potentially arising from changes in the binding proteins and blood lactate. These findings support the use of saliva as a medium for steroid determination in sport [10072].

In hair

The measurement of anabolic steroid levels in human hair is possible in order to distinguish between pharmaceutical steroids and natural steroids. It was now presented the first investigation into the physiological concentrations of anabolic steroids in human hair in Chinese subjects. A gas chromatography-tandem mass spectrometry (GC/MS/MS) method was developed for the simultaneous identification and quantitation of five endogenous anabolic steroids (testosterone, epitestosterone, androsterone, etiocholanolone and dehydroepiandrosterone) in hair. After basic hydrolysis, hair samples were extracted with diethyl ether, derivatized and then detected using GC/MS/MS in the multiple-reaction monitoring mode. The one precursor/two product ion transitions for each anabolic steroid were monitored. The limits of detection for the five endogenous anabolic steroids were in the 0.1-0.2 pg/mg range. Within-day and between-day precisions were less than 20 percent. This method was applied to the analysis of testosterone, epitestosterone, androsterone, etiocholanolone, and dehydroepiandrosterone in human hair. Full-length hair samples were taken at the skin surface from the vertex of 39 males, 30 females and 11 children from China. None of the subjects were professional athletes. Testosterone and dehydroepiandrosterone were detected in all the hair segments. The physiological concentrations of testosterone were in the range 0.8-24.2 pg/mg, 0.1-16.8 pg/mg and 0.2-11.5 pg/mg in males, females and children, respectively, however, the mean values of dehydroepiandrosterone were much higher than the concentrations of testosterone. These data are suitable reference values and are the basis for the interpretation of results from investigations into the abuse of endogenous anabolic steroids [09074].

A simple and sensitive gas chromatography/tandem mass spectrometry (GC/MS/MS) method is described for the detection of anabolic steroids, usually found in keratin matrix at very low concentrations. Hair samples from seven athletes who spontaneously reported their abuse of anabolic steroids, and in a single case cocaine, were analyzed for methyltestosterone, nandrolone, boldenone, fluoxymesterone, cocaine and its metabolite benzoylecgonine. Anabolic steroids were determinate by digestion of hair samples in 1 m NaOH for 15 min at 95 degrees C. After cooling, samples were purificated by solid-phase and liquid-liquid extraction, then anabolic steroids were converted to their trimethylsilyl derivative and finally analyzed by GC/MS/MS. For detection of cocaine and benzoylecgonine, hair samples were extracted with methanol in an ultrasonic bath for 2 h at 56 degrees C then overnight in a thermostatic bath at the same temperature. After the
incubation, methanol was evaporated to dryness, and benzoylecgonine was converted to its trimethylsilyl derivative prior of GC/MS/MS analysis. Results obtained are in agreement with the athletes' reports, confirming that hair is a valid biological matrix to establish long-term intake of drugs [07082].

New highly sensitive, specific, reliable, reproducible and robust LC-MS/MS methods were developed to detect the anabolic steroids, nandrolone and stanozolol, in human hair for the first time. Hair samples from 180 participants (108 males, 72 females, 62 % athletes) were screened using ELISA which revealed 16 athletes as positive for stanozolol and 3 for nandrolone. Positive samples were confirmed on LC-MS/MS in selective reaction monitoring (SRM) mode. The assays for stanozolol and nandrolone showed good linearity in the range 1-400 pg/mg and 5-400 pg/mg, respectively. The methods were validated for LLOD, interday precision, intraday precision, specificity, extraction recovery and accuracy. The assays were capable of detecting 0.5pg stanozolol and 3.0 pg nandrolone per mg of hair, when approximately 20 mg of hair were processed. Analysis using LC-MS/MS confirmed 11 athletes' positive for stanozolol (5.0 pg/mg to 86.3 pg/mg) and 1 for nandrolone (14.0 pg/mg) thus avoiding false results from ELISA screening. The results obtained demonstrate the application of these hair analysis methods to detect both steroids at low concentrations, hence reducing the amount of hair required significantly. The new methods complement urinalysis or blood testing and facilitate improved doping testing regimes. Hair analysis benefits from non-invasiveness, negligible risk of infection and facile sample storage and collection, whilst reducing risks of tampering and cross-contamination. Owing to the wide detection window, this approach may also offer an alternative approach for out-of-competition testing [10073].

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A method for the screening of various anabolic steroids and their esters in human hair, based on liquid-chromatography-high resolution mass spectrometry using an Exactive benchtop Orbitrap mass spectrometer, has been set up and validated. This method involved methanolic incubation of 30 mg of hair and analysis of the relevant extract in HPLC using a C18 column. The mass detector, with nominal resolving power of 100,000, operated in full scan mode in APCI under positive ionization mode. Analytes were identified by exact mass, correspondence of isotopic cluster and retention times. The limits of detection obtained varied from 10 to 50 pg/mg, and limits of quantitation were 0.5 ng/mg for all compounds. The
method was linear for all analytes in the ranges from the LOQ to 6 ng/mg, giving correlation coefficients >0.99 for all analytes. Also accuracy (intended as %E) and repeatability (%CV) were always lower than 15 percent. Specificity was assessed by analysing ten blank samples and fifteen samples from polidrug abusers. This method was applied to a real-life case, resulting in the identification of testosterone undecanoate in the hair of a suspect. The analyte identity was confirmed by the analysis of its in-source fragmentation and comparison to a certified standard. Thanks to the scan acquisition, this method also enables retrospective re-analysis of the acquired datafile in case a further analyte needs to be screened [13179].

**Faecal analyses**

Faeces, which could be a potential alternative medium for doping control, have been used for the detection of 1,4-androstadiene-3,17-dione administration to horses. Semi-quantitative analyses of 1,4-androstadiene-3,17-dione, testosterone, 17alpha- and 17beta-boldenone have been conducted in pre- and post-administration faeces, and in controls (untreated stallions, geldings and mares). Sample preparation comprised diethyl ether extraction, lipid removal, HPLC purification and derivatisation. 1,4-Androstadiene-3,17-dione, testosterone, 17alpha- and 17beta-boldenone were analysed by GC-EI/MS/MS. Quantitative limits of detection were 0.1 ng/g for 1,4-androstadiene-3,17-dione, and 0.025 ng/g for testosterone, 17alpha- and 17beta-testosterone. In post-administration samples from geldings and mares, peak levels of 1,4-androstadiene-3,17-dione, 17alpha-, 17beta-boldenone and testosterone were attained 24 h after administration. In untreated geldings and mares (in dioestrus), 17alpha- and 17beta-boldenone and testosterone were not detected. Faeces from females in oestrus had detectable levels of boldenone isomers and testosterone. 1,4-Androstadiene-3,17-dione was undetectable in faeces collected from untreated horses, but the presence of this androgen was recently reported in faeces from untreated swine and it would therefore be advisable to check for its possible presence in a larger number of individual faecal samples [08191].

**In food**

Anabolic steroids are banned from use in food-producing animals in the European Union (Council Directive 96/22/EC). To control the zero-tolerance concept, an LC-MS/MS method for the screening and confirmation of most of the relevant natural and synthetic estrogenic and androgenic steroids in bovine and porcine blood plasma was developed and validated. The method permits confirmation and quantification of all analytes above a concentration of 0.65 microg/L. The validation was carried out according to Commission Decision 2002/657/EC, Chapter 3.1.3 "Alternative Validation", by applying a matrix-comprehensive in-house validation concept. Decision limit CCalpha, detection capability CCbeta, recovery, repeatability, within-laboratory reproducibility and the uncertainty of measurement were calculated. Furthermore, a factorial effect analysis was carried out to identify factors that have a significant influence on the method. Factors considered to be relevant for the method in routine analysis (e.g. operator, storage duration of the extracts before measurement and different cartridge lots) were systematically varied on two levels [13182].

**In egg**

A cheap, reliable and practical high-performance liquid chromatography-tandem mass spectrometric method was developed for the simultaneous determination of seven anabolic steroids in eggs, including trenbolone, boldenone, nandrolone, stanozolol, methandienone, testosterone and methyl testosterone. The analytes were extracted from the egg samples using methanol. The extracts were subjected to the removal of fat by freezing-lipid filtration
and then further purified by liquid-liquid extraction using tert-butyl methyl ether. The analytes were separated on a Luna C18 column by a gradient elution program with 0.1% formic acid and acetonitrile. This method was validated over 1.00-100 ng/g for all steroids of interest. The correlation coefficients (r) for each calibration curve are higher than 0.99 within the experimental concentration range. The decision limits of the steroids in eggs ranged from 0.20 to 0.44 ng/g, and the detection capabilities were below 1.03 ng/g. The average recoveries were between 66.3 and 82.8% in eggs at three spiked levels of 1.00, 1.50 and 2.00 ng/g for each analyte. The between-day and within-day relative standard deviations were in the range of 2.4-11 percent. High matrix suppression effects were observed for all compounds of interest [13183].

**Musk extracts**

The relevance of IRMS analyses in sports drug testing was highlighted in a recent case report concerning the administration of musk pod extracts to female elite athletes. Numerous steroidal components relevant to doping controls and steroid profiling were influenced by the preparation commonly used in traditional Chinese medicine (TCM) regimens and triggered GC/C/IRMS analyses that confirmed the non-human origin of the urinary steroid metabolites. In a follow-up study, the diversity of musk preparations concerning steroid content and respective CIR was demonstrated. Four batches of musk grains were purchased including two specimens from wild musk deer and two from domesticized musk deer, outlining substantial differences in both amounts of steroids and isotopic signatures. In administration studies with two preparations (100 mg of musk grains) however no significant change in urinary steroid profiles and CIR were found [13009].

Musk is widely used as a traditional drug in Asia for the treatment of stroke, tumour, and cardiopathy with an oral dosage of 0.03-0.1 g per day. Because of the potential anabolic effect, musk preparations have been included in the list of medical products containing prohibited substances employed for doping. The application of musk pod formulation was regarded as the reason of some adverse analytical findings in the 2011 FIFA Women's World Cup. In order to investigate the influence of musk administration on the doping test, we executed a chemical analysis and excretion study. The gas chromatography/mass spectrometry (GC-MS) analysis demonstrated the diversity of steroid concentrations in musk samples. Furthermore, the delta-13C-values of steroids from wild deer musk showed more depleted than those of domestic deer musk by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) analysis. Because the steroids from some musk had delta-13C-values in the range of naturally produced steroids in human body, the possible abuse of this kind of musk is very hard to be detected by isotope ratio mass spectrometry (IRMS) in doping control. Musk grains from wild and domestic deer were administrated for the excretion study respectively. Spot urine samples were collected from two male volunteers before and after 100 mg musk grains administration. The profiles and carbon isotope ratios of urinary steroids were determined by GC-MS and GC/C/IRMS. The ingestion of either wild or domestic deer musk did not lead to the adverse analytical finding of doping control in the single dosage of 100 mg [13184].

Musk is the dried secretion from the preputial follicles of the male musk deer, which are located in a small sac (resulting from an infolding of the skin) in close proximity to the preputial orifice. This sac (or pod), which contains the brownish musk, comprises a small canal debouching close to the preputial orifice that allows the controlled release of the unctuous product by the animal. Upon removal of moisture, the material converts into small, dark, reddish-brown musk grains. In comprehensive studies, the lipid constituents of musk were elucidated and numerous steroidal components were characterized. The administration
of musk extract, that is, ingredients obtained by extraction of the liquid secreted from the preputial gland or resulting grains of the male musk deer (e.g. Moschus moschiferus), has been recommended in traditional chinese medicine (TCM) applications and was listed in the Japanese pharmacopoeia for various indications requiring cardiovascular stimulation, anti-inflammatory medication or androgenic hormone therapy. Numerous steroidal components including cholesterol, 5alpha-androstane-3,17-dione, 5beta-androstane-3,17-dione, androsterone, etiocholanolone, epiandrosterone, 3beta-hydroxy-androst-5-en-17-one, androst-4-ene-3,17-dione and the corresponding urea adduct 3alpha-ureido-androst-4-en-17-one were characterised as natural ingredients of musk over several decades, implicating an issue concerning doping controls if used for the treatment of elite athletes. In the present study, the impact of musk extract administration on sports drug testing results of five females competing in an international sporting event is reported. In the course of routine doping controls, adverse analytical findings concerning the athletes’ steroid profile, corroborated by isotope-ratio mass spectrometry (IRMS) data, were obtained. The athletes’ medical advisors admitted the prescription of TCM-based musk pod preparations and provided musk pod samples for comparison purposes to clarify the antidoping rule violation. Steroid profiles, IRMS results, literature data and a musk sample obtained from a living musk deer of a local zoo conclusively demonstrated the use of musk pod extracts in all cases which, however, represented a doping offence as prohibited anabolic-androgenic steroids were administered [13185].

A user-friendly library

One work presents a novel database search engine - MLibrary - designed to assist the user in the detection and identification of androgenic anabolic steroids (AAS) and its metabolites by matrix assisted laser desorption/ionization (MALDI) and mass spectrometry-based strategies. The detection of the AAS in the samples was accomplished by searching the mass spectrometric (MS) spectra against the library developed to identify possible positives and by comparison of the tandem mass spectrometric (MS/MS) spectra produced after fragmentation of the possible positives with a complete set of spectra that have previously been assigned to the software. The urinary screening for anabolic agents plays a major role in anti-doping laboratories as they represent the most abused drug class in sports. With the help of the MLibrary software application, the use of MALDI techniques for doping control is simplified and the time for evaluation and interpretation of the results is reduced. To do so, the search engine takes as input several MALDI-TOF-MS and MALDI-TOF-MS/MS spectra. It aids the researcher in an automatic mode by identifying possible positives in a single MS analysis and then confirming their presence in tandem MS analysis by comparing the experimental tandem mass spectrometric data with the database. Furthermore, the search engine can, potentially, be further expanded to other compounds in addition to AASs. The applicability of the MLibrary tool is shown through the analysis of spiked urine samples [13180].

Reference values from South America

The urinary steroid profile has been used in clinical endocrinology for the early detection of enzyme deficiencies. In the field of doping, its evaluation in urine samples is used to diagnose the abuse of substances prohibited in sport. This profile is influenced by sex, age, exercise, diet, and ethnicity, among others; laboratories own reference ranges might compensate for ethnic differences among population and inter-laboratory biases. One paper shows the reference ranges obtained in the Antidoping Laboratory of Havana for the following steroid profile parameters: ten androgens (testosterone, epitestosterone, androsterone, etiocholanolone, 5alpha-androst-3alpha,17beta-diol, 5beta-androstan-
3alpha,17beta-diol, dehydroepiandrosterone, epiandrosterone, 11beta-hydroxyandrostenedione and 11beta-hydroxyetiocholanolone), three estrogens (estradiol, estriol and estrone), two pregnanes (pregnanediol and pregnanetriol) and two corticosteroids (cortisol and tetrahydrocortisol). The urine samples (male: n=2454 and female: n=1181) and data obtained are representative of population from Latin-American countries like Cuba, Venezuela, Mexico, Dominican Republic, Guatemala and Chile. Urine samples were prepared by solid-phase extraction followed by enzymatic hydrolysis and liquid-liquid extraction with an organic solvent in basic conditions. Trimethylsilyl derivatives were analyzed by gas chromatography coupled to mass spectrometry. Reference ranges were established for each sex, allowing the determination of abnormal profiles as a first diagnostic tool for the detection of the abuse of androgenic anabolic steroids. The comparison with the Caucasian population confirms that the urinary steroid profile is influenced by ethnicity [13181].

**Experimental**

**Contextual interaction**

Seasonal changes in steroid hormones are known to have a major impact on social behavior, but often are quite sensitive to environmental context. In the bi-directionally sex changing fish, Lythrypnus dalli, stable harem groups exhibit baseline levels of interaction. Status instability follows immediately after male removal, causing transiently elevated agonistic interactions and increase in brain and systemic levels of a potent fish androgen, 11-ketotestosterone (KT). Coupling KT implants with a socially inhibitory environment for protogynous sex change induces rapid transition to male morphology, but no significant change in social behavior and status, which could result from systemically administered steroids not effectively penetrating into brain or other tissues. Here, it was first determined the degree to which exogenously administered steroids affect the steroid load within tissues. Second, it was examined whether coupling a social environment permissive to sex change would influence KT effects on agonistic behavior. It was implanted cholesterol (Chol, control) or KT in the dominant individual (alpha) undergoing sex change (on d0) and determined the effects on behavior and the degree to which administered steroids altered the steroid load within tissues. During the period of social instability, there were rapid (within 2h), but transient effects of KT on agonistic behavior in alphas, and secondary effects on betas. On d3 and d5, all KT, but no Chol, treated females had male typical genital papillae. Despite elevated brain and systemic KT 5days after implant, overall rates of aggressive behavior remained unaffected. These data highlight the importance of social context in mediating complex hormone-behavior relationships [13186].

**Prepuberal induction**

Few data are available on adolescent users because most behavioral studies on anabolic-androgenic steroids (AAS) abuse have been performed in adults. Studies evaluating the impact of long-term effects of AAS abuse on the prepubertal phase are even more uncommon. Accordingly, this study was developed to test the hypothesis that changes induced by the use of AAS during the adolescent phase may be noted in the adult phase even when the AAS treatment cycle is discontinued. Therefore, not only behavioral changes but also possible autonomic and electrolyte disorders were evaluated. For this purpose, we used male prepubertal, 26-day-old (P26) Wistar rats that were treated with vehicle (control, n=10) or testosterone propionate (TP; 5 mg/kg intramuscular (IM) injection, AAS, n=10) five times per week for 5 weeks, totaling 25 applications during the treatment. Aggression tests were performed at the end of the cycle (P54-56), whereas open-field tests (OFTs), elevated plus maze (EPM) behavioral tests and measurements of heart rate variability (HRV), fluid intake and pathology were conducted in the adult phase (P87-92). The AAS group showed
greater aggressiveness in the pubertal phase and higher levels of horizontal and vertical exploration and anxiety-related behavior in the adult phase than the control group. HRV tests showed an increase in sympathetic autonomic modulation, and hydroelectrolytic assessment showed lower basal intake levels of hypertonic saline than the control group, without statistically significant changes in the basal intake of water. These data together suggest that the use of AAS during the prepubertal phase induces behavioral, autonomic and hydroelectrolytic changes that manifest in the adult phase even when treatment is discontinued in late adolescence in rats [13187].

Markers for anabolic steroids

The screening of testosterone misuse in the doping control field is normally performed by the measurement of the ratio between the concentrations of testosterone and epitestosterone excreted as glucuronides (T/E). Despite the satisfactory results obtained with this approach, the measurement of T/E presents some limitations like the long-term detection of oral testosterone administration. Recently, several testosterone metabolites released after basic treatment of the urine have been reported (androsta-1,4-dien-3,17-dione, androsta-4,6-dien-3,17-dione, 17beta-hydroxy-androsta-4,6-dien-3-one and 15-androsten-3,17-dione). In one work, the usefulness of these metabolites for the detection of oral testosterone misuse were evaluated and compared with the conventional T/E measurement. For this purpose, 173 urine samples collected from healthy volunteers were analysed in order to obtain reference concentrations for the four metabolites released after alkaline treatment. On the other hand, urine samples collected from five volunteers before and after testosterone undecanoate administration were also analysed. Concentrations of androsta-4,6-dien-3,17-dione and 17beta-hydroxy-androsta-4,6-dien-3-one showed a similar behaviour as the T/E, allowing the detection of the misuse for several hours after administration. More promising results were obtained by quantifying androsta-1,4-dien-3,17-dione and 15-androsten-3,17-dione. The time in which the concentrations of these analytes could be differentiated from the basal level was between 3 and 6 times longer than the obtained with T/E, as a result, an improvement in the detection of testosterone abuse can be achieved. Moreover, several ratios between these compounds were evaluated. Some of them improved the detection of testosterone misuse when comparing with T/E. The best results were obtained with those ratios involving androsta-1,4-dien-3,17-dione [11576].

The aim of one study was to explore effectors of the pituitary-testicular axis suitable as potential biochemical markers to screen for testosterone doping. It was a pilot study with male bodybuilding athletes with a self-reported history of testosterone doping (repeated intramuscular administration of testosterone preparations, last injection 8 weeks or less ago) compared with an equal sized control group matched for sex, age, and body mass index. Fifteen healthy young men of white background had inhibin B, testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH) tested. Although the levels of testosterone, LH, and FSH did not differ between the 2 groups, the serum concentrations of inhibin B in individuals with a history of testosterone doping were exclusively at or below the lower limit of the normal range for adult men (100-400 pg/mL). Inhibin B was significantly lower in those men who used testosterone for weight lifting. It was concluded that low concentration of serum inhibin B may reflect the application of exogenous testosterone and appears to be a potential marker associated with anabolic androgenic steroid doping [10095].

AICAR

Influencing the endurance in elite sports is one of the key points in modern sports science. Recently, a new class of prohibited substances reached in the focus of doping control
laboratories and their misuse was classified as gene doping. The adenosine monophosphate activated protein kinase activator 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR) was found to significantly enhance the endurance even in sedentary mice after treatment. Due to endogenous production of AICAR in healthy humans, considerable amounts were present in the circulation and, thus, were excreted into urine. Considering these facts, the present study was initiated to fix reference values of renally cleared AICAR in elite athletes. Therefore a quantitative analytical method by means of isotope-dilution liquid chromatography (analytical column: C6-phenyl) coupled to tandem mass spectrometry, after a sample preparation consisting of a gentle dilution of native urine, was developed. Doping control samples of 499 athletes were analysed, and AICAR concentrations in urine were determined. The mean AICAR value for all samples was 2,186 ng/mL with a standard deviation of 1,655 ng/mL. Concentrations were found to differ depending on gender, type of sport and type of sample collection (in competition/out of competition). The method was fully validated for quantitative purposes considering the parameters linearity, inter- (12 %, 7 % and 10 %) and intraday precision (14 %, 9 % and 12 %) at low, mid and high concentration, robustness, accuracy (approx. 100 %), limit of quantification (100 ng/mL), stability and ion suppression effects, employing an in-house synthesised 13C5-labelled AICAR as internal standard [10096].

Activity on carboanhydrases

The in vitro effects of the anabolic compounds, zeranol, 17beta-estradiol, diethylstilbestrol (DES), and trenbolone, on the activity of purified human carbonic anhydrase I and II were evaluated. In vitro carboanhydrase enzyme activity was determined colorimetrically using the CO2 hydration method of Maren. IC50 values of the compounds that caused inhibition were determined by means of activity percentage diagrams. The IC50 concentrations of zeranol, 17beta-estradiol, DES and trenbolone on human carbonic anhydrase I were 94, 55, 10, 898 microM and for human carboanhydrase II 89, 159, 439 and 101 microM, respectively [11110].

Purchase over the Internet

A case of hypogonadotropic hypogonadism due to the chronic abuse of anabolic steroids purchased over the Internet was reported. It was described the clinical symptoms, androgen normalization, levels of serum testosterone, follicle-stimulating hormone, and luteinizing hormone, and after withdrawal, within 1 month, the patient's serum testosterone was in the normal range, and he reported a return to normal energy and libido [10454].

Anabolic steroid use and condom use

Previous research has revealed a significant bivariate relationship between anabolic-androgenic steroid (AAS) use and reduced condom use among adolescent boys. However, to date, no known studies have explored the psychological mechanisms that may explain this relationship. Thus, the current study sought to examine two possible mediators in the association between AAS and condom use: depressive symptoms and substance use. Data were extracted from a nationally representative sample of US adolescents. Participants were 3,780 U.S. high school boys who responded to self-report items assessing a number of health behaviors, including symptoms of depression, substance use, AAS use, and use of condoms during their most recent act of intercourse. Both depression and substance use
were significant mediators in the relationship between AAS and condom use. However, when these effects were contrasted, the indirect effect of substance use was significantly stronger in magnitude than the effect of depression. Although AAS use was associated with sexual risk behaviors among adolescent boys, significant variance in this relationship was accounted for by elevated levels of depression and substance use, with substance use demonstrating a particularly salient pathway [13161].

Case reports

Most users of anabolic androgenic steroids (AAS) are male, but the prevalence of such use appears to be increasing in females. It was presented a sudden unexpected death in a female fitness athlete with a possible connection to use of doping agents [08192].

It was presented a case of a 19-year-old male athlete with protein C deficiency who developed proximal deep venous thrombosis and pulmonary embolism while abusing anabolic-androgenic steroids. Anabolic-androgenic steroids have been reported to have anticoagulatory and profibrinolytic effects in patients with protein C deficiency. Despite these antithrombotic effects, the patient developed repeated venous thromboembolism during treatment with low-molecular-weight heparin. The net effect of anabolic-androgenic steroids on the haemostatic system may change from antithrombotic to prothrombotic in male abusers of anabolic steroids with protein C deficiency [08193].

An increase in the use of anabolic and amino acid supplements has been linked to a diverse array of pathologies. It was reported multi-organ damage resulting from the abuse and uncontrolled use of anabolic steroid supplements, mainly testosterone. A 24-year-old white man presented with abdominal pain concomitant with nausea and vomiting. Laboratory analysis revealed hypercalcemia, elevated liver enzymes and high levels of amylase, lipase and creatine protein kinase [08194].

Experimental

GABA type A receptors

Anabolic androgenic steroids (AAS) can promote detrimental effects on social behaviors for which GABA type A (GABA_A) receptor-mediated circuits in the forebrain play a critical role. While all AAS bind to androgen receptors (AR), they may also be aromatized to estrogens and thus potentially impart effects via estrogen receptors (ER). Chronic exposure of wild-type male mice to a combination of chemically distinct AAS increased action potential frequency, selective GABA_A receptor subunit mRNAs, and GABAergic synaptic current decay in the medial preoptic area. Experiments performed with pharmacological agents and in AR-deficient mutant mice suggest that the AAS-dependent enhancement of GABAergic transmission in wild-type mice is AR-mediated. In AR-deficient mice, the AAS elicited dramatically different effects, decreasing AP frequency, spontaneous IPSC amplitude and frequency and the expression of selective GABA_A receptor subunit mRNAs. Surprisingly, in the absence of AR signaling, the data indicate that the AAS do not act as ER agonists, but rather suggest a novel in vivo action in which the AAS inhibit aromatase and impair endogenous ER signaling. These results show that the AAS have the capacity to alter neuronal function in the forebrain via multiple steroid signaling mechanisms and suggest that effects of these steroids in the brain will depend not only on the balance of AR- versus ER-mediated regulation for different target genes, but also on the ability of these drugs to alter
steroid metabolism and thus the endogenous steroid milieu [09075].

**Effect of subcutaneous testosterone on emotionality**

The androgenic steroid testosterone is well known for its function in reproduction, sexual differentiation and sexual behavior. A growing number of human and animal studies suggest a modulatory role of testosterone in the regulation of emotionality and associated psychiatric disorders, including depressive-like disorders. However, most of the studies have been carried out in subjects deficient in androgenic steroid levels. Here, it was tested potential beneficial effects of subcutaneously applied testosterone on emotionality and depressive-like behavior in healthy male rats. For this purpose, male Wistar rats (3-4 months) received either vehicle or testosterone (1.0, 2.0, 4.0mg/kg) subcutaneously and were tested for potential effects on motor activity and anxiety-like behavior in a novel open field and elevated plus-maze. The forced swim test was used for assessing potential beneficial effects of testosterone on depressive-like behavior. The results show, that, while subcutaneous application of testosterone failed to influence spontaneous motor activity as well as anxiety-like behavior in the open field, a trend for an increase in the time spent on the open arms in the elevated plus-maze with the highest dose was found. Furthermore, in the forced swim test, testosterone application induced a dose-dependent reduction of immobility behaviour, indicating antidepressant-like action of testosterone in healthy animals [09076].

**Effect of testosterone in castrated guinea pigs**

Anabolic steroids are widely used to increase skeletal muscle mass and improve physical performance. Some dietary supplements also include potent steroid precursors or active steroid analogs such as nandrolone. One previous study reported the anabolic steroid effects on skeletal muscle mass in a castrated guinea pig model with muscle measured using a highly quantitative magnetic resonance imaging (MRI) protocol. The aim of one study was to apply this animal model and in vivo MRI protocol to evaluate the growth effects of four widely used over-the-counter testosterone and nandrolone precursors: 4-androstene-3 17-dione (androstenedione), 4-androstene-3beta 17beta-diol (4-androsdiol), 19-nor-4-androstene-3beta-17beta-diol (bolandiol) and 19-nor-4-androstene-3 17-dione (19-norandrostenedione). The results showed that providing precursor to castrated male guinea pigs led to plasma steroid levels sufficient to maintain normal skeletal muscle growth. The anabolic growth effects of these specific precursors on individual and total muscle volumes, sexual organs, and total adipose tissue over a 10-week treatment period, in comparison with those in the respective positive control testosterone and nandrolone groups, were documented quantitatively by MRI [09077].

**Apoptosis and NOS2 (nitric-oxide synthase 2)**

Anabolic-androgenic steroids (AAS) are synthetic derivatives of testosterone (T) predominantly taken as drugs of abuse. Using in vivo treatment of adult male rats we investigated the effects of testosterone enanthate (TE) a widely abused AAS, on apoptosis of Leydig cells. Increased T and decreased luteinizing hormone levels in serum and decreased intra-testicular T values were found in 2 and 10 weeks treated groups. Two weeks of TE-treatment stimulated the expression of inducible nitric oxide synthase (NOS2) followed by increased NO production, decreased mitochondrial membrane potential and increased prevalence of Leydig cell apoptosis. This was prevented by in vivo administration of androgen receptor blocker. The induced NOS2 level and apoptosis returned to control levels after 10 weeks of TE-treatment but testes contained fewer Leydig cells. Overall, AAS in addition to reduced steroidogenesis induce transient increase of Leydig cells apoptotic rate
through mechanism associated with androgen receptor, most likely involving NOS2 induction [12158].

Androgen-induced cardiac autonomic dysfunction

One study aimed to evaluate the combined effects of exercise and antagonists of the angiotensin II and aldosterone receptors on cardiac autonomic regulation and ventricular repolarization in rats chronically treated with nandrolone decanoate (ND), a synthetic androgen. Thirty male Wistar rats were divided into six groups: sedentary, trained, ND-treated, trained and ND-treated, trained and treated with both ND and spironolactone, and trained and treated with both ND and losartan. ND (10 mg/kg weekly) and the antagonists (20 mg/kg daily) of the angiotensin II AT(1) (losartan) and aldosterone (spironolactone) receptors were administered for 8 weeks. Exercise training was performed using a treadmill five times each week for 8 weeks. Following this 8-week training and treatment period, electrocardiogram recordings were obtained to determine the time and frequency domains of heart rate variability (HRV) and corrected QT interval (QTc). Nandrolone decanoate treatment increased the QTc interval and reduced the parasympathetic indexes of HRV (RMSSD, pNN5 and high-frequency power) in sedentary and trained rats. The ratio between low- and high-frequency power (LF/HF) was higher in ND-treated groups. Both losartan and spironolactone treatments prevented the effects of ND on the QTc interval and the HRV parameters (RMSSD, pNN5, high-frequency power, and the LF/HF ratio). The results show that chronic treatment with a high dose of ND induces cardiac parasympathetic dysfunction and disturbances in ventricular repolarization in both sedentary and exercised rats. Furthermore, inhibiting the renin-angiotensin-aldosterone system using losartan, or spironolactone, prevented these deleterious effects [12159].

Interaction of testosterone with cocaine

Abuse of cocaine and androgenic-anabolic steroids (AASs) has become a serious public health problem. Despite reports of an increase in the incidence of simultaneous abuse of these substances, potential toxic interactions between cocaine and AASs are poorly known. In one study, it was investigated the effects of either single or combined administration of testosterone and cocaine for one or 10 consecutive days on autonomic (arterial pressure, heart rate and tail cutaneous temperature) and neuroendocrine (plasma corticosterone) responses induced by acute restraint stress in rats. Combined administration of testosterone and cocaine for 10 days reduced the increase in heart rate and plasma corticosterone level, as well as the fall in tail skin temperature induced by restraint stress. Furthermore, repeated administration of cocaine inhibited the increase in arterial pressure observed during restraint, and this effect was not affected by coadministration of testosterone. Ten-day combined administration of testosterone and cocaine increased basal values of arterial pressure. Moreover, chronic administration of testosterone induced rest bradycardia and elevated basal level of plasma corticosterone. One-day single or combined administration of the drugs did not affect any parameter investigated. In conclusion, the present study demonstrated that combined administration of testosterone and cocaine changed the autonomic and neuroendocrine responses to acute restraint stress. These findings suggest that interaction between AASs and cocaine may affect the ability to cope with stressful events [12160].
SIDE EFFECTS OF TESTOSTERONE AND OTHER ANABOLIC STEROIDS

Overviews

Depending on duration and dosage of administration, AAS can cause various adverse effects marked by virilization and hirsutism in women, deepening of voice (permanent) as well as testicular atrophy (reversible) and gynaecomastia in men. Furthermore, ASS abuse in adolescence may induce premature epiphyseal fusion resulting in stunted growth. Also, via both genomic and non-genomic pathways, AAS can trigger aggressive behaviour and hostility as well as mood disturbances such as depression and hypomania. However, the development of side-effects induced by supraphysiological doses of AAS correlate, at least as regards the psychiatric symptoms, to the dose and duration of abuse. Considerable changes of lipid profile result in decrease in HDL and increase in LDL concentration, as well as diabetes mellitus, arterial hypertension and cardiac morbidity have been associated with AAS abuse. The most typical cardiac abnormality is left ventricular hypertrophy with fibrosis, while rare cardiovascular substrate is eosinophilic myocarditis. Nevertheless, the cause–effect relationship between AAS abusers and cardiac death has not been conclusively established, though ventricular arrhythmias and congestive heart failure have often been reported, and thrombotic complications (intracardial thrombosis, stroke, venous thromboembolism, cerebral venous sinus thrombosis) have been markedly associated with AAS abusers [12011].

Anabolic steroids have been linked to many adverse health effects, including cardiomyopathy, cerebrovascular events, hypertension, aggression, prostatic hypertrophy, and cholestatic liver injury. In one study, 96 percent of users of anabolic steroids reported at least one side effect. However, the absolute risks have not been fully evaluated. Users typically take them in “cycles” that vary in length (often 8-12 weeks) to minimise side effects, with a similar amount of time “off cycle” [12025].

Anabolic-androgenic steroids (AAS) administration has been shown to be associated with cardiovascular side effects, urogenital problems, that is, gynecomastia, impotency, hepatotoxicity, hepatocellular carcinoma, and neuropsychiatric disorders, that is, aggressiveness and depression. Cardiovascular adverse effects of AAS abuse have been reported sporadically as case reports of hypertension, myocardial infarction (MI) and stroke, dysrhythmia, cardiomyopathy, and sudden cardiac death in body builders with long-term AAS abuse in the recent years. Case reports on hard atherosclerotic endpoints (sudden cardiac death, MI or stroke) comprise young AAS abusers without preexistent cardiac risk factors, suggesting that a high AAS dose imposes additional independent risk to conventional cardiovascular risk factors. It has been reported a more than four times higher incidence of early death in professional athletes abusing AAS compared to the age- and sex-matched general population, in a 12-years prospective observation. In addition to the potential risk associated with AAS abuse, it is notable that therapeutic treatment with AAS in hypogonadic men has recently been shown to be linked with a higher cardiovascular event rate. This important finding underscores that there is a delicate balance between benefits and risks related to AAS use as a treatment option for patients suffering from androgen deficiency. Adding up clinical AAS use and illegal AAS abuse rates represent a new wave of AAS-associated cardiovascular adverse consequences, in which early diagnosis can reduce health burden. The wave of now middle-aged ex-AAS abusers and the increasing group of the elderly AAS users necessitates more detailed documentation of the underlying pathophysiology to enhance insight into the delicate balance between benefit and harm. Owing to the obvious ethical reasons, prospective double-blinded human studies are not
easily justified. Accordingly, retrospective case-control studies of cohorts and prospectively follow-up of such cohorts seem to be the most feasible strategy for human studies to obtain more conclusive epidemiologic data [12125].

There are limited data on the short-term complication of AAS use with most data involving case reports. The long-term health effects of chronic AAS abuse are not well defined because of the difficulty in studying illicit drug use and the highly-variable dosages involved with AAS abuse. Clinical and laboratory studies indicate that the administration of AASs causes physiologic changes, primarily in the liver, reproductive system, and serum lipids. The use of AAS produces an unfavorable change in the blood lipid profile (i.e. elevated low-density lipoprotein, reduced high-density lipoprotein) with potentially increased risk of coronary heart disease; although conflicting, some data suggest left ventricular hypertrophy may persist after cessation of AAS use as a result of elevated blood pressure during use. Other potential adverse effects include glucose intolerance (i.e. increased peripheral insulin resistance), hyperinsulinism, behavioral and mood changes, cerebrovascular accidents, prostate abnormalities, edema, and immune dysfunction. Most physiologic changes associated with AAS use are reversible within several months of the cessation of AAS use [13003].

**Short-term side effects**

The attraction of anabolic agents apparently continues to be unconfined among cheating athletes and recreational sportsmen and women despite numerous comprehensive and new reports on health risks attributed to the abuse of AAS, ranging from acne fulminans over cardiovascular issues to increased risk of breast and Leydig cell cancer as well as psychic disorders and dependence [13009].

Most of the adverse effects following the use of AASs result from the enhancement of normal physiologic response to testosterone by either direct receptor agonist activity or suppression of steroid biosynthesis. In general, toxic effects associated with AAS abuse involve the following:

- anabolic side effects
- enhanced androgenic effects
- estrogenic side effects
- antiandrogenic effects from the suppression of the hypothalamus-pituitary-adrenal/gonadal axes
- hepatotoxicity
- neuropsychiatric effects

Methodological issues limit the determination of the toxic effects of illicit AAS use including the extraordinary doses and types of AAS used by athletes compared with medical use, reporting bias of self-reports, the paucity of well-documented pathologic findings, and the lack of well-defined postmortem markers of AAS use. Most medical data on the toxic effects of AAS abuse involve case reports rather than epidemiologic studies. Pathologic abnormalities from AAS abuse are best-documented in the cardiovascular system, reproductive system, liver, and serum lipids. Animal studies suggest that AAS can cause dysplasia of collagen fibrils and decreased tensile strength, and potentially the use of these drugs could cause disruption of connective tissue [13003].

**Anesthesia risk**
A strong tendency toward body enhancement and body forming in western industrial societies makes it more likely for each anesthesiologist to get involved in the care of bodybuilders. These patients quite frequently consume androgenic anabolic steroids, human growth hormone and other drugs or substances which are believed to accelerate muscle gain. Cardiovascular, hepatic, psychiatric, hormonal and infectious side effects or complications are common and rarely monitored by health care professionals. The anesthesia risk is not exactly known but seems to be determined mainly by cardiovascular events like myocardial ischemia and dysrhythmias [08143].

**Different effects of different anabolic steroids**

All synthetic AAS are derived from testosterone. They have a carbon skeleton with 4 fused rings; most have 19 carbons. Modifications include hydroxylation at the C10 position to increase receptor binding affinity (e.g. nandrolone) esterification to slow release into circulation (e.g. testosterone cypionate), or alkylation at the C17 position to permit moral delivery by reducing first-pass metabolism in the liver (e.g. oxymetholone). AAS can be converted to highly-androgenic or estrogenic metabolites. For testosterone, dihydroteosterone is the principle androgenic product; estradiol is the major estrogenic metabolite. Non-aromatizable AAS (e.g. drostanolone) have fewer estrogenic side-effects such as gynecomastia. Non-reducible AAS (e.g. oxandrolone) have fewer androgenic side-effects such as acne, baldness, and prostatic hypertrophy because they have lower binding affinity for the androgen receptor. For athletes subject to drug testing, a key drawback of synthetic AAS is that their use is easily detected, since their metabolites are not normally present. An extended precompetition wash-out period is necessary to avoid a positive test. This varies with the route of administration and the half-life of the individual AAS. However, long-acting AAS such as nandrolone can be detected for at least 6 months. By contrast, the urinary metabolites of exogenous and endogenous testosterone are virtually identical. Many athletes take long-acting testosterone esters such as testosterone propionate. Although esterification prolongs the half-life in circulation, the active steroid is still testosterone [12100].

Doping is becoming an everyday problem in sports medicine. Its main feature is its universality: it concerns all sports, even the most unexpected, from cycling to billiards; all countries are affected with certain continental preferences with regards to the substances used; it is seen in all levels of competition, both in amateurs and professionals. Doping is observed early on, even in childhood. Many substances are used and they are increasingly available: all bodily functions are targeted: cerebral, metabolic, cardiovascular, respiratory, haematological and, in the near future, genetic. Detection of doping is difficult and unpredictable in a legislative environment which is gradually improving. The different modes of action of the doping substances often target the cardiovascular system, especially with regards to their potential complications: hypertension, arrhythmias, thrombosis, coronary artery and peripheral artery diseases and also cardiomyopathies. Every cardiologist should therefore be aware of the problem, even outside the context of sport, as it may impact on daily cardiological practice [06065].

**Toxicokinetics**

Most of the data on the kinetics of testosterone and AASs is derived from the pharmacokinetics of these compounds in animals or in hypogonadal males receiving therapeutic doses of AASs. There are few data on the toxicokinetics of AASs in individuals abusing AASs at doses up to 10-100 times the therapeutic dose. Despite the rapid
absorption of testosterone, the systemic bioavailability of oral testosterone is low as a result of extensive first-pass hepatic metabolism. Structural modifications of testosterone produce synthetic testosterone derivatives (anabolic-androgenic steroids), which increase bioavailability and prolong the duration of action. Alkylation of the 17-alpha position of testosterone produces oral AAS, whereas esterification of the 17-beta position results in injectable AAS (e.g. lipid-soluble cypionate or enanthat). The duration of action of these esters depends on the rate of absorption from the site of administration as determined by the chain length of the acid moiety and the formulation. Hydrolysis of these esters in vivo prolongs the duration of action compared with testosterone. Anabolic androgenic steroids can diffuse across the skin and mucous membranes, allowing other delivery modes including transdermal patches, nasal sprays, and buccal tablets. Following oral administration of 120 mg testosterone undecanoate, volunteer studies in dicate that plasma concentrations of testosterone are detectable for about 1-6 h after administration using gas chromatography–tandem massspectrometry. There are dramatic individual variations (i.e.10-fold) in the peak total plasma testosterone concentrations. In a study of 61 eugonadal men receiving long-acting gonadotropin-releasing hormone agonist to suppress endogenous testosterone secretion, the mean nadir testosterone concentrations ranged from 2.53 to 23.7 ng/mL following weekly injections of testosterone enanthate doses of 25-600 mg for 20 weeks [13003].

Anabolic-androgenic steroids are bound in the plasma to sex-hormone-binding globulins. Although testosterone is highly protein bound (i.e. 98 %) in plasma, the binding of AAS to sex-hormone-binding globulins is highly variable base done animal studies. The metabolism of endogenous testosterone involves the conversion to the estrogenic compound, estradiol, via steroid aromatase and the androgenic compound, 5alpha-dihydrotestosterone, via 5alpha–steroid-reductase. Comparatively, the biotransformation of AASs is quite complex. The initial and rateliming step in testosterone metabolism is reduction of the C4-C5 double bond on the A-ring with 5alpha-reductase and 5beta-reductase. Hydroxylation of testosterone by CYP450 isoenzymes results in the formation of a variety of minor urinary metabolites of testosterone. Single-dose human excretion studies indicate that 6-beta-hydroxylation is also a minor pathway for the biotransformation of boldenone (17beta-hydroxyandrost-1,4-dien-3-one) and 17alpha-methyl-testosterone. However, 6beta-hydroxylation of the B-ring is the major metabolic pathway for 4-chloro-1,2-dehydro-17alpha-methyltestosterone, methandienone, and fluoxymesterone because the presence of a C1-C2 double bond in the former 2steroids and the C9 alpha-fluorine atom in the latter compound blocks A-ring reduction. Metabolic changes (e.g. 12-hydroxylation) of AASs at the C-ring are minor. D-ring metabolism by the enzymatic oxidation of 17beta-hydroxysteroid-dehydrogenase to form the corresponding 17-ketosteroid is a major metabolic pathway for testosterone and all AAS swith secondary 17beta-hydroxy groups (e.g. boldenone, clostebol, drostanolone, mesterolone, methenolone, nandrolone, norclostebol, and stenbolone). The main urinary metabolites of testosterone are androstanone (3alpha-hydroxy-5alpha-androstan-17-one), etiocholanolone (3alpha-hydroxy-5beta-androstan-17-one), epiandrosterone (3beta-hydroxy-5alpha-androstan-17-one), 5alpha-androstane-3alpha, 17beta-diol, and 5beta-androstane-3alpha,17beta-diol [13003].

In individuals without AAS use, only small amounts (i.e. about 1 %) of endogenous testosterone appear unchanged in the urine. Phase II conjugation reactions couple AASs and associated metabolite swith glucuronic acid or sulfate before excretion in the urine. The vast majority (i.e. about 90 %) of the absorbed dose of testosterone appears in the urine as glucuronide or sulfate conjugates. In a study of 8 hypogonadal males, the terminal elimination half-lives of 500 mg and 1000 mg intramuscular doses of testosterone undecanoate were 18.3 and 2.3 days and 23.7 and 2.7 days, respectively. The mean residence times were 21.7 and 1.1 days and 23.0 and 0.8 days, respectively. Not all anabolic
steroids undergo phase II reactions. Unconjugated AASs in human urine include oxandrolone, fluoxymesterone, 4-chloro-1,2-dehydro-17alpha-methyltestosterone, and formebolone, along with metabolites of oxandrolone, methandienone, and stanozolol. There is very limited (i.e., about 5%) enterohepatic recirculation of testosterone. Anabolic-androgenic steroids readily cross the placenta [13003].

**Impurities in illicit samples of anabolic steroids**

There are few data on the purity of illicit samples of AASs as a result of the lack of regulation. Consequently, there are no assurances that the chronic AAS abuser knows the dose or type of AAS. The difficulty determining doses used by AAS abusers limits the ability of studies to elucidate the effect of AAS abuse. Frequently, illicit samples of AASs do not contain declared ingredients or concentrations of ingredients. Analysis of 70 products confiscated from illegal sources demonstrated 17 (35%) of the 48 steroidal compounds did not contain labeled ingredients as measured by liquid chromatography-tandem mass-spectrometry, gas chromatography mass-spectrometry with nitrogen-phosphorus detection, gel-electrophoresis, and immunological tests. Visual inspection did not distinguish original products from counterfeits [13003].

**Anabolic steroids’ impact on the cardiovascular system**

**Overview**

The use of doping substances and methods is extensive not only among elite athletes, but also among amateur and recreational athletes. Many types of drugs are used by athletes to enhance performance, to reduce anxiety, to increase muscle mass, to reduce weight or to mask the use of other drugs during testing. However, the abuse of doping substances and methods has been associated with the occurrence of numerous health side-effects. The adverse effects depend on the type of the consumed drug, as well as the amount and duration of intake and the sensitivity of the body, since there is a large inter-individual variability in responses to a drug. Usually the doses used in sports are much higher than those used for therapeutic purposes and the use of several drugs in combination is frequent, leading to higher risk of side-effects. Among biomedical side-effects of doping, the cardiovascular ones are the most deleterious. Myocardial infarction, hyperlipidemia, hypertension, thrombosis, arrhythmogenesis, heart failure and sudden cardiac death have been noted following drug abuse. This paper reviews the literature on the adverse cardiovascular effects after abuse of prohibited substances and methods in athletes, aiming to inform physicians, trainers and athletes and to discourage individuals from using drugs during sports [06063].

The intake of anabolic-androgenic steroids (AAS) leads to an increase in skeletal muscle mass and is prohibited as a doping measure in sport. AAS abuse is not limited to competitive athletes. It is also prevalent in subjects who do body building or resistance training for cosmetic reasons only. Out of the numerous and partly serious side effects, the cardiovascular ones maybe most important. An increase in left ventricular muscle mass is well documented, and some researchers have even reported concentric hypertrophy. By contrast, resistance training without AAS intake does not lead to increased ventricular wall thickness. AAS do not affect the systolic function of the left ventricle, whereas diastolic function might be impaired. Different ultrastructural myocardial alterations have been documented in animal studies. In addition, AAS can induce arterial hypertension. Blood clotting and fibrinolysis are negatively affected, and several case studies of thrombi exist in
young strength athletes. Changes in the concentration of blood lipoproteins, particularly a reduction in vessel-protective HDL cholesterol, can lead to early atherosclerosis. Many case reports exist about cardiac deaths in seemingly healthy subjects-most often body builders and other strength athletes. In fatal and nonfatal myocardial infarctions patent coronary arteries were proven frequently. Besides the prothrombotic effects of AAS, an impaired endothelial function and vasospasms are discussed hypothetically as pathomechanisms. Also, cardiomyopathies can occur due to AAS abuse. On the basis of the described possible cardiovascular side effects, it can be concluded that in cases of sudden cardiac deaths in young athletes, a misuse of AS should be excluded [06064].

Scientific data on the cardiac and metabolic complications of AAS abuse are divergent and often conflicting. A total of 49 studies describing 1,467 athletes were reviewed to investigate the cardiovascular effects of the abuse of AAS. Although studies were typically small and retrospective, some associated AAS abuse with unfavorable effects. Otherwise healthy young athletes abusing AAS may show elevated levels of low-density lipoprotein and low levels of high-density lipoprotein. Although data are conflicting, AAS have also been linked with elevated systolic and diastolic blood pressure and with left ventricular hypertrophy that may persist after AAS cessation. Finally, in small case studies, AAS abuse has been linked with acute myocardial infarction and fatal ventricular arrhythmias. In conclusion, recognition of these adverse effects may improve the education of athletes and increase vigilance when evaluating young athletes with cardiovascular abnormalities [10305].

Abuse of anabolic androgenic steroids (AAS) has been linked to a variety of different cardiovascular side effects. In case reports, acute myocardial infarction is the most common event presented, but other adverse cardiovascular effects such as left ventricular hypertrophy, reduced left ventricular function, arterial thrombosis, pulmonary embolism and several cases of sudden cardiac death have also been reported. However, to date there are no prospective, randomized, interventional studies on the long-term cardiovascular effects of abuse of AAS. In one review it was studied the relevant literature regarding several risk factors for cardiovascular disease where the effects of AAS have been scrutinized:

- echocardiographic studies show that supraphysiologic doses of AAS lead to both morphologic and functional changes of the heart. These include a tendency to produce myocardial hypertrophy, a possible increase of heart chamber diameters, unequivocal alterations of diastolic function and ventricular relaxation, and most likely a subclinically compromised left ventricular contractile function
- AAS induce a mild, but transient increase of blood pressure. However, the clinical significance of this effect remains modest
- AAS confer an enhanced pro-thrombotic state, most prominently through an activation of platelet aggregability. The concomitant effects on the humoral coagulation cascade are more complex and include activation of both pro-coagulatory and fibrinolytic pathways
- users of AAS often demonstrate unfavorable measurements of vascular reactivity involving endothelial-dependent or endothelial-independent vasodilatation. A degree of reversibility seems to be consistent, though
- there is a comprehensive body of evidence documenting that AAS induce various alterations of lipid metabolism. The most prominent changes are concomitant elevations of LDL and decreases of HDL, effects that increase the risk of coronary artery disease
- the use of AAS appears to confer an increased risk of life-threatening arrhythmia leading to sudden death, although the underlying mechanisms are still far from being elucidated
Taken together, various lines of evidence involving a variety of pathophysiologic mechanisms suggest an increased risk for cardiovascular disease in users of anabolic androgenic steroids [10064].

Athletes use androgenic-anabolic steroids but it may lead to dilated cardiomyopathy. It was report a case of a 41-year-old bodybuilder with severe systolic dysfunction and Class IV heart failure despite maximal medical therapy. He used anabolic steroids and insulin growth factor, and did not have any other risk factors for cardiomyopathy. It was briefly reviewed the literature and summarize other reported cases with similar scenarios. In most of them cardiomyopathy was at least partially reversible after discontinuation of anabolics. Abuse of anabolic steroids may be an uncommon cause of cardiomyopathy in young and otherwise healthy individuals [09057].

Chronic heart failure (CHF) involves derangements in multiple neurohormonal axes leading to a procatabolic state and wasting syndrome associated with significant mortality. Catabolic abnormalities include excess catecholamines and glucocorticoids. Anabolic defects include deficiencies of sex steroids, insulin resistance, and growth hormone (GH) resistance. These abnormalities are also correlated with increased morbidity and mortality in CHF. Anabolic axes have been augmented in pilot studies in CHF with testosterone, GH, insulin-like growth factor-1, and GH secretagogues. Results have been varied although some treatments have been associated with improved surrogate endpoints. One review article explores the current understanding of metabolic derangements in CHF and highlights potential neuroendocrine treatment strategies [09058].

Recent surveys and reports suggest that many athletes and bodybuilders abuse anabolic-androgenic steroids (AAS). However, scientific data on the cardiac and metabolic complications of AAS abuse are divergent and often conflicting. A total of 49 studies describing 1,467 athletes were reviewed to investigate the cardiovascular effects of the abuse of AAS. Although studies were typically small and retrospective, some associated AAS abuse with unfavorable effects. Otherwise healthy young athletes abusing AAS may show elevated levels of low-density lipoprotein and low levels of high-density lipoprotein. Although data are conflicting, AAS have also been linked with elevated systolic and diastolic blood pressure and with left ventricular hypertrophy that may persist after AAS cessation. Finally, in small case studies, AAS abuse has been linked with acute myocardial infarction and fatal ventricular arrhythmias. In conclusion, recognition of these adverse effects may improve the education of athletes and increase vigilance when evaluating young athletes with cardiovascular abnormalities [10431].

The most common cardiovascular consequences of AAS include atherosclerosis (secondary to changes in cholesterol metabolism and platelet function), hypertension, cardiac hypertrophy, impaired cardiac function, and sudden death. AAS use causes metabolic derangements that increase the risk for atherosclerosis and thrombus formation. Studies using animal models and various steroid regimens have demonstrated changes in serum cholesterol levels with decreased high-density lipoprotein and increased low-density lipoprotein, both promoting atherosclerotic and peripheral vascular disease. Cholesterol alterations vary among different AASs; alkylated agents (e.g. stanozolol) cause greater changes than testosterone [07058].

AAS use also increases platelet reactivity without an associated thrombocytosis; this has been proposed as an etiology for some of the myocardial infarctions, strokes, and peripheral vascular disease events reported in otherwise healthy individuals. AAS use also increases serum C-reactive protein (CRP), reflecting an inflammatory state that may contribute to atheroma formation and peripheral vascular disease. Conversely, changes in lipid
metabolism may be protective from atheroma formation because of a reduction in lipoprotein A. Many studies show that AASs cause abnormal cholesterol profiles, increased CRP, and increased platelet reactivity. It is difficult to quantify the change in risk, but one study estimates AASs triple the cardiovascular risk [07058].

Recent evidence suggests that low, rather than high, testosterone (T) is associated with increased male morbidity and mortality. It was reviewed relationships between hypogonadism, metabolic syndrome (MetS) and cardiovascular (CV) disease (CVD), along with erectile dysfunction (ED), a common condition in the three diseases. Although several experimental data indicate that T exerts a protective effect on vascular function, epidemiological studies do not support a link between hypogonadism and CVD and three meta-analyses found no significant effect of testosterone replacement therapy (TRT) on CV events. Low T is associated with increased risk of CV death in community-dwelling men, and in men with ED. It is possible that both low T and CVD are associated with another, still unknown (or not assessed) factor, thus explaining the association, in the absence of any causal relationship. A meta-analysis on the effect of TRT in MetS-associated hypogonadism demonstrated positive effects of T on some of the components of MetS. Large-scale interventional studies with TRT are therefore advisable [11092].

Cardiovascular disorders are known to be the most common cause of sudden death during exercise. In younger athletes (below 45 years) this is due in the majority of cases to congenital heart diseases, while in older people atherosclerosis is the primary cause. There is, however, a non-negligible percentage of disorders of the cardiovascular system, even sudden cardiac death (SCD), that are attributable to the use of performance-enhancing drugs, either prohibited (doping) or legal. The users can be athletes, professional or amateur, or just people engaging in exercise in gyms or fitness and leisure centres, while both sexes and all age groups are involved. Seeking to improve their performance, according to the event in which they participate, most athletes use a combination of prohibited substances and methods, or of prohibited and non-prohibited drugs, so as to alleviate the complications and/or to avoid being detected by screening. The most common and serious consequences of almost all illicit drugs in sport concern the cardiovascular system. These disorders, such as hypertension, cardiac arrhythmias, and acute myocardial infarction, may be manifested either directly, or as the result of long-term use. Frequent complications may also occur in other organs. Specifically, anabolic steroids have been implicated in liver cirrhosis and liver or kidney cancer, growth hormone in diabetes mellitus, erythropoietin in thromboembolic episodes, central nervous system stimulants in psychotic syndromes, and so on. Apart from the prohibited substances, however, cardiovascular disorders may be caused by other substances commonly used in sports, such as dietary supplements [12126].

A search of the English-language scientific literature from 1976 through March 2012 was performed primarily by searching the MEDLINE, Embase, and Google databases. The key words used in the search included androgens, anabolic, androgenic, steroids, exercise, athlete, cardiovascular, effects. The bibliographies of articles from the above search also were searched for relevant articles; links on Web sites containing published articles were searched for pertinent information [12119].

Potential adverse effects of AAS on the cardiovascular system include atherogenesis, thrombosis, vasospasm, myocarditis, concentric left ventricular hypertrophy, myocardial fibrosis, hypertrophic cardiomyopathy with ventricular dysrhythmias, and direct myocardial injury. However, the contribution of AAS use to these potential adverse cardiovascular effects remains unclear. Chronic AAS use enhances hepatic triglyceride lipase activity, resulting in reduction of high-density lipoproteins and elevation of low-density lipoproteins. Although these changes are reversible within several months of cessation of AAS use,
chronic AAS use theoretically increases the risk of cardiac disease. Potentially, the chronic abuse of AAS enhances coagulability and thrombosis, but the clinical importance of this potential adverse effect also remains unclear. Studies of chronic AAS abuse in weight lifters suggest that some anabolic-androgenic steroid using weight lifters have accelerated activation of their hemostatic system as evidenced by increased generation of both thrombin and plasmin. A study of AAS-positive steroid using weight lifters indicated that these individuals had a higher percentage of abnormally high plasma thrombin-antithrombin complexes along with elevated plasma concentrations of prothrombin fragment 1, antithrombin II, and protein S, when compared with non-AAS using controls. Additionally, the plasma concentrations of tissue plasminogen activator and its inhibit or were lower in AAS users than in controls. Clinical studies on body builders suggest that chronic AAS use impairs vascular eactivity independent of the smooth muscle hypertrophy and vascular stiffness associated with bodybuilding. Anabolic-androgenic steroids decrease the production of cyclic guanosine monophosphate (cGMP) by inhibiting guanylyltransferase. As a result, AAS spottetently inhibit the ability of nitric oxide store lax smooth muscles in the coronary arteries resulting in coronary artery vasospasm and potentially sensitizing AAS users to sudden death. Case reports associate the chronic use of anabolic steroids with sudden death and contraction band necrosis in the myocardium. In these cases, no other cause of death was apparent, but the role of chronic, high-dose anabolic steroid use in these deaths remains unclear. Athletes with certain genetic mutations and structural abnormalities may be particularly vulnerable to the use of anabolic steroids including athletes with accessory AV pathways, latent structural heart diseases (dilated cardiomyopathy, arrhythmogenic right ventricular dysplasia type II, myocarditis, segmental arrhythmogenic ventricular cardiomyopathy, and coronary artery anomalies), latent Brugada syndrome, mutations of the long QT syndrome genes, and other genetic mutations of ion channels (cardia cryanodine receptor gene defects and calsequestrin gene defects). Pathologic evidence of some of these abnormalities may not appear on postmort emexamination. The use of diuretics to mask the use of anabolic steroids may predispose these athletes to serious ventricular dysrhythmias from hypokalemia and dehydration [13003].

Experimental
The aim of one study was the investigation of effects of the metenolone enanthate (ME) that is used among athletes as doping and muscle amplifier, on hearts of male and female rats that are in puberty using morphometrical methods. A total of 36 rats which were divided into three separate groups (Experiment, ME; vehicle, PO; control, C) each consisting of 6 male and 6 female rats were used. 0.5mg/kg metenolone enanthate was applied intraperitoneally into experiment subjects 5 times a week over a period of 4 weeks. At the end of experiment, rats were euthanized and their hearts were cut at the level of musculus papillaris after the fixation in formalin. Hearts were taken out and embedded in paraffin wax. Photos were taken at cut surfaces, and thickness, diameters and surface area levels were measured. Left ventriculus mass (LVM) and left ventriculus mass index (LVMI) were calculated. In the study LVM and LVMI were found to be significantly higher in the ME group in females whereas left ventricular lumen diameter (LVLD) were found to be significantly lower. Thus left ventricular hypertrophy development was observed. LVM and LVMI were found to be similar in ME and C groups among male rats and the highest level of these data were found in the group. LVM and LVMI were higher among females. In conclusion, it has been shown that the adverse effects of ME on heart were developing starting from puberty and resulting with the enlargement of the heart and left ventricular hypertrophy and especially among females this condition was more evident. It has also been discussed that the continuous use of drugs may further enhance this condition [12127].

Cardiac aldosterone might be involved in nandrolone decanoate (ND) deleterious effects on the heart. Therefore, we investigated the involvement of cardiac aldosterone, by the
pharmacological block of AT1 or mineralocorticoid receptors, on cardiac hypertrophy and fibrosis. Male Wistar rats were randomized into 8 groups (n=14/group). Nandrolone (10 mg/kg/week), was administered during 10 weeks of swimming training (5 times/wk). Losartan (20 mg/kg/day) and spironolactone (10mg/kg/day) were administered in drinking water. Cardiac hypertrophy was increased 10 percent by using nandrolone and 17 percent by nandrolone plus training. In both groups, there was a significant increase in the collagen volumetric fraction (CVF) and cardiac collagen type III expression. The nandrolone treatment increased: LV-ACE activity, AT1 receptor expression, aldosterone synthase (CYP11B2) and 11-beta hydroxysteroid dehydrogenase 2 (11betaHSD2) gene expression and inflammatory markers, TGFbeta and osteopontin. Both losartan and spironolactone inhibited the increase of CVF and collagen type III. In addition, both treatments inhibited the increase in LV-ACE activity, CYP11B2, 11betaHSD2, TGFbeta and osteopontin induce by the nandrolone treatment. The results suggest that these effects may be associated to TGFbeta and osteopontin. Thus, it was conclude that the cardiac aldosterone has an important role on the deleterious effects on the heart induced by nandrolone [11065].

To investigate the effects of exercise training and anabolic androgenic steroids (AAS) on hemodynamics, glycogen content, angiogenesis, apoptosis and histology of cardiac muscle. Forty rats were divided into 4 groups; control, steroid, exercise-trained and exercise-trained plus steroid groups. The exercise-trained and trained plus steroid groups, after one week of water adaptation, were exercised by jumping into water for 5 weeks. The steroid and trained plus steroid groups received nandrolone decanoate, for 5 weeks. Systolic blood pressure and heart rate (HR) were monitored weekly. Heart weight/body weight ratio (HW/BW ratio) was determined. Serum testosterone, vascular endothelial growth factor (VEGF), cardiac caspase-3 activity and glycogen content were measured. Compared with control, the steroid group had significantly higher blood pressure, HR, sympathetic nerve activity, testosterone level, HW/BW and cardiac caspase-3 activity. Histological examination revealed apoptotic changes and hypertrophy of cardiomyocytes. In exercise-trained group, cardiac glycogen, VEGF and testosterone levels were significantly higher while HR was significantly lower than control. HW/BW was more than control confirmed by hypertrophy of cardiomyocytes with angiogenesis on histological examination. Trained plus steroid group, had no change in HR, with higher blood pressure and HW/BW than control, cardiac glycogen and serum VEGF were higher than control but lower than exercise-trained group. Histological examination showed hypertrophy of cardiomyocytes with mild angiogenesis rather than apoptosis. Thus, when exercise is augmented with AAS, exercise-associated cardiac benefits may not be fully gained with potential cardiac risk from AAS if used alone or combined with exercise [13129].

One study focused on the short term effects of repeated low level administration of turinabol and methanabol on cardiac function in young rabbits (4 months). The experimental scheme consisted of two oral administration periods, lasting 1 month each, interrupted by 1 month wash-out period. Serial echocardiographic evaluation at the end of all three experimental periods was performed in all animals. Oxidative stress markers have also been monitored at the end of each administration period. Treated animals originally showed significantly increased myocardial mass and systolic cardiac output, which normalized at the end of the wash out period. Re-administration led to increased cardiac output, at the cost though of a progressive myocardial mass reduction. A dose-dependent trend towards impaired longitudinal systolic, diastolic and global myocardial function was also observed. The adverse effects were more pronounced in the methanabol group. For both anabolic steroids studied, the low dose had no significant effects on oxidative stress markers monitored, while the high dose created a hostile oxidative environment. In conclusion, anabolic administration has been found to create a possible deleterious long term effect on the growth of the immature heart and should be strongly discouraged especially in young human subjects [13130].
High doses of anabolic androgenic steroids (AAS) impair the cardioprotective effects of exercise against ischemia/reperfusion (I/R) insult, possibly through cellular redox imbalance. Here, the effect of nandrolone decanoate (DECA) treatment on heart redox metabolism was investigated during I/R in sedentary and exercised rats. DECA treatment significantly reduced superoxide dismutase and glutathione reductase activities in exercised rats after heart reperfusion. Catalase and glutathione peroxidase activities were not affected by DECA in both sedentary and trained rats, regardless the I/R period. DECA also induced myocardial oxidative stress, as evidenced by the reduced levels of total reduced thiols after heart reperfusion in exercised rats treated with the anabolic steroid. These results indicate that cardiotoxic effects of supraphysiological doses of AAS involve reduced heart antioxidant capacity [13131].

One study focuses on the short term effects of repeated low level administration of turinabol and methanabol on cardiac function in young rabbits (4 months-old). The experimental scheme consisted of two oral administration periods, lasting 1 month each, interrupted by 1-month wash-out period. Serial echocardiographic evaluation at the end of all three experimental periods was performed in all animals. Oxidative stress markers have also been monitored at the end of each administration period. Treated animals originally showed significantly increased myocardial mass and systolic cardiac output, which normalized at the end of the wash out period. Re-administration led to increased cardiac output, at the cost though of a progressive myocardial mass reduction. A dose-dependent trend towards impaired longitudinal systolic, diastolic and global myocardial function was also observed. The adverse effects were more pronounced in the methanabol group. For both anabolic steroids studied, the low dose had no significant effects on oxidative stress markers monitored, while the high dose created a hostile oxidative environment. In conclusion, anabolic administration has been found to create a possible deleterious long term effect on the growth of the immature heart and should be strongly discouraged especially in young human subjects [13133].

Metabolic syndrome

Obesity is one of the constellations of factors that make up the definition of the metabolic syndrome. Metabolic syndrome is also associated with insulin resistance, dyslipidemia, hypertriglyceridemia, and type 2 diabetes mellitus. The presence of obesity and metabolic syndrome in men and women is also associated with increased risk of cardiovascular disease and hypertension. In men, obesity and metabolic syndrome are associated with reductions in testosterone levels. In women, obesity and metabolic syndrome are associated with increases in androgen levels. In men, reductions in androgen levels are associated with inflammation, and androgen supplements reduce inflammation. In women, increases in androgens are associated with increases in inflammatory cytokines, and reducing androgens reduces inflammation [11093].

Coronary artery calcifications

The authors measured coronary artery calcification as a means of examining the impact of anabolic steroids on the development of atherosclerotic disease in body builders using anabolic steroids over an extended period of time. Fourteen male professional body builders with no history of cardiovascular disease were evaluated for coronary artery calcium, serum lipids, left ventricular function, and exercise-induced myocardial ischemia. Seven subjects had coronary artery calcium, with a much higher than expected mean score of 98. Six of the 7 calcium scores were >90th percentile. Mean total cholesterol was 192 mg/dL, while mean
high-density lipoprotein was 23 mg/dL and the mean ratio of total cholesterol to high-density lipoprotein was 8.3. Left ventricular ejection fraction ranged between 49 percent and 68 percent, with a mean of 59 percent. No subject had evidence of myocardial ischemia. This small group of professional body builders with a long history of steroid abuse had high levels of coronary artery calcium for age. The authors conclude that in this small pilot study there is an association between early coronary artery calcium and long-term steroid abuse. Large-scale studies are warranted to further explore this association [06069].

Myocardial infarction

Anabolic-androgenic steroids are associated with numerous side effects, including acute myocardial infarction. It was reported a case of myocardial infarction in a young 31-year-old bodybuilder. Because of the serious cardiovascular complications of anabolic steroids, physicians should be aware of their abuse and consequences [08142].

Sudden cardiac deaths secondary to myocardial infarction and related to AAS use in previously healthy athletes have been reported; however, it must be pointed out that these effects are reported in individual case reports and no large, randomized study has been conducted to verify these results. Exposure to AAS alters endothelial cell growth with a strong antiproliferative effect, induces apoptosis, and modifies intracellular levels of calcium. These observed endothelial alterations may be considered events that predispose to serious damage at the cell vasculature level. Androgens impair arterial vasomotor response and consequently increase collagen and other fibrous proteins in arterial vascular tissue and they impair flow-mediated, endothelium-dependent vasodilation. This effect may improve after the discontinuation of agents. Moreover, experimental studies have shown that androgens potentiate platelet aggregation in vitro and in vivo. Androgens may exert their effect on platelets through an effect on the prostaglandin system and lead to increasing platelet production of thromboxane A2 (a potent platelet aggregator), decreasing the production of prostacycline (prostaglandin I2, an inhibitor of platelet aggregation) and increasing fibrinogen levels. They also increase human platelet thromboxane A2 receptor density and their aggregation responses. The above-mentioned physiological changes may predispose individuals to be at higher risk for myocardial infarction. It also has been shown that aortic distensibility decreases in athletes who use AAS. This went against previously reported increases in aortic distensibility in athletes in comparison with age-matched sedentary volunteers. Aortic stiffness by increasing ventricular load predisposes to the development of LVH, progressing to left ventricular dysfunction and cardiac failure, and creating an unfavorable oxygen supply/demand ratio. It also causes a reduction in arterial pressure during diastole, which decreases the coronary perfusion pressure and contributes to myocardial ischemia even in the absence of coronary artery atherosclerotic narrowing [12119].

Cardiac arrhythmias

Sudden cardiac arrhythmia resulting from inflammatory process and myocardial fibrosis has been suggested to be the cause of death in athletes using AAS. AAS cause the deepest and most prolonged depression of stimulation threshold in the heart muscles. Long-term use may be responsible for an alteration in the electrophysiology of the myocardium that may predispose to reentry mechanism. It also has been shown that QTc interval and dispersion are increased in individuals who abuse androgens, suggesting the presence of ventricular repolarization abnormalities that could increase the risk of cardiac arrhythmias and sudden cardiac death. Atrial fibrillation secondary to high-dose steroids was reported in two cases of athletes who were using AAS and had no other known cause of atrial fibrillation [12119].
In an electrophysiological study power athletes taking AAS were found to have increased QT dispersion and short QT intervals. The authors associated these changes with the manifestation of arrhythmias. In addition, in an experimental study it was found that the administration of nandrolone decanoate to rats led to a disturbance of cardiac autonomic nervous system function. It was also found analogous results in a study of athletes who used anabolic steroids, who presented a reduction in baroreflex sensitivity and maintained that anabolic steroids lead to degenerative changes in endomyocardial sympathetic neurons, resulting in the appearance of malignant arrhythmias. This degenerative mechanism is referred to as catecholamine myotoxicity [12126].

Cardiac arrhythmias are associated with AAS abuse. Most commonly, atrial fibrillation but also ventricular tachycardia and ventricular fibrillation has been described secondary to AAS abuse in human case reports. In a study on rats treated with high-dose nandrolone for 10 weeks, heart rate variability measurements revealed a reduction in parasympathetic activity compared with the vehicle-treated group. Sympathetic indices were also higher in the AAS-treated group. It was also shown that AAS-treated animals show prolonged action potentials as a result of reduced density of transient potassium outward current in the left ventricle [12125].

The current management of athletes with cardiac arrhythmias has become complicated by the widespread use of illicit drugs, which can be arrhythmogenic. The World Anti-Doping Agency annually updates a list of prohibited substances and methods banned by the International Olympic Committee that includes different classes of substances namely, anabolic androgenic steroids, hormones and related substances, beta2-agonists, diuretics, stimulants, narcotics, cannabinoids, glucocorticosteroids, alcohol, beta-blockers and others. Almost all illicit drugs may cause, through a direct or indirect arrhythmogenic effect, a wide range of cardiac arrhythmias (focal or reentry type, supraventricular and/or ventricular) that can even be lethal and which are frequently sport activity related. A large use of illicit drugs has been documented in competitive athletes, but the arrhythmogenic effect of specific substances is not precisely known. Precipitation of cardiac arrhythmias, particularly in the presence of a latent electrophysiologic substrate including some inherited cardiomyopathies, at risk of sudden death or due to long-term consumption of the substances, should raise the suspicion that illicit drugs may be a possible cause and lead cardiologists to investigate carefully this relationship and appropriately prevent the clinical consequences [07063].

**QT-interval**

The association between physiologic levels of sex hormones and QT-interval duration in humans was evaluated using data from 727 men enrolled in the Third National Health and Nutrition Examination Survey and 2,942 men and 1,885 postmenopausal women enrolled in the Multi-Ethnic Study of Atherosclerosis. Testosterone, estradiol, and sex hormone-binding globulin levels were measured in serum and free testosterone was calculated from those values. QT interval was measured using a standard 12-lead electrocardiogram. In men from the Third National Health and Nutrition Survey, the multivariate adjusted differences in average QT-interval duration comparing the highest quartiles with the lowest quartiles of total testosterone and free testosterone were -8.5 ms (95% confidence interval: -15.5 to -1.4) and -8.0 ms (95% confidence interval -13.2, -2.8), respectively. The corresponding differences were -1.8 ms and -4.7 ms, respectively. Estradiol levels were not associated with QT-interval duration in men, but there was a marginally significant positive association in postmenopausal women. The findings suggest that testosterone levels may explain differences in QT-interval duration between men and women and could be a contributor to population variability in QT-interval duration among men [11330].

**Maximal heart rate**
Previous study showed that muscle sympathetic nerve activity (MSNA) was augmented in anabolic steroids users (AASU). In one study, it was tested the hypothesis that the heart rate (HR) responses after maximal exercise testing would be reduced in AASU. Ten male AASU and 10 AAS nonusers (AASNU) were studied. Cardiopulmonary exercise was performed to assess the functional capacity and heart rate recovery. MSNA was recorded directly from the peroneal nerve by microneurography technique. Peak oxygen consumption (VO₂) was lower in AASU compared to AASNU (44 ± 2 vs 53 ± 2 mL/kg/min). HR recovery (HRR) at first and second minute was lower in AASU than AASNU (21 vs 27 bpm, and 37 vs 45 bpm, respectively). MSNA was higher in AASU than AASNU (29 vs 20 bursts/min). Further analysis showed a correlation between HRR and MSNA, HRR at first minute and peak VO₂ and HRR at second minute and peak VO₂. The exacerbated sympathetic outflow associated with a lower parasympathetic activation after maximal exercise, which impairs heart rate recovery, strengthens the idea of autonomic imbalance in AASU [13132].

Heart rate recovery

Previous study showed that muscle sympathetic nerve activity (MSNA) was augmented in anabolic steroids users (AASU). In the present study, we tested the hypothesis that the heart rate (HR) responses after maximal exercise testing would be reduced in AASU. Ten male AASU and 10 AAS nonusers (AASNU) were studied. Cardiopulmonary exercise was performed to assess the functional capacity and heart rate recovery. MSNA was recorded directly from the peroneal nerve by microneurography technique. Peak oxygen consumption (VO₂) was significantly lower in AASU compared to AASNU. HR recovery (HRR) at first and second minute was significantly lower in AASU than AASNU. MSNA was higher in AASU than AASNU. Further analysis showed a correlation between HRR and MSNA, HRR at first minute and peak VO₂ and HRR at second minute and peak VO₂. The exacerbated sympathetic outflow associated with a lower parasympathetic activation after maximal exercise, which impairs heart rate recovery, strengthens the idea of autonomic imbalance in AASU [13136].

Myocardial fibrosis caused of anabolic steroids

Experimental studies have demonstrated that AAS abuse leads to skeletal muscle hypertrophy and increased collagen accumulation; changes that are similarly detected in the myocardium. Studies in rats and mice have shown that AAS abuse leads to both myocardial hypertrophy and fibrosis, destruction of the mitochondria and other elements of the cardiomyocytes, disturbances of the microcirculation, and to a deterioration in systolic function and to diastolic dysfunction. Post-mortem studies of athletes who used AAS have found infiltration of eosinophils into myocardial cells, as well as destruction of myofibrils. Endothelial dysfunction was also observed [12126].

A 2005 study reported two cases of sudden cardiac death in young male athletes related to AAS abuse. Both cases involved healthy individuals without any history of coronary artery disease (CAD) and with no evidence of significant abnormality in arterial microscopic examination. Autopsy of both hearts showed focal myocardial fibrosis suggestive of prior myocardial injury. In a study of a sudden unexpected death in a female fitness athlete using steroids and ephedrine, the only pathological finding was a few small foci of granulation tissue, which was interpreted as evidence of earlier myocardial necrosis. Sudden cardiac arrhythmia resulting from inflammatory process and myocardial fibrosis was suggested to be the cause of death in these cases. Other researchers have reported sudden cardiac deaths related to steroids that also showed myocardial fibrosis in the absence of CAD [12119].
**Left ventricular myocardial dysfunction and cardiac hypertrophy**

Chronic anabolic steroid use suppresses left ventricular functions. However, there is no information regarding the chronic effects of anabolic steroids on right ventricular function which also plays a key role in global cardiac function. The main objective of one study was to investigate the effects of androgenic anabolic steroids usage among athletes on remodeling the right part of the heart. Androgenic-anabolic steroids-using bodybuilders had smaller diastolic velocities of both ventricles than drug-free bodybuilders and sedentary counterparts. This study shows that androgenic anabolic steroids-using bodybuilders exhibited depressed diastolic functions of both ventricles [08141].

Anabolic steroids cause a variety of side effects, among them a slight concentric left ventricular hypertrophy. The objective of the present study was to clarify if they also induce alterations in left ventricular function. Fourteen male body builders with substantial intake of anabolic steroids (users) were examined by standard echocardiography and cardiac tissue Doppler imaging. They were compared to 11 steroid-free strength athletes (non-users) and 15 sedentary control subjects. Users showed an increased left ventricular muscle mass index. The ratio of peak transmitral blood flow velocities during early diastolic filling and atrial contraction did not differ between groups. In contrast an analogous tissue Doppler parameter, the ratio of myocardial velocities during early and late ventricular filling in the basal septum, was significantly lower in users when compared to non-users or controls. The velocity gradient during myocardial E-wave in the posterior wall showed significantly lower values in users as compared to controls. There were no differences in systolic function. Summarizing strength athletes abusing anabolic steroids show negative alterations in diastolic function [07064].

Recent echocardiographic studies of AAS users demonstrate an increase in septal and left ventricular posterior wall thickness. This hypertrophy is greater in weight-trained individuals using AASs than in weight-trained individuals provided placebo or not using AASs and persists for years among former AAS users. Cardiac wall hypertrophy may not occur after short-term ASA use. AAS use impairs measures of diastolic function (e.g. isovolumetric relaxation time and altered tissue Doppler imaging of the left ventricle) that reflect impaired relaxation and altered filling during diastole. Possible etiologies for impaired diastolic function include increased collagen content or areas of focal necrosis, seen at autopsy of AAS users. Cardiovascular performance also can be assessed by way of formal exercise testing; although AASs may increase bulk and strength, they do not improve endurance. Despite having similar aerobic and weight-training schedules as control subjects, AAS users had a significantly decreased maximum oxygen consumption (VO₂max; an index of metabolic and cardiovascular endurance ability). Impaired diastolic function could contribute to decreased VO₂max [07058].

Biopptic data have shown that in athletes under the pharmacological effects of AAS, a focal increase in myocardial collagen content might occur as a repair mechanism against myocardial damage. To investigate the potential underlying left ventricular myocardial dysfunction after chronic misuse of AAS in athletes by use of Doppler myocardial imaging (DMI) and strain rate imaging (SRI). Standard Doppler echocardiography, DMI, SRI and ECG treadmill test were undertaken by 45 bodybuilders, including 20 athletes misusing AAS for at least 5 years (users), by 25 anabolic-free bodybuilders (non-users) and by 25 age-matched healthy sedentary controls, all men. The mean number of weeks of AAS use per year was 31 in users, compared with 9 years in non-users, and the mean weekly dosage of AAS was 525 mg. The groups were matched for age. Systolic blood pressure was higher in athletes (145 vs 130 mm Hg) than in controls. Left ventricular mass index did not significantly differ
between the two groups of athletes. In particular, both users and non-users showed increased wall thickness and relative wall thickness compared with controls, whereas left ventricular ejection fraction, left ventricular end-diastolic diameter and transmitral Doppler indexes were comparable for the three groups. Colour DMI analysis showed significantly lower myocardial early: myocardial atrial diastolic wave ratios in users at the level of the basal interventricular septum (IVS) and left ventricular lateral wall, in comparison with both non-users and controls. In addition, in users, peak systolic left ventricular strain rate and strain were both reduced in the middle IVS and in the left ventricular lateral free wall. By stepwise forward multivariate analyses, the sum of the left ventricular wall thickness, the number of weeks of AAS use per year and the weekly dosage of AAS were the only independent determinants of middle IVS strain rate. In addition, impaired left ventricular strain in users was associated with a reduced performance during physical effort. Several years after chronic misuse of AAS, power athletes show a subclinical impairment of both systolic and diastolic myocardial function, strongly associated with mean dosage and duration of AAS use. The combined use of DMI and SRI may therefore be useful for the early identification of patients with more diffused cardiac involvement, and eventually for investigation of the reversibility of such myocardial effects after discontinuation of the drug [07065].

Anabolic androgenic steroids (AAS) are used by some athletes to enhance performance despite the health risk they may pose in some persons. This work was carried out to evaluate the possible structural and functional alterations in the heart using two-dimensional, M-mode, tissue Doppler imaging (TDI) and strain rate imaging (SRI) in athletes using supraphysiological doses of AAS. Additionally, the histological and ultrastructural changes in cardiac muscles of adult albino rats after injection of sustanon, as an example of AAS, were studied. Fifteen male bodybuilders using anabolic steroids constituted group 1, five male bodybuilders who are not using anabolic steroids constituted group 2, and five nonathletic males constituted negative control group (group 3). They were investigated by two-dimensional, M-mode, TDI and SRI. Moreover, a study was performed on 30 adult albino rats. They were divided into two groups. Group I (Control group) (n=10) was subdivided into negative control, subgroup 1a (n=5), and subgroup 1b (n=5), which received 0.8 ml olive oil intramuscular once a week for 8 weeks. Group II (Experimental group) (n=20) received sustanon 10 mg/kg intramuscularly once a week for 8 weeks. The heart specimens were prepared for light microscopy and transmission electron microscopy. Echocardiographic results showed that bodybuilders who use steroids have smaller left ventricular dimension with thicker walls, impaired diastolic function, as well as higher peak systolic strain rate in steroid-using bodybuilders as compared to the other two groups. Light microscopy examination of cardiac muscle fibers showed focal areas of degeneration with loss of striations and vacuolation in the experimental group. Ultrastructural examination showed disturbance of the banding pattern of the cardiac muscle fiber with disintegration, loss of striations, dehiscent intercalated disc, and interrupted Z-bands. Administration of supraphysiological doses of AAS caused severe deleterious effects in the myocardium both in athletes and in experimental animals. The SRI shows promise in the early detection of systolic dysfunction in those athletes who use steroids [09056].

Anabolic androgenic steroids (AAS) are sometimes used by power athletes to improve performance by increasing muscle mass and strength. Recent bioptical data have shown that in athletes under the pharmacological effects of AAS, a focal increase in myocardial collagen content might occur as a repair mechanism against myocardial damage. To investigate the potential underlying left ventricular myocardial dysfunction after chronic misuse of AAS in athletes by use of Doppler myocardial imaging (DMI) and strain rate imaging (SRI). Standard Doppler echocardiography, DMI, SRI and ECG treadmill test were undertaken by 45 bodybuilders, including 20 athletes misusing AAS for at least 5 years (users), by 25 anabolic-
The effects of anabolic androgenic steroids (AASs) on left ventricular (LV) diastolic function in strength-trained athletes are controversial. The main objective of this study was to evaluate the effects of regular AAS administration in bodybuilders using pulsed tissue Doppler imaging (TDI) to evaluate LV relaxation properties. Fifteen male bodybuilders with a history of intensive, long-term strength training and 16 age-matched sedentary controls were recruited. Six of the bodybuilders reported regular use of AASs, and 9 were drug free. To assess LV diastolic function, each subject underwent standard Doppler echocardiography and pulsed TDI. Drug-using bodybuilders exhibited altered LV diastolic filling characterized by a smaller contribution of passive filling to LV filling compared with their drug-free counterparts. TDI measurements indicated that drug-using bodybuilders had smaller peak E(m) than drug-free bodybuilders and sedentary controls, except at the level of the anterior wall, at which peak E(m) was significantly smaller than in drug-free bodybuilders only. The E/E(m) ratio, an index of LV filling pressures, was not affected by strength training or by AAS use. Drug-using bodybuilders exhibited larger LV end-diastolic diameters, volumes, and masses than their drug-free counterparts. However, no difference was found in LV wall thickness between the groups. In conclusion, drug-using bodybuilders showed a decrease in the contribution in LV passive filling to LV filling associated with a decrease in LV relaxation properties. Because no wall thickening was obtained in drug-using bodybuilders, the decrease in LV relaxation properties might have been due to an alteration in the active properties of the myocardium, but that has yet to be confirmed [06067].

It was compared cardiac parameters in weightlifters reporting long-term AAS use to those in otherwise similar weightlifters without prior AAS exposure. It was performed 2D tissue-Doppler and speckle-tracking echocardiography to assess left ventricular (LV) ejection fraction, LV systolic strain, and conventional indices of diastolic function in long-term AAS users (n=12) and otherwise similar AAS nonusers (n=7). AAS users (median [quartile 1, quartile 3] cumulative lifetime AAS exposure, 468 [169, 520] weeks) closely resembled nonusers in age, prior duration of weightlifting, and current intensity of weight training. LV structural parameters were similar between the two groups; however, AAS users had significantly lower LV ejection fraction (51 % [48, 54] versus 59 % [58 %, 62 %]), longitudinal...
strain (17 % [14 %, 19 %] vs 21 % [20 %, 23 %]), and radial strain (38 % [29 %, 44 %] vs 50 % [44 %, 62 %]). Ten of the 12 AAS users showed LV ejection fractions below the accepted limit of normal (>55 %). AAS users also demonstrated decreased diastolic function compared to nonusers as evidenced by a markedly lower early peak tissue velocity (7.4 [6.8, 7.9] cm/s vs 9.9 [8.3, 10.5] cm/s) and early-to-late diastolic filling ratio (0.93 [0.88, 1.39] vs 1.80 [1.48, 2.00]). It was concluded that cardiac dysfunction in long-term AAS users appears to be more severe than previously reported and may be sufficient to increase the risk of heart failure [10327].

An increase in LV mass is an independent risk factor for CV disease. AS use has been associated with an increase in LV mass, but there is conflicting data. There are some data in AS users that suggest a reduction in systolic cardiac function although this is not a consistent finding between studies. A reduction in diastolic function has been observed more frequently and it has been suggested that a reduction in myocardial relaxation/elastance is associated with AS use [12114].

Left ventricular hypertrophy (LVH) has been reported in androgen abusers. Several groups have shown that athletes using AAS have reduced end diastolic dimension, a thicker posterior wall and interventricular septum, and a larger left ventricular mass than athletes not using AAS. Cardiac muscle cells have receptors for androgens, and both testosterone and dihydrotestosterone produce a hypertrophic response by acting directly on cardiac muscle cells, increasing amino acid incorporation into protein. The problem is that LVH may persist after discontinuation of AAS [12119].

In athletes who are mainly involved in bodybuilding, echocardiographic studies have derived conflicting results regarding the effects of AAS on left ventricular mass and function. Most studies compared the echocardiographic results between AAS users and nonusers or healthy controls. It has been found significant left ventricular wall thickening in elite power athletes using AAS compared to non AAS-users. Indeed, in one case the wall thickness was 16 mm. However, none of them demonstrated diastolic dysfunction. In contrast, a number of studies found no significant difference in left ventricular hypertrophy between AAS users and non-users. In most cases, the hypertrophy observed was concentric, as would be expected after long-term static exercise training, while only a few showed eccentric hypertrophy due to dilatation of the cardiac cavities. It is noteworthy that studies until early 2000 found no particular evidence for systolic and diastolic dysfunction in athletes using AAS. However, with the use of the latest echocardiographic techniques, such as tissue Doppler, some researchers detected left ventricular diastolic dysfunction in athletes who are AAS users. In a study of ours the use of pulsed tissue Doppler was helpful in the early detection of diastolic dysfunction caused by AAS abuse, which was not detectable using the classical estimation of transmural flow. Moreover, the diastolic dysfunction was found to be correlated with the dosage and the duration of use. Apart from left ventricular diastolic dysfunction, it was found using Doppler myocardial imaging and strain rate imaging, also recorded early findings of deteriorated systolic function in drug users. It is likely that studies using the latest non-invasive diagnostic techniques will confirm the possibility that AAS lead to cardiomyopathy in athletes, mainly due to a direct toxic effect on the myocardium. There are reports of athletes with dilated cardiomyopathy and heart failure after AAS abuse [12126].

The use of anabolic androgenic steroids (AASs) has been associated with hypertrophy of the left cardiac ventricle (LVH) as diagnosed by echocardiography. Case reports suggest that AAS-related LVH may lead to sudden death. It was performed an investigation of the gross cardiac pathological findings in deceased male AAS users in order to further elucidate the proposed role of AAS in cardiac hypertrophy. Eighty-seven deceased males who tested positive for AAS at autopsy and 173 age-adjusted control deceased males without suspected
AAS use were studied for cardiac hypertrophy. The AAS-positive subjects had been examined at any of the six departments of forensic medicine in Sweden during the period from 1989 to 2009. Data were assessed employing multivariate analyses controlling for body weight, height, age, bleeding after trauma, and the impact of weight training. The analysis of the logarithm of heart mass by multivariate statistics implied that strong correlations existed between body mass and heart mass, height and heart mass, age and heart mass, and trauma (bleeding) and heart mass. After controlling for these factors, a significantly higher heart mass was found among the AAS-positive males. The findings suggest that use of AAS may lead to cardiac hypertrophy with a direct cardiotropic effect [12128].

The role of AAS abuse in myocardial hypertrophy has been shown in animal and human studies. In a recent investigation on rats treated with high-dose nandrolone for 8 weeks, electrical remodelling and increasing myocytes nuclei diameter in the AAS group suggested early stages of myocardial hypertrophy. Significant increase in left ventricular mass index, ranging from 7 to 24 percent, has been shown in studies on rats treated with low-dose and high-dose AAS for 8-10 weeks. Another study on the AAS treatment for 3 weeks in mice subjected to aerobic training and sedentary mice showed that high-dose AAS treatment in sedentary mice results in increased ventricular mass index by 25 percent. Also, many case reports of sudden cardiac death in athletes who abused AAS have shown clinically important left ventricular hypertrophy. Association between AAS abuse and echocardiographical detected myocardial hypertrophy has been shown in a study on athletes who chronically abused AAS (median 24 months). In this study, hypertrophic index (interventricular septum plus posterior wall thickness divided by the internal diameter) was significantly higher in AAS (ex-)abusers compared with nonuser athletes. Moreover, the extent of AAS abuse was linearly correlated with mean left ventricular wall thickness. It has been shown that long-term AAS abuse increases peripheral vascular resistance, blood pressure, and myocardial sympathetic nerve activity, which can explain mechanical stress-induced myocardial hypertrophy in AAS abusers. Moreover, androgen receptors which are responsible for AAS-induced hypertrophic effects on skeletal muscles are also present in myocytes and result in increased protein anabolism within myocardial cells and interstitium [12125].

Anabolic androgenic steroids (AAS) abuse for improving physical appearance and performance in body builders is common and has been considered responsible for serious cardiovascular effects. Due to disagreement about cardiovascular side effects of these drugs in published articles, this case control study was designed to evaluate the echocardiographic findings in body builder athletes who are current and chronic abusers of these drugs. Body builder athletes with continuous practice for the preceding two years and were training at least twice weekly were selected and divided into AAS abuser and non user and compared with age and BMI matched non athletic healthy volunteers (15 cases in each group). There was no significant difference in left ventricular size or function either systolic or diastolic in comparison to cases and control groups. The only difference was in diastolic size of septum and free wall but observed differences were only significant between first (athletic with AAS abuser) and third group (non athletic and nonuser). The difference between the above-mentioned indexes was not significant between two groups of athletes. Observed differences in diastolic size of septum and free wall is in favor of that long term abuse of anabolic steroid results in accentuation of physiologic hypertrophy due to long term sport most probably due to higher rate pressure product. Furthermore long term abuse and supra pharmacologic doses do not have significant effect in size and left ventricular function [13798].

Concentric left ventricular myocardial hypertrophy is a common pathologic finding following the chronic use of AAS. A 21-year-old, previously healthy weight lifter collapsed during a benchpress workout, and paramedics found him in ventricular fibrillation. For the preceding several months, he used parenteral AASs (nandrolone, 19-nortestosterone). Postmortem
findings included marked cardiac hypertrophy, regional myocardial fibrosis, and focal myocardial necrosis along with hepatosplenomegaly and renal hypertrophy. There was no evidence of recent myocardial inflammation. Other autopsies of AAS abusers have not demonstrated cardiac hypertrophy, but histologic examination of cardiac tissue also detected focal myocardial necrosis. The postmortem examination of 2 cases of sudden death in 2 previously healthy chronic AAS abusers (i.e. bodybuilders) demonstrated also focal myocardial necrosis (contraction band necrosis) without evidence of significant coronary artery disease or myocardial hypertrophy. Other pathologic changes associated with cardiac arrest in previously healthy athletes following AAS use (e.g. oxymesterone) include hypertrophic cardiomyopathy, acute cellular ecrosis, interstitial fibrosis, and myocarditis. Typically, there is no evidence of coronary artery disease in these cases of sudden death. However, evidence of recent (i.e. 2 weeks) myocardial infarction may occur in these cases of sudden death [13003].

Cardiac (autonomic) dysfunction

To date no published data exist regarding the effects of chronic high-dose anabolic-androgenic steroid administration on tonic cardiac autonomic control. The aim of this study was to evaluate, by power spectral analysis of heart rate variability (HRV), the effects of chronic treatment with supraphysiological doses of nandrolone decanoate (DECA) on tonic cardiac autonomic regulation in sedentary rats. Male Wistar rats were treated weekly with 10 mg/kg of DECA (n=7) or vehicle (CONTROL, n=7) for 10 weeks. At the 8th week of treatment, electrocardiogram was recorded in the conscious state, for time- and frequency-domain HRV analysis. Parasympathetic indexes were reduced in DECA group: high-frequency power, RMSSD, and pNN5. The sympathetic index LF/HF tended to be higher in DECA group. In conclusion, chronic treatment with DECA, in rats, impairs tonic cardiac autonomic regulation, which may provide a key mechanism for anabolic steroid-induced arrhythmia and sudden cardiac death [06068].

Anabolic-androgenic steroids abuse has been shown to affect the cardiomyocyte survival and heart function in cell cultures, animal models and humans. A recent study reported that both diastolic and systolic functional parameters are impaired in AAS abuser athletes comparing with nonabuser athletes. In this study, echocardiography in AAS abusers showed a significantly lower ejection fraction (50 % vs 59 %) and longitudinal strain compared to AAS nonabusers. A similar trend was observed in diastolic functional parameters. The mechanisms of high-dose AAS-associated heart dysfunction are still not thoroughly investigated. However, some studies showed deleterious molecular and cellular effects of high-dose AAS administration on myocardium which overlap early injury pathways of heart failure. It is known that in hypertrophic myocardium, hypertrophy can be linked with any of the heart failure signalling pathways, resulting in heart failure. It has also been shown that AAS indirectly mediates the processes that precede mitochondrial damage, apoptosis and sarcomere disruption. It has also been reported that high-dose AAS treatment in small animal models is associated with interstitial collagen deposition and fibrosis. Fibrosis is assumed to occur initially as an adaptation in myocardial hypertrophy to preserve the function of the ventricles and, thereafter, as a repair mechanism to compensate apoptotic myocardial cell loss [12125].

An echocardiographic study of 47 strength-training individuals (46 male subjects), 28 of whom were regular AAS users, revealed a lower systolic function in AAS users versus nonusers, ejection fractions 58 versus 63 percent, respectively. In addition, there was evidence of reduced diastolic function by tissue Doppler measurement in the AAS users (i.e. their hearts were weaker and stiffer). Another study of 12 long-term AAS users noted that compared with controls, they were noted to have significant systolic cardiac dysfunction as
measured by lower left ventricular ejection fraction (50 % vs 59 %). An Italian Doppler imaging study also showed reduced systolic function but in a regional distribution [12119].

One study aimed to evaluate if androgenic-anabolic steroids (AAS) abuse may induce cardiac autonomic dysfunction in recreational trained subjects. Twenty-two men were volunteered for the study. The AAS group (n=11) utilized AAS at mean dosage of 410 ± 78.6 mg/week. All of them were submitted to submaximal exercise testing using an Astrand-Rhyming protocol. Electrocardiogram (ECG) and expired gas analysis were monitored at rest, during, and post-effort. Mean values of VO2 , VCO2 , and VE were higher in AAS group only at rest. The heart rate variability variables were calculated from ECG using MATLAB-based algorithms. At rest, AAS group showed lower values of the standard deviation of R-R intervals, the proportion of adjacent R-R intervals differing by more than 50 ms (pNN50), the root mean square of successive differences (RMSSD), and the total, the low-frequency (LF) and the high-frequency (HF) spectral power, as compared to Control group. After submaximal exercise testing, pNN50, RMSSD, and HF were lower, and the LF/HF ratio was higher in AAS group when compared to control group. Thus, the use of supraphysiological doses of AAS seems to induce dysfunction in tonic cardiac autonomic regulation in recreational trained subjects [13134].

**Vascular reactivity**

Anabolic androgenic steroids are used by some bodybuilders to enhance performance. While the cardiovascular implications of supraphysiological androgen levels requires further clarification, use is associated with sudden death, left ventricular hypertrophy, thromboembolism and cerebro-vascular events. To further understand the effect of androgenic anabolic steroid abuse on vascular function, this study assessed vascular stiffness (pulse-wave analysis) and cardiovascular risk factors in 28 male, bodybuilding subjects, of whom ten were actively receiving anabolic agents (group A; 26 years) and eight had undergone a 3-month "wash-out" period (group B; 32 years). The remaining ten bodybuilding subjects (group C; 24 years) denied any past use of anabolic steroids or other performance enhancing drugs. Comparisons were made with ten sedentary male controls (group D, 29 years). Endothelial independent dilatation in response to glycerol trinitrate was significantly impaired in the group currently using anabolic steroids (group A) compared with the other three groups, whereas no significant differences in endothelial-dependent dilatation were detected between the groups. Previous studies described a decline in vascular reactivity occurring in bodybuilding subjects which is independent of anabolic steroid use and may result from smooth muscle hypertrophy with increased vascular stiffness. This study revealed impaired vascular reactivity associated with anabolic agents and that improvement in vascular function may occur following their discontinuation [06070].

**Cholesterol changes**

An association between premature cardiovascular events and the misuse of AAS in athletes has been observed. This is believed to be primarily mediated through changes in the lipid profile. Although endurance exercise favorably affects the lipid profile primarily through an antiatherogenic effect of raising high-density lipoprotein (HDL) cholesterol and lowering triglycerides, heavy resistance training alone fails to show a similar effect. This implies that the effect of AAS on lipid profile may confounded by the type of training the athlete pursues. Multiple prospective studies of AAS effects on cholesterol have yielded varied results. The majority of results (studies ranging from 3 to 26 weeks) show no overall change in levels of total serum cholesterol; however, some individual studies show increases and others show a decrease in total cholesterol. Despite the varied results on total cholesterol, the effects on
HDL seem more consistent. Reductions in HDL range from 39 to 70 percent depending on the type of AAS and also appear to be dose dependent. Several studies show reductions of HDL down into the teens, which, based on Framingham data, places these patients at a three times greater risk for coronary artery disease compared with men with HDL above 50 mg/dL. There even appears to be some variation in the dose effect based on gender. In one study of hemodialysis patients, weekly administration of nandrolone resulted in a reduction of HDL-2 and apolipoprotein A-1 levels, complemented by a corresponding increase in apolipoprotein B and triglycerides. The oral 17-alpha alkylated steroids, as opposed to parenteral nandrolone, seem to exert the greatest effects on lipids and lipoproteins, which can be seen as early as the first few days of administration. This reduction often reaches a plateau effect after 8 weeks of use. Although the direct mechanisms of action and impact on cardiovascular disease remain unproven, this negative effect on HDL suggests a higher risk of atherogenesis. The alteration in lipid profile seems completely reversible upon discontinuation, but may take at least 4 to 12 weeks, often depending on dose and duration of steroid use [06031].

In addition to HDL effects, an increase in low-density lipoprotein (LDL) appears to parallel the HDL reduction. Significant LDL increases were appreciated in just 8 weeks of anabolic steroid use in one study, and often had not returned to baseline 6 weeks after cessation of AAS use. Because HDL acts as a primary scavenger of LDL particles, LDL changes possibly reflect a secondary rather than primary effect. These alterations in HDL and LDL cholesterol are more profound in athletes engaged in heavy resistance sports taking AAS as compared with endurance athletes, possibly reflecting the influence of the athlete’s training regimen. In one arm (self-administered, prospective, nonblinded portion) of the study from the Netherlands, reductions in lipoprotein (a), which is an independent risk factor for vascular disease, seem to provide a slightly beneficial effect on the lipid profile. Reductions of as much as 50 percent of lipoprotein (a) were observed in as little as 8 weeks of AAS use, and remained decreased at 6 weeks postcessation. Longer duration of AAS did not correlate directly with further serum reductions, but did demonstrate a more prolonged return to baseline of lipoproteins. In the second phase (randomized controlled trial, double-blinded portion) of the Dutch study, both placebo groups and nandrolone decanoate both demonstrated reductions (19 % and 40 %, respectively) in lipoprotein (a) that was nonsignificant. One explanation for the difference is that the oral 17-alpha alkylated steroids, taken in the first portion, seem to exert the greatest effects on lipids and lipoproteins as opposed to the parenterally administered nandrolone used in the second arm. This is mediated by the first-pass metabolism of the orally administered drugs through the liver. These effects can be seen as early as the first few days of administration, and seem to be more dependant on the type of steroid as opposed to the duration, although no long-term studies exist currently. Concentrations of lipoprotein (a) have been shown to have a close correlation with deposition in vascular walls, are often genetically determined, and seem resistant to current lipid [06031].

**Impaired exercise-induced cardioprotection of antioxidant enzymes**

High doses of anabolic androgenic steroids (AAS) impair the cardioprotective effects of exercise against ischemia/reperfusion (I/R) insult, possibly through cellular redox imbalance. Here, the effect of nandrolone decanoate (DECA) treatment on heart redox metabolism was investigated during I/R in sedentary and exercised rats. DECA treatment significantly reduced superoxide dismutase and glutathione reductase activities in exercised rats after heart reperfusion. Catalase and glutathione peroxidase activities were not affected by DECA in both sedentary and trained rats, regardless of the I/R period. DECA also induced myocardial oxidative stress, as evidenced by the reduced levels of total reduced thiols after heart reperfusion in exercised rats treated with the anabolic steroid. These results indicate that
Cardiotoxic effects of supraphysiological doses of AAS involve reduced heart antioxidant capacity [13126].

Uncertainty remains about possible cardiac adaptation to resistance training. Androgenic anabolic steroids (AAS) use plays a potential role and may have adverse cardiovascular effects. To elucidate the effect of resistance training and of AAS-use on cardiac dimensions and function cardiac magnetic resonance (CMR) were performed in 156 male subjects aged 18-40 years: 52 non-athletes (maximum of 3 exercise hours/week), 52 strength-endurance (high dynamic-high static, HD-HS) athletes and 52 strength (low dynamic-high static, LD-HS) trained athletes (athletes ≥ 6 exercise hours/week). Twenty-eight LD-HS athletes denied and 24 admitted to AAS use for an average duration of 5 years (range 3 months-20 years). No significant differences were found between non-athletes and non-AAS-using LD-HS athletes. AAS-using LD-HS athletes had significantly larger LV and RV volumes and LV wall mass than non-AAS-using LD-HS athletes, but lower than HD-HS athletes. In comparison to all other groups AAS-using LD-HS athletes showed lower ejection fractions of both ventricles (LV/RV EF 51/48 % versus 55-57/51-52 %) and lower E/A ratios (LV/RV 1.5/1.2 versus 1.9-2.0/1.4-1.5) as an indirect measure of diastolic function. Linear regression models demonstrated a significant effect of AAS-use on LV EDV, LV EDM, systolic function and mitral valve E/A ratio. It was concluded that strength athletes who use AAS show significantly different cardiac dimensions and biventricular systolic dysfunction and impaired ventricular inflow as compared to non-athletes and non-AAS-using strength athletes. These findings may help raise awareness of the consequences of AAS use [13127].

The beneficial effects of exercise in reducing the incidence of cardiovascular diseases are well known and the abuse of anabolic androgenic steroids (AAS) has been associated to cardiovascular disorders. Previous studies showed that heart protection to ischemic events would be mediated by increasing the antioxidant enzyme activities. Here, we investigated the impact of exercise and high doses of the AAS nandrolone decanoate (DECA), 10 mg/kg body weight during 8 weeks, in cardiac tolerance to ischemic events as well as on the activity of antioxidant enzymes in rats. After a global ischemic event, hearts of control trained (CT) group recovered about 70 percent of left ventricular developed pressure, whereas DECA trained (DT), control sedentary (CS) and DECA sedentary (DS) animals recovered only about 20 percent. Similarly, heart infarct size was significantly lower in the CT group compared to animals of the three other groups. The activities of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) were significantly higher in CT animals than in the other three groups, whereas catalase activity was not affected in any group. Together, these results indicate that chronic treatment with DECA cause an impairment of exercise induction of antioxidant enzyme activities, leading to a reduced cardioprotection upon ischemic events [06071].

**Sudden death**

Among 15,000 forensic post-mortem examinations performed on the coroner's order over a 24-year period (1981-2004) in the area of Lyon, France (population: 2,000,000), 2250 cases of unexpected cardiac sudden death were identified retrospectively according to WHO criteria. Of these, 108 occurred during recreational sport and 12 occurred in athletes. In the latter category, a history of anabolic steroid abuse was found in 6 cases, whereas pre-existing ordinary cardiac lesions were observed in the 6 remaining cases. To shed light on the possible role of anabolic steroids in the induction of cardiac lesions, an experimental study was conducted in rabbits that were treated orally with norethandrolone 8mg/kg/day for 60 days, and sacrificed at day 90. The histopathological examination of the heart from treated animals showed coronary thrombosis associated with left ventricle hypertrophy in 3
cases, and lesions analogous to toxic or adrenergic myocarditis in all other treated animals. These findings were very similar to those observed after cardiac sudden death in the 6 athletes with a history of anabolic steroid abuse. In addition, elevated caspase-3 activity in the heart of treated rabbits as compared to controls suggests that apoptosis is involved in the induction of norethandrolone-induced cardiac lesions. These results confirm the cardiotoxic potential of anabolic steroid abuse [08140].

There have been several cases described of sudden death, SCD, in athletes using AAS. However, the frequency and the pathophysiological mechanism of SCD remain unknown. Some researchers have concluded that AAS have an arrhythmogenic action, while others believe that SCD is a secondary event, resulting from the cardiovascular side effects caused by their abuse. Cases of atrial fibrillation and ventricular tachycardia have also been described in athletes users. It has been suggested that the chronic administration of anabolic agents prolongs and increases the inhomogeneity of repolarisation, thus creating an arrhythmogenic substrate. These disturbances are more apparent in athletes with significant cardiac hypertrophy as an adaptation to long term exercise training (“athlete’s heart”) or to the use of anabolic substances [12126].

Anabolic androgenic steroids (AAS) are the main class of doping agents and their consumption produces adverse effects involving several organs and systems. Three cases of sudden cardiac death (SCD) and one of death due to congestive heart failure of previously healthy athletes who were AAS users are herein reported. Concentric cardiac hypertrophy with focal fibrosis (one case), dilated cardiomyopathy with patchy myocyte death (two cases) and eosinophilic myocarditis (one case) were observed and most probably relate to the final event. Molecular investigation for viral genomes was positive in one case (Ebstein virus). The data confirm previous findings, showing that the most typical cardiac abnormality in AAS abusers is left ventricular hypertrophy, associated with fibrosis and myocytolysis. An exceptional cardiovascular substrate was represented by the case with drug induced eosinophilic myocarditis. These features are at risk of ventricular arrhythmias as well as congestive heart failure. The cause-effect relationship between AAS abuse and cardiac death can be established only by a rigorous methodology with the use of standardized protocols, including precise morphological studies of all target organs to search for chronic toxic effects. Laboratory investigations should focus on AAS searching on a wide range of biological matrices to demonstrate type, magnitude and time of exposure [12129].

Sudden cardiac death related to sports in young patients can have many causes. Hypertrophic cardiomyopathy, congenital coronary abnormalities, and myocarditis make up about half of the causes of sudden cardiac death after sports. Screening for all athletes is important to prevent such episodes. This involves yearly examinations including clinical examinations, stress echocardiograms, echocardiography, and laboratory investigations. Also, behavioral follow up should be addressed, as cocaine administration and doping can both lead to cardiac problems and sudden cardiac death after sports. It was presented a case of a 17-year-old boy who collapsed after an ice hockey competition as a result of an acute myocardial infarction, which was first represented by ventricular fibrillation. It was also reviewed the main causes of sudden cardiac death in such young athletes and the main investigations that have to be performed to reach the proper diagnosis and etiology of the condition [06072].

Sudden death among athletes is very rare (1:50,000-1:100,000 annually) but it is still 2-4 times more frequent than in the age-matched control population and attracts significant media attention. We propose a mechanism underlying sudden cardiac death in athletes that does not relate to myocardial ischemia but is based on repolarization abnormalities due to potassium channel downregulation and can also be best explained by the concurrent...
presence of several factors such as cardiac hypertrophy (athlete's heart), and/or hypertrophic cardiomyopathy, increased sympathetic tone, genetic defects, drugs, doping agents, food, or dietary ingredients. These factors together can increase the repolarization inhomogeneity of the heart ("substrate") and an otherwise harmless extrasystole ("trigger") occurring with a very unfortunate timing may sometimes induce life-threatening arrhythmias. The effective and possible prevention of sudden cardiac death requires the development of novel cost effective cardiac electrophysiological screening methods. Athletes identified by these tests as individuals at higher proarrhythmic risk should then be subjected to more costly genetic tests in order to uncover possible underlying genetic causes for alterations in ionic channel structure and/or function [10066].

It was reported two cases of sudden cardiac death (SCD) involving previously healthy bodybuilders who were chronic androgenic-anabolic steroids users. In both instances, autopsies, histology of the organs, and toxicologic screening were performed. The findings support an emerging consensus that the effects of vigorous weight training, combined with anabolic steroid use and increased androgen sensitivity, may predispose these young men to myocardial injury and even SCD [07066].

Several case reports associate the chronic use of AAS with serious cardiovascular complications including acute myocardial infarction, cardiac arrest, and hypertrophic cardiomyopathy without significant cardiac valvular or coronary artery disease. Case reports associate chronic AAS abuse with myocardial infarctions in young men with and without evidence of coronary artery occlusion; the presence of coronary artery disease in these athletes occurs despite the lack of known risk factors for coronary artery disease. The development of an acute myocardial infarction was associated with high-dose AAS abuse (e.g. 6 weeks daily, years intermittently) by a 44-year-old recreational bodybuilder with diffuse coronary artery disease and multiple myocardial risk factors including polycythemia, smoking, and family history of early coronary artery disease. Left ventricular hypertrophy is a common structural abnormality in bodybuilders with AAS abuse. A study of 21 bodybuilders with reported AAS abuse suggests that concentric hypertrophy of the left ventricular wall and impaired diastolic function are common complications of steroid abuse. Echocardiographic studies of these athletes demonstrated increased left ventricular posterior wall thickness and end-diastolic volumes as well as decreased ratios of ventricular end-diastolic diameter to body mass. A case report of 2 previously healthy bodybuilders associated sudden cardiac death with chronic AAS abuse. There was evidence of focal myocardial necrosis without clinically significant coronary artery disease, but the role of chronic AAS abuse in the cardiac arrest remains unclear. Ventricular dysrythmias are not commonly associated with chronic AAS abuse. Several case reports associate persistent atrial fibrillation with chronic AAS use. A 22-year-old man developed generalized weakness, diaphoresis, anxiety, and dyspnea. The electrocardiogram revealed rapid atrial fibrillation and the echocardiogram indicated an early cardiomyopathy. He had gynecomastia, and he admitted to the recent injection of anabolic steroids. Although there is no direct evidence that AASs are thrombogenic in humans, case reports suggest a possible causal relationship between AAS use and thrombogenic events (e.g. massive pulmonary embolus, cerebral thrombosis, cardiomyopathy with congestive heart failure, biventricular thrombi, and hepatorenal dysfunction). Studies on the association between chronic AAS use and hypertension or left ventricular hypertrophy are inconsistent [13003].

Sudden death is the most frightening consequence of AAS use. The etiology of these events likely is multifactorial, with AAS use contributing to the observed pathology. There are case reports of myocardial infarctions, stroke, and peripheral vascular obstruction from thrombus that likely are related to the changes in platelet function, inflammation, and cholesterol metabolism discussed above. Autopsies of 34 users of AASs found chronic cardiac changes
consisting of cardiac hypertrophy, myocardial fibrosis, and coronary artery atheromatous changes in 12 victims, although these were believed to contribute to the deaths of only 2 victims. Many sudden death events among AAS users have been due to ischemia secondary to coronary artery disease; however, there is a report of ventricular tachycardia during exercise testing of an AAS user who had myocardial fibrosis on biopsy. Other case reports of sudden death demonstrate diffuse, patchy fibrotic changes in the myocardium of AAS users without coronary artery atherosclerosis. The presence of scar or infiltrative processes is commonly believed to be a cause for arrhythmia. The exact cause of sudden death in AAS users is unclear but likely is due to ischemia or arrhythmia [07058].

*Multiple organ failure*

It was a report of a 42 year old male amateur body builder using anabolic androgenic steroids, who developed acute respiratory distress syndrome, acute kidney injury and refractory supraventricular tachycardia. He required extracorporeal membrane oxygenation, continuous veno-venous hemodialysis, and catheter ablation. It was thought that long-term anabolic androgenic steroid abuse predisposed the patient to developing multiple organ dysfunction syndromes from its immunomodulatory effects in an otherwise healthy patient. Anabolic androgenic steroid use should be part of the history taking process since it may complicate patient outcomes [13139].

*Altered lipid profile*

Altered lipid profiles in AAS users are reflected in increased low-density lipoprotein and decreased high-density lipoprotein. The oral C-17 alkylated steroids seem to exert the greatest effects on the lipid profile. Thrombus formation has been postulated by way of these adverse lipid changes and is supported further by findings of AAS-induced increased platelet aggregation, enhanced coagulation enzyme activity, and coronary vasospasm. Hypertension in AAS users has been reported and is likely the result of blood volume increases and fluid retention. This effect, as well as the finding of increased septal thickness and left ventricular mass reported in AAS users, can lead to significant detrimental cardiac remodeling [07008].

*Lipid profiles in rats*

Dietary protein amount and source, hypertrophy resistance training (RT) and anabolic androgenic steroids (AAS) may affect body weight and plasma and hepatic lipid profile. 157 adult male Wistar rats were randomly distributed in 16 experimental groups resulting in: normal-protein (NP) or high-protein (HP) diets, whey or soy-protein diets, with or without RT and with or without AAS, for 3 months. Final body weight was lower in the RT and AAS groups compared to sedentary and non-AAS groups, respectively. Plasma total cholesterol (TC) was lower for the HP compared to the NP diets, for the whey compared to the soy-protein diets and for the AAS compared to the non-AAS groups. Plasma HDL-cholesterol was higher in the RT groups but lower for the AAS groups, the HP and the soy-protein diets. Plasma triglycerides (TAG) were lower for the HP diet, for the RT and the non-AAS groups. Liver TC was lower for the NP, for the soyprotein and for the AAS groups. Liver TAG were lower for the whey-protein diet, RT and non-AAS groups. Some interactions were found, such as the greater effect of AAS on reducing body weight of rats that performed RT or ingested a HP diet. HDL-cholesterol was higher when RT was combined with HP diets or non-AAS and when HP diets were combined with non-AAS. Groups that combined RT with non-AAS administration obtained the lowest hepatic TAG. Among all the interventions tested, AAS was the factor that most negatively affected plasma and hepatic lipid profile, whereas HP diets and RT could benefit lipid profile, especially when combined [13143].

*Abnormal plasma lipoprotein*
Many studies have shown that AAS can cause dyslipidemia by increasing low-density lipoprotein as high as 596 mg/dL and decreasing high-density lipoprotein as low as 5 mg/dL. Alterations in high- and low-density lipoprotein levels occur in a dose-dependent manner within 9 weeks of self-administration of steroids. These changes could accelerate coronary artery atherosclerosis over the long term, resulting in an increased risk of coronary heart disease three to six times that of normal. The effects of androgens on lipid profile have been shown to be reversible after the discontinuation of administration [12119]

AS have been associated with negative alterations in lipid profiles. Changes reported include a decrease in high-density lipoprotein (HDL), an elevation in low-density lipoprotein (LDL) and reduced apolipoprotein levels, possibly through up-regulation of hepatic triglyceride lipase. The changes in lipid profiles indicate an increase in atherosclerotic risk. Increases in homocysteine, a naturally occurring amino-acid thought to have a role in vaso-control, and C-reactive proteins (CRP), an acute-phase protein that rises in response to inflammation, have been implicated as risk factors for CV disease. It has been demonstrated a significant increase in CRP in AS users. It was noted a significant elevation in homocysteine in AS users as well as those who had abstained from AS use for 3 months, indicating a possible effect of AS on vitamin B absorption. Previous studies have also suggested a possible link between AS use and thrombotic risk through alterations in haemoglobin levels [12114].

To evaluate the effects of anabolic androgenic steroids (AAS) on chylomicron metabolism an artificial lipid emulsion labeled with radioactive cholesteryl ester (CE) and triglycerides (TG) mimicking chylomicrons was intravenously injected into individuals who regularly weight trained and made regular use of AAS (WT+AAS group), normolipidemic sedentary individuals (SDT group) and individuals who also regularly weight trained but did not use AAS (WT group). Fractional clearance rates (FCR) were determined by compartmental analysis for emulsion plasma decay curves. FCR-CE for the WT+AAS group was reduced (0.0073 ± 0.0079 per min, 0.0155 ± 0.0100 per min, 0.0149 ± 0.0160 per min, respectively), FCR-TG was similar for both the WT and SDT groups. HDL-C plasma concentrations were lower in the WT+AAS group when compared to the WT and SDT groups (22 ± 13; 41 ± 7; 38 ± 13 mg/dL, respectively). Hepatic triglyceride lipase activity was greater in the WT+AAS group when compared to the WT and SDT groups (7243 ± 1822; 3898 ± 1232; 2058 ± 749, respectively). However, no difference was observed for lipoprotein lipase activity. Data strongly suggest that AAS may reduce the removal from the plasma of chylomicron remnants, which are known atherogenic factors [12130].

**Chylomicron metabolism**

An artificial lipid emulsion labeled with radioactive cholesteryl ester (CE) and triglycerides (TG) mimicking chylomicrons was intravenously injected into individuals who regularly weight trained and made regular use of AAS (WT+AAS group), normolipidemic sedentary individuals (SDT group) and individuals who also regularly weight trained but did not use AAS (WT group). Fractional clearance rates (FCR) were determined by compartmental analysis for emulsion plasma decay curves. FCR-CE for the WT+AAS group was reduced, FCR-TG was similar for both the WT and SDT groups. HDL-C plasma concentrations were lower in the WT+AAS group when compared to the WT and SDT groups. Hepatic triglyceride lipase activity was greater in the WT+AAS group when compared to the WT and SDT groups. However, no difference was observed for lipoprotein lipase activity. Data thus strongly suggest that AAS may reduce the removal from the plasma of chylomicron remnants, which are known atherogenic factors [12146].

**Trombocyte function**
Several mechanisms have been implicated in atherothrombosis of the coronary and other arteries in steroid users: atherogenesis, thrombosis, vasospasm, and direct myocardial damage. Another cause of atherothrombosis may be the increase in erythrocytosis caused by testosterone. A number of studies have demonstrated a significant disturbance of lipid metabolism in athletes who have used anabolic steroids. The beneficial effect of AAS on platelet aggregation and the mechanism of thrombosis, mainly through the activation of prostaglandins and plasminogen, is well known from experimental studies. However, there are conflicting results regarding the effects of anabolic steroids on the mechanism of thrombosis in athletes. In addition, there are only a few reports on the effect of anabolic steroids on vascular function in athletes [12126].

**Adhesion molecules expression and platelets aggregation**

Although therapeutic and physiological dosages of AAS seem to have beneficial effects on platelet aggregation, deleterious effects of supraphysiological AAS dosages in promoting expression of adhesion molecules in vessel walls and facilitating platelet-endothelium binding have been reported as a mechanism that contributes to AAS-induced atherosclerosis. Additionally, the role of AAS abuse in thrombogenicity has been reported in some studies. Detection of adhesion molecules expression as an upstream process leading to binding of platelets to the arterial wall can depict atherosclerotic plaque formation at early stages. Vascular cell adhesion molecule-1 (VCAM-1) and integrins provide suitable targets for molecular imaging of adhesion molecules expression. VCAM-1 is expressed by endothelial cells, macrophages and smooth muscle cells. Integrins, that is, avb3 integrin, are adhesion molecules that are expressed following endothelial cell injury, as well as at more progressed stages of atherosclerotic plaque formation during neo-angiogenesis. avb3 integrin has high binding affinity to arginine-glycine-aspartate amino acid sequence facilitating cell to extracellular matrix interactions [12125].

**Hypercoagulability**

One study evaluated the short-term effects of oxandrolone, an anabolic androgenic synthetic steroid, on blood coagulation and the hemostatic/fibrinolytic system in healthy individuals. Subjects (n=14) were administered oxandrolone (10 mg twice daily) for 14 days. Blood was obtained on days 0, 1, 3, 7, 9, 14, and then at day 42 (28 days after discontinuation of the drug). Samples were analyzed for the plasma plasminogen, plasminogen activator inhibitor (PAI-1), fibrinogen, and coagulation factors (II, V, VII, VIII, and X). After 7 days of administration of oxandrolone, the plasma plasminogen level significantly increased significantly. PAI-1 was significantly decreased at day 3. Coagulation factors II and V significantly increased at day 14, respectively. Factor VII level decreased (not significantly) by day 3, but after 14 days factor VII level returned to baseline. The increase of factor VIII level was not significant. Factor X increased steadily over 14 days of drug treatment and after discontinuation, decreased and returned to baseline by day 42. Fibrinogen decreased by 22 ± 12 percent. Administration of oxandrolone, to healthy young men was thus associated with a significant increase in select blood coagulation factors and plasminogen. These changes create a state of potential hypercoagulability that appears to be counterbalanced by increased fibrinolytic activity to maintain homeostasis [06073].

Suppression of clotting factors II, V, VII, and X and bleeding in patients receiving concomitant anticoagulant therapy have been reported with testosterone. Case reports demonstrated that coadministration of oral anticoagulants and 17-alkylated androgens (fluoxymesterone, oxandrolone, oxymetholone, methyltestosterone, methandrostenolone, stanozolol) resulted
in a prolonged prothrombin time and hemorrhages; AASs may reduce the need for therapeutic anticoagulants by 25 percent [07058].

Anabolic androgenic steroids (AAS) are synthetic derivatives of testosterone with thrombogenic potential in high doses and long-term administration. Taurine, a widely distributed amino-sulfonic acid, is known for its beneficial effects in hypercoagulable states. In order to assess the impact of chronic administration of high doses of AAS and taurine upon haemostasis process in rats, 40 male Wistar rats were divided into four equal groups: control group (group C) – no treatment; androgen group (group A) – received 10 mg/kg per week of nandrolone decanoate (DECA); taurine (group T) – received oral supplementation of 2 percent taurine in drinking water; androgen and taurine group (group AT) – concomitant administration of DECA and taurine. After 12 weeks, blood samples were collected and haemostasis parameters were assessed with the thrombelastographic (TEG) analysis system: reaction time, clot kinetics (K, alpha), final clot strength, coagulation index and the clot lysis (Ly30). Nandrolone significantly decreased reaction time in group A compared with control, whereas taurine significantly increased reaction time, the effect was maintained in group AT compared with group A. Similar differences between groups have been recorded for the clot kinetics parameters K, alpha. The final clot strength and coagulation index were significantly increased in group A versus group C, but not in group AT versus group C. There were no differences in clot lysis, as shown by Ly30. Nandrolone produces an accelerated clot development and an increased clot firmness in Wistar rats. Taurine association ensures a protective effect against this hypercoagulable state, partially restoring the altered parameters of the coagulation profile [12131].

**Altered coagulation profile**

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**Arterial thrombosis**

The use of supraphysiological doses of anabolic androgenic steroids can have serious side effects. One article reports the case of a young man who suffered potentially life-threatening arterial thromboses following the use of these drugs [13135].
Thrombosis

It was presented a case of a 19-year-old male athlete with protein C deficiency who developed proximal deep venous thrombosis and pulmonary embolism while abusing anabolic-androgenic steroids. Anabolic-androgenic steroids have been reported to have anticoagulatory and profibrinolytic effects in patients with protein C deficiency. Despite these antithrombotic effects, the patient developed repeated venous thromboembolism during treatment with low-molecular-weight heparin. The net effect of anabolic-androgenic steroids on the haemostatic system may change from antithrombotic to prothrombotic in male abusers of anabolic steroids with protein C deficiency [10065].

Cerebral venous thrombosis

There are only a few reports of patients developing cerebral venous sinus thrombosis (CVST) after androgen therapy. It was presented a young man who developed cortical venous thrombosis after using androgens to increase muscle mass. He was hospitalised for paraesthesia and dyspraxia in the left hand followed by a generalised tonic-clonic seizure. At admission, he was drowsy, not fully orientated, had sensory inattention, pronation drift and a positive extensor response, all on the left side. The patient had been using anabolic steroids (dianabol 20 mg/day) for the last month for bodybuilding. CT angiography showed a right cortical venous thrombosis. Anticoagulation therapy was started with intravenous heparin for 11 days and oral anticoagulation (warfarin) thereafter. A control CT angiography 4 months later showed resolution of the thrombosis. He recovered fully [13148].

Pulmonary embolism

It was presented case of a 56-year-old man with deep vein thrombosis (DVT) and pulmonary embolism (PE). He had been given intramuscular injections of testosterone and the anabolic-androgenic steroid nandrolone, due to a muscle injury, a total of three times prior to manifestation of the symptoms. An ultrasonographic examination of the right leg revealed a DVT and computed tomography of the pulmonary arteries showed PE. The thromboembolic episodes in this previously healthy patient were in all probability associated with intramuscular injections of testosterone and nandrolone, to which there is a clear correlation in time [07070].

Endothelial cells

The aim of one study was to investigate the effects in vitro induced by androgenic anabolic steroids (AAS) (testosterone, nandrolone, androstenedione, norandrostenedione, and norandrostenediol) used illicitly in sport competitions, on the proliferation ability, apoptosis and the intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) in human umbilical vein endothelial cells (HUVECs), selected as a prototype of a biological target system whose structure and function can be affected by steroids. For this purpose, it was evaluated the proliferation inhibition by cytotoxic assay expressed as the concentration of drug inducing a 50 percent decrease in growth (IC\(_{50}\)). The IC\(_{50}\) was reached for testosterone at 100 microM, androstenedione at 375 microM, nandrolone at 9 microM, norandrostenedione at 500 microM. The IC\(_{50}\) value for norandrostenediol was not reached until a concentration of 6000 microM. The apoptotic effect was evaluated by flow cytometry at IC\(_{50}\) for each drug. It was observed that testosterone induced 31 percent of apoptotic cells, norandrostenedione 25 percent, androstenedione 15 percent and nandrolone 18 percent. It was analyzed the effects of these drugs on [Ca\(^{2+}\)]\(_i\) both in the immediate and long-term continuous presence of each compound. The data show a statistically significant increase of [Ca\(^{2+}\)]\(_i\) in the acute condition and in long-term treated cultures, suggesting that androgen steroids modulate intracellular
levels of calcium independent of incubation time or compound identity. As a whole, the study demonstrates that AAS might alter endothelial homeostasis, predisposing to the early endothelial cell activation that is responsible for vascular complications observed frequently in AAS users [07067].

Atherothrombotic markers and endothelial dysfunction
The use of androgenic anabolic steroids (AAS) may be associated with changes in atherothrombotic markers and endothelial function. The purpose of one study was to compare atherothrombotic markers and endothelial function of AAS users and non-users. Ten athletes who were users of AAS (confirmed by urine analysis) and 12 non-user athletes were evaluated. Body weight, blood pressure, exercise load (hours/week), complete blood count (CBC), platelets, fibrinogen, lipids, high-sensitivity C-reactive protein (hs-CRP), follicle-stimulating hormone, testosterone and estradiol were measured. Endothelium-dependent and independent functions were assessed by brachial artery ultrasound. AAS users had significantly higher body mass and blood pressure. Platelet count was higher whereas HDL-cholesterol was lower in AAS users compared with non-users. Levels of hs-CRP were higher in AAS users. Follicle-stimulating hormone was suppressed in all users and not suppressed in non-users. Compared with non-users, flow-mediated dilation was significantly reduced in AAS users, whereas endothelium-independent function was similar in both groups. Additionally, flow-mediated dilation was positively associated with levels of HDL-cholesterol. AAS users present important changes in blood lipids as well as in inflammatory markers, which are compatible with increased cardiovascular risk. Furthermore, this profile is accompanied by a reduction in the endothelial function [13137].

Increased intima-media thickness
AS use has also been associated with reduced endothelial function in conduit arteries. It was noted a reduced flow-mediated dilation in AS users as well as a reduced vasodilator response to glyceryl-trinitrate [12114].

It has been measured carotid intima-media thickness and radial and brachial artery reactivity in bodybuilders using AAS. It was found a non-significant increase in the thickness and diameter of the arteries in users compared to non-users, which was attributed mainly to fluid retention. A small degree of endothelial dysfunction was also reported by other investigators. It was also found changes of aortic wall elasticity in athletes who used steroids. Moreover, using an electron beam tomography system, it was found increased calcium deposition in the coronary arteries of bodybuilders using AAS. The authors hypothesised that this was due to a direct toxic or inflammatory effect of steroids on the vascular endothelium [12126].

Aortic elasticity
The use of anabolic-androgenic steroids (AAS) has been linked to acute cardiovascular events in athletes. The purpose of the present study was to investigate the aortic elastic properties in athletes who had been self-administering AAS compared with a group of athletes not using these drugs. Fourteen male bodybuilders using AAS and 27 male wrestlers (non-users) volunteered to the study. All subjects were placed in a mild recumbent position and the ascending aorta was recorded in the two-dimensional guided M-mode tracings. The aortic distensibility was found to be reduced in user athletes. The results of this study indicate that aortic stiffness is increasing in athletes using AAS [07068].

Arterial hypertension
Significant research attention has focused on the impact of AS use on cardiovascular (CV) disease risk factors namely blood pressure, lipid profile, left ventricular (LV) mass, cardiac function and arterial function. Elevated systemic arterial blood pressure is associated with an increased CV disease risk. Compared to healthy controls, AS users have increased resting and exercise systolic blood pressure. Conversely, other studies have not observed increased blood pressure in AS user. Differences in the training level of the participants along with age could be responsible for the differences seen in these studies [12114].

Although not shown in all studies, an association between elevated blood pressure and AAS abuse has been reported. Enhanced reactivity of the vasculature to norepinephrine, increased plasma renin activity, stimulation of aldosterone production by testosterone, and sodium retention by the kidneys are suggested mechanisms for high blood pressure following AAS use in athletes. Blood pressure response to androgen use typically shows a dose-response relation. The effects of AAS abuse on blood pressure may persist for long periods; some studies have shown persistent elevations for 5 to 12 months after discontinuing steroids [12119].

In experimental studies it has been found that the administration of AAS leads to hypertension. An increase in the secretion of 11-deoxycorticosterone, norepinephrine, renin, or aldosterone has been implicated as a possible mechanism, while others have noted an increase in cardiac output and peripheral resistances. However, clinical studies in athletes have led to conflicting results. Some observed a significant increase in both systolic and diastolic blood pressure, whereas others noted only the latter. It has been attributed the increase in blood pressure in steroid-using athletes to an increase in plasma volume. In contrast, other authors found no significant increase in blood pressure at rest or during exercise in athletes who used steroids compared to non-users [12126].

Systemic hypertension is a side effect of medical steroid administration and may require antihypertensive therapy; therefore, high-dose ASA use also should result in systemic hypertension. This is found in some reports, but not consistently. AAS-induced hypertension may be related to vascular endothelial response, increased responsiveness to catecholamines, and increased renin production. The magnitude and incidence of hypertension likely are related to dosage and to the specific AAS [07058].

Other vascular effects

Reported severe adverse effects of anabolic-androgenic steroid use include cerebral venous sinus thrombosis, ischemic cerebral stroke, and cardiovascular events in the absence of risk factors. Two cases of limb-threatening arterial thrombosis were reported with the use of danazol (Danocrine®), an antigonadotropin steroid-like compound with weak anabolic properties [07031].

Lipid profile influence early atherogenesis. Therapeutic use of AAS has been shown in many studies to affect the individuals’ lipid profile. A meta-analysis including 19 studies and comprising 272 hypogonadal men showed that substitution therapy with intramuscularly administered testosterone results in a decrease in plasma HDL cholesterol levels. The same results were also demonstrated in a recent meta-analysis including 51 studies on men with low or low-to-normal plasma testosterone levels who received testosterone in different doses as therapy. Moreover, high-dose AAS abuse has been demonstrated to exert unfavourable direct and indirect effects, through AAS-associated hyperhomocysteinaemia, on plasma lipid levels. In a nonblinded investigation on 19 bodybuilders, short-term (8 weeks) and long-term (> 14 weeks) high dosages of AAS administration markedly reduced HDL cholesterol. The
suppressive effects of AAS administration on HDL plasma levels are dose dependent and depending on the type of AAS and route of administration can result in decrement of 40-70 percent. The adverse effects of high AAS dosages on plasma levels of LDL cholesterol have been shown in animal and human studies. Lipid profile impairment is causally implicated in vascular wall injury by promoting inflammatory processes in the arterial wall, macrophage recruitment, and uptake of LDL and oxidized LDL by macrophages which results in foam cell formation. The aforementioned processes, which contribute to establishment and progression of atherosclerotic plaques, can be depicted by molecular imaging techniques. $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) positron emission tomography (PET) has been studied in a notable number of investigations and has been shown to correlate with the macrophage density in atherosclerotic plaques in humans and in animal models. Additionally, $^{18}$F-FDG PET depicts MI subsequent to coronary atherosclerosis. Molecular targeting of oxidized LDL and macrophage uptake of radiolabelled LDL has verified promising targets for visualizing vulnerable atherosclerotic plaques. Moreover, a recent pilot study reported feasibility of ultrasmall superparamagnetic particles of iron oxide (USPIO) in detecting inflammation in endothelial cells during atherogenesis with magnetic resonance imaging (MRI) [12125].

**Impaired vasodilatation**

Although endogenous testosterone has been shown to exert vasodilatory effects, AAS use in hypogonadal men has been shown to result in paradoxical pro-atherogenic vasoconstrictive effects. It was shown that testosterone therapy in hypogonadal men is correlated with impaired vasodilation, independently from lipid profile measures. Supraphysiological doses of AAS have also shown to exert similar effects on vasoreactivity in human and animal studies. In a study on male bodybuilders who abused AAS for 3-4 years, vasodilatation was significantly lower than that of ex-abusers and controls. AAS abuse in bodybuilders independently of the other factors impaired endothelium-independent vasodilator pathways. It was also shown that a 3-month period of abstinence results in a degree of improvement in vascular function. Moreover, longterm therapy with supraphysiological doses of AAS in female-to-male transsexuals has shown to result in decreased vasodilation independent of the effects of age, lipid profile and vessel size. The mechanisms through which AAS induces deleterious effects on vasodilatation are not sufficiently investigated. However, endothelial injury as a result of lipid profile alterations and establishment of atherosclerosis could explain the impairment in endothelium-dependent pathway through decreased NO production [12125].

**Stroke**

Anabolic-androgenic steroids are synthetic substances derived from testosterone that are employed for their trophic effect on muscle tissue, among other uses. Their consumption can give trigger a series of adverse side effects on the body, including the suppression of the hypothalamus-pituitary-gonadal axis as well as liver, psychiatric and cardiovascular disorders. The most common effects are altered fat profiles and blood pressure values, cardiac remodelling, arrhythmias or myocardial infarcts. It was reported a case of a young male, with a background of anabolic-androgenic steroids abuse, who visited because of an acute neurological focus in the right hemisphere related with an ischaemic stroke. The aetiological study, including cardiac monitoring, echocardiograph and imaging studies (magnetic resonance and arteriography) and lab findings (thrombophilia, serology, autoimmunity, tumour markers) showed no alterations. Thus, the association between consumption of anabolic-androgenic steroids and cardiovascular pathologies is known, but its relation with cerebrovascular disease has not received so much attention from researchers [13128].

**Summary of metabolic and vascular effects of anabolic steroids**

In summary, metabolic and vascular adverse effects of AAS abuse can be classified as
alterations in the lipid profile, especially decreased serum HDL levels and hyperhomocysteinaemia contributing to endothelial damage
- increased platelets adhesion to vascular wall
- vasospastic effects and impaired vasodilatation

**Experimental**

The objectives of one study were to investigate the time-course and the cellular, ionic and molecular processes underlying ventricular repolarization in rats chronically treated with AAS. Male Wistar rats were treated weekly for 8 weeks with 10 mg/kg of nandrolone decanoate (n=21) or vehicle (n=20). ECG was recorded weekly. Action potential and transient outward potassium current (I_{to}) were recorded in rat hearts. Expression of KChIP2, Kv1.4, Kv4.2, and Kv4.3 was assessed by real-time PCR. Hematoxylin/eosin and Picrosirius red staining were used for histological analysis. QTc was greater in the DECA group. After nandrolone treatment the left, but not right, ventricle showed a longer AP duration than did the control. I_{to} current densities were 48 percent lower in the left but not in the right ventricle after nandrolone. In the right ventricle the I_{to} inactivation time-course was slower than in the control group. After nandrolone the left ventricle showed lower KChIP2 (approximately 26 %), Kv1.4 (approximately 23 %) and 4.3 (approximately 70 %) expression while the Kv 4.2 increased in 4 (approximately 250 %) and diminished in 3 (approximately 30 %) animals of this group. In the right ventricle the expression of I_{to} subunits was similar between the treatment and control groups. Nandrolone-treated hearts had 25 percent fewer nuclei and greater nuclei diameters in both ventricles. The results strongly suggest that supra-physiological doses of AAS induce morphological remodeling in both ventricles. However, the electrical remodeling was mainly observed in the left ventricle [10328].

**Cardiovascular effects of low androgens**

Interestingly, if androgen levels are too low, cardiac risk may increase. Androgen-deprivation therapy (ADT) is a widely used treatment for prostate cancer, and several studies have reported an association between ADT and an increased risk of myocardial infarction and cardiovascular mortality. Antiandrogens (e.g. flutamide, bicalutamide) block the binding of androgen to its receptor, and they are often coupled with gonadotropin-releasing hormone (GnRH) agonists (e.g. leuprolide, goserelin, triptorelin). In one population-based Medicare study, the use of a GnRH agonist in men with prostate cancer for at least 1 to 4 months was associated with an increased risk of incident coronary heart disease (adjusted hazard ratio, HR, 1.16), myocardial infarction (adjusted HR), and sudden cardiac death or life-threatening ventricular arrhythmia (adjusted HR 1.16). Another population based study noted that the use of ADT was associated with a 20 percent higher risk of cardiovascular morbidity (HR 1.20) during 5 years of follow-up. In addition, androgen deficiency has been associated with cardiovascular risk factors by causing increased serum total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides. There is also an association between low androgen levels and an adverse metabolic profile (insulin resistance, metabolic syndrome, and diabetes) [12119].

**Summaries of effects of anabolic steroids on the heart**

To sum up, the myocardial effects of AAS abuse can be summarized in three different categories including [125]:

[12125]:

- alterations in the lipid profile, especially decreased serum HDL levels and hyperhomocysteinaemia contributing to endothelial damage
- increased platelets adhesion to vascular wall
- vasospastic effects and impaired vasodilatation
- myocardial hypertrophy as result of
  o elevated muscle sympathetic nerve activity
  o direct anabolic effects of AAS
  o renin-angiotensin system activity induced collagen deposition and interstitial fibrosis
- left ventricular dysfunction as result of
  o AAS-induced myocardial hypertrophy
  o mitochondrial damage and apoptosis as consequences of Ca\(^{2+}\) signaling
  o rennin-angiotensin system activity and fibrosis
- cardiac arrhythmias as result of increased myocardial mass and reduction

Some physiological effects of anabolic-androgen steroids were summarized [12119]:

<table>
<thead>
<tr>
<th>System</th>
<th>Physiological effect</th>
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</thead>
<tbody>
<tr>
<td>Musculoskeletal skeletal</td>
<td>muscle hypertrophy and formation of new muscle cells, especially in muscles of thorax, neck, shoulders, and upper arms. Increased bone remodeling and growth, closure of the epiphyseal growth centers</td>
</tr>
<tr>
<td>Hematological</td>
<td>stimulation of bone marrow with increased production of red blood cells</td>
</tr>
<tr>
<td>Dermatological</td>
<td>hirsutism (i.e. increased growth of androgen-sensitive hair: pubic, beard, chest, limb</td>
</tr>
<tr>
<td>Sebaceous gland</td>
<td>stimulation, resulting in acne</td>
</tr>
<tr>
<td>Ear, nose, throat</td>
<td>vocal cord hypertrophy (deepening of the voice)</td>
</tr>
<tr>
<td>Genitourinary</td>
<td>hypertrophy of clitoris and penis. Testicular atrophy (oligospermia, decreased ejaculatory volume). Prostate hypertrophy</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>retention of sodium and fluid</td>
</tr>
<tr>
<td>Endocrine</td>
<td>gynecomastia</td>
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**Increased risk of diabetes**

Anabolic steroids decrease glucose tolerance and increase insulin resistance, which lead to hyperinsulinism and secondary diabetes mellitus with type II symptoms [07058].

The case of a 36-year-old male professional bodybuilder was reported. He presented to the accident and emergency department with right upper quadrant pain. This was on the background of a 15-year history of anabolic steroid and growth hormone misuse. Examination revealed mild hepatomegaly and a random blood sugar of 30.2 mmol/L. There was no evidence of ketonuria or acidosis. Biochemical evidence of hepatitis was found, and the patient was in acute renal failure. He was given a sliding scale of insulin and an intravenous infusion of crystalloid. The hepatitis and hyperglycaemia settled with conservative treatment. It is believed that this is the first reported case of frank diabetes precipitated by supraphysiological recreational growth hormone misuse [07069].

A 33-year-old male presented to the emergency department with complaints of polydipsia, polyuria, nausea, headaches, blurry vision and malaise. Lab work revealed a serum glucose level of 1166 mg/dl (64.8 mmol/L). The patient admitted to completing a cycle of androgenic anabolic steroids (AASs) for bodybuilding. His regimen consisted of supraphysiologic intramuscular injections of a bovine growth hormone, trenbolone acetate and testosterone. The patient received intravenous fluids and insulin to restore metabolic balance. Previously healthy with a non-contributory family history, he was diagnosed with new onset diabetes.
Discussion: It has been demonstrated that AAS use, specifically growth hormone, can affect glucose homeostasis through increasing cellular insulin resistance and reducing glucose uptake. Excess growth hormone has been shown to cause symptoms of acromegaly which predisposes up to 40% of patients to diabetes. As trenbolone acetate is not indicated for human use and athletes are known to use supraphysiologic doses of this underground, performance enhancing drug, the correlation of the timing of events and the use of this veterinary growth hormone likely exacerbated an underlying condition or caused this new onset diabetes. It was reported a case of a young bodybuilder with no significant past medical history who was diagnosed with new onset diabetes associated with supraphysiologic self-injections of the bovine growth hormone, trenbolone acetate, combined with testosterone. AAS have the potential to induce or exacerbate diabetic conditions due to decreased glucose tolerance and increased insulin resistance [11094].

Effects on the brain

The misuse of anabolic androgenic steroids has in several reports been associated with effects resulting in altered behavior. One study used the Morris water maze task to investigate the effect of high doses of the anabolic androgenic steroid nandrolone on spatial learning and memory in male rats. During the experiment, it was observed a significantly impaired Morris water maze performance in the nandrolone-treated rats compared with controls. The hippocampus, a brain region associated with cognitive function, was analyzed for mRNA expression of prodynorphin, the precursor of dynorphinergic peptides. The results indicated that the transcription levels of prodynorphin were significantly elevated in the animals treated with nandrolone compared with controls. Thus, the findings suggest that administration of nandrolone to male rats impairs memory function, possibly via dynorphinergic actions [09059].

Anabolic androgenic steroids and high testosterone doses have been reported to induce impulsive behavior in man and behavioral disinhibition in rats. The purpose of the present study was to investigate whether aromatization of testosterone to estradiol is of importance for the behavioral disinhibiting effect of a high testosterone dose in adult male rats. Testosterone administered via five testosterone-filled silastic capsules implanted subcutaneously to non-castrated, group-housed rats for six days induced behavioral disinhibition in a modified Vogel's drinking conflict model and yielded supraphysiological serum levels of testosterone and increased accessory sex organ weights. Moreover, concurrent administration of the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD; 60 mg/kg/day s.c.) decreased behavioral disinhibition in testosterone-treated rats (without affecting accessory sex organ weights) while behavior was not significantly affected in sham-treated animals. Since some reports indicate that ATD, in addition to inhibit aromatase, also may affect the binding of testosterone to the androgen receptor, the effect of the non-steroidal androgen receptor antagonist flutamide was investigated. Flutamide treatment did not affect disinhibited behavior in testosterone-treated rats. However, in sham-treated animals, flutamide (50 mg/kg/day) produced behavioral disinhibition. These results suggest that estradiol is of importance in the mechanisms underlying behavioral disinhibition in non-castrated rats treated with a high testosterone dose. Speculatively, aromatization may be involved in pro-impulsive effects of high testosterone doses in humans [09060].

Several case reports associated chronic AAS abuse with the development of seizures, ischemic midbrain lesions with residual dysfunction (hemiparesis, aphasia, and dysarthria), and thrombotic strokes, but the causal link between cerebrovascular accidents and chronic AAS abuse is unproven. A 34-year old bodybuilder developed an acute right hemiparesis is a dysarthria after using various AASs for 4 years. He had a simple partial seizure. At discharge,
he had mild motor weakness in the right upper extremity with no sensory changes. A 21-year old man developed a generalized tonic-clonic seizure, left-sided hemiparesis after using 6-8 mg ethylestrenol daily for 6 weeks. Angiography demonstrated an occlusion of the right anterior cerebral and right middle cerebral arteries. In a retrospective cohort study of 248 patients who tested positive for the presence of AAS in connection with receiving medical care, the incidence of unspecified convulsions was increased in the AAS-positive group (RR 54) when compared with controls (i.e. patients testing negative for AAS at the same institution). One of the AAS-positive patients with seizures died. However, there are few data from cohort or case-control studies to confirm the causal link between AAS abuse and seizures [13003].

**Brain development**

Puberty is a critical period for brain maturation that is highly dependent on gonadal sex hormones. Modifications in the gonadal steroid environment, via the use of anabolic androgenic steroids (AAS), have been shown to affect brain development and behavior. Studies in both humans and animal models indicate that AAS exposure during adolescence alters normal brain remodeling, including structural changes and neurotransmitter function. The most commonly reported behavioral effect is an increase in aggression. Evidence has been presented to identify factors that influence the effect of AAS on the expression of aggression. The chemical composition of the AAS plays a major role in determining whether aggression is displayed, with testosterone being the most effective. The hormonal context, the environmental context, physical provocation and the perceived threat during the social encounter have all been found to influence the expression of aggression and sexual behavior. All of these factors point toward an altered behavioral state that includes an increased readiness to respond to a social encounter with heightened vigilance, and enhanced motivation. This AAS-induced state may be defined as emboldenment. The evidence suggests that the use of AAS during this critical period of development may increase the risk for maladaptive behaviors along with neurological disorders [12133].

**Cerebral infarction**

Anabolic androgenic steroid (AAS) abuse has increased among athletes in recent years. However, AAS abuse can increase hypercoagulopathy and cause cerebrovascular disease. We report a case of a 27-year-old man who had right hemiparesis, hemianopia, dysarthria, and double vision in the middle of muscle training. He suspected acute disseminated encephalomyelitis at first, because of a preceding respiratory infection. However, extensive work-up was performed, including brain magnetic resonance imaging, transcranial Doppler and transesophageal echocardiography, confirming the final diagnosis of cardioembolic stroke. Physicians should be aware that cerebrovascular disease may be a side effect of AAS, even in younger populations [12134].

**Brain nerve growth factor**

Anabolic androgenic steroids (AASs) are synthetic androgen-like compounds which are abused in sport communities despite their side effects. Nerve growth factor (NGF) influences neuronal differentiation and survival, and also mediates higher brain functions such as learning and memory. Changes in NGF expression have been implicated in neurodegenerative disorders, including Alzheimer's disease. Hence, we decided to study the effect of chronic AASs exposure on brain NGF profile, NGF-dependent cholinergic function and related behavioral performance. Male Wistar rats were injected for 4 weeks with either nandrolone or stanozolol at daily doses (5.0 mg/kg, s.c.) that are considered equivalent to
those abused by humans. NGF levels and NGF receptor (TrkA and p75NTR) expression were measured in the hippocampus and in the basal forebrain. Choline acetyltransferase expression was evaluated in basal forebrain. Spatial learning and memory were assessed using the Morris water maze. AASs treatment caused region-specific changes in the expression of NGF and its receptors. Both nandrolone and stanozolol increased NGF levels in the hippocampus and reduced NGF levels in the basal forebrain, reduced p75NTR expression in the hippocampus, and failed to affect TrkA expression in the basal forebrain. Finally, AASs treatment reduced the expression of choline acetyltransferase in the basal forebrain and impaired the behavioral performance in the Morris water maze. The evidence that supraphysiologic doses of AASs cause neurotrophic unbalance and related behavioral disturbances, raises the concern that AASs abuse in humans may affect mechanisms that lie at the core of neuronal plasticity [12132].

Decreased memory

Chronic exposure to the anabolic androgenic steroids (AAS) nandrolone decanoate (ND) in supra-physiological doses is associated with learning and memory impairments. Given the well-known beneficial effects of voluntary exercise on cognitive functions, it was examined whether voluntary exercise would improve the cognitive deficits induced by chronic administration of ND. It was also investigated the effects of ND and voluntary exercise on hippocampal BDNF levels. The rats were randomly distributed into 4 experimental groups: the vehicle-sedentary group, the ND-sedentary group, the vehicle-exercise group, and the ND-exercise group. The vehicle-exercise and the ND-exercise groups were allowed to freely exercise in a running wheel for 15 days. The vehicle-sedentary and the ND-sedentary groups were kept sedentary for the same period. Vehicle or ND injections were started 14 days prior to the voluntary exercise and continued throughout the 15 days of voluntary exercise. After the 15-day period, the rats were trained and tested on a water maze spatial task using four trials per day for 5 consecutive days followed by a probe trial two days later. Exercise significantly improved performance during both the training and retention of the water maze task, and enhanced hippocampal BDNF. ND impaired spatial learning and memory, and this effect was not rescued by exercise. ND also potentiated the exercise-induced increase in hippocampal BDNF levels. These results seem to indicate that voluntary exercise is unable to improve the disruption of cognitive functions by chronic ND. Moreover, increased levels of BDNF may play a role in ND-induced impairments in learning and memory. The harmful effects of ND and other AAS on learning and memory should be taken into account when athletes decide to use AAS for performance or body image improvement [12135].

Anabolic-androgenic steroids (AAS) are used in the medical treatment of many disorders. Erythropoietin (EPO) is a hematopoietic cytokine that has anti-apoptotic, anti-oxidative, and anti-inflammatory effects. The aim of one study was to investigate the neuroprotective effects of EPO in the hippocampus, parietal cortex and prefrontal cortex, in brain damage due to nandrolone decanoate. Thirty-five Wistar male rats were randomly divided into: (1) control group, (2) sham group, (3) nandrolone decanoate group (ND, intramuscular, 10mg/(kg week), 8 weeks), (4) ND+low dose EPO treated group (ND+L-EPO) and (5) ND+high dose EPO treated group (ND+H-EPO). EPO was administrated by intraperitoneal injection at a dose of 100U/(kg day) for L-EPO treatment and at a dose of 500 U/(kg day) for H-EPO treatment during 8 weeks. The number of neurons of CA1, CA2, CA3 and dentate gyrus of hippocampus, parietal cortex and prefrontal cortex were significantly less in the ND group compared with the control group. Treatment with H-EPO significantly preserved the number of neurons in hippocampus when compared with ND administrated. Besides, H-EPO treatment decreased the number of TUNEL-positive and active caspase-3 positive cells and MDA levels and increased GPx levels when compared to ND group. In conclusion, abuse of AAS causes reduction in the number of neurons in hippocampus, parietal cortex and
prefrontal cortex regions and increases oxidative damage and therefore H-EPO may be useful as a neuroprotective agent in brain injury [12136].

Mania

Anabolic androgenic steroids (AASs have been associated with several major psychiatric symptoms and disorders such as violence, aggression, suicidal tendency, psychotic deterioration, cognitive impairment, depression, and mania. Among these, manic symptoms were considered to be the most frequent, as shown by a clinical trial with testosterone cypionate and a controlled study that analyzed a population of AAS-using athletes. The synthetic male sex hormone mesterolone has even been tested as an antidepressant in clinical studies in the 1980s. These studies, however, yielded inconsistent results. Here it was reported the development of a manic episode subsequent to oral intake of mesterolone in a previously mentally healthy person. The 38-year-old white patient was admitted to a psychiatric ward because of a maniform syndrome that was present for approximately 2 weeks. On admission, he demonstrated with disturbed contact behavior, logorrhea, incoherent thinking with flight of ideas, reduced attention and appetite, increase of impulse, euphoric mood, and reduced need to sleep. There were no overt psychotic phenomena in terms of megalomania or delusion. Physical examination was unremarkable (body weight, 96 kg; height, 178 cm; body mass index, 30.3 kg/m²) apart from hypertrophic upper body musculature. Evidence for previous psychiatric disorders, especially history of an addictive disorder or abuse of psychotropic substances, was absent. Family history revealed major depressive disorder of the patient’s sister, mother, and aunt on the mother’s side. The patient was working as a car mechanic and had a stable partnership without children. On admission, routine blood examination including thyroid tests and urine drug screening (amphetamines, benzodiazepines, cannabinoids, cocaine, 3,4-methylenedioxymethamphetamine, opiates, tricyclic antidepressants) were unremarkable. Blood glucose levels as well as glycated hemoglobin (HbA₁₀) were within reference limits. Four days later, the patient suddenly reported to have used Proviron (mesterolone 25 mg/d) for muscle gain during the last 21 days before admission until the day of admission. He reported that he had been bodybuilding for more than 15 years and that he had never used any illicit substances, that is, from the group of AAS to enhance training effects before the use of mesterolone. As the initial urine samples were already discarded, urine was collected again (4 days after the last self-reported mesterolone intake) and analyzed for mesterolone metabolites (1alpha-methyl androsterone, 1alpha-methyl-5alpha-androstane-3alpha, 17beta-diol), but as expected in view of the short window for drug detection, these substances could no longer be detected by means of gas chromatography combined with high-resolution mass spectroscopy and liquid chromatography combined with mass spectroscopy. In addition, pituitary hormones were measured normal. To rule out an underlying somatic disease affecting the central nervous system (eg, encephalitis), electroencephalogram, magnetic resonance imaging of the brain, and examination of the cerebrospinal fluid were performed without pathological findings. Hence, a diagnosis of (putative) mesterolone-induced mania was made. Initially, the patient was treated with olanzapine that was gradually increased up to 30 mg/d. Because of a remarkable increase in liver enzymes, olanzapine was discontinued, and amisulpride was administered. Because the patient developed parkinsonism under amisulpride at a dosage of 800 mg/d and the antimanic effect was still insufficient, it was reduced amisulpride and administered lithium carbonate. Under treatment with lithium carbonate (lithium carbonate 1800 mg/d) and amisulpride (400 mg/d), the patient developed complete remission within 2 months of in-patient treatment. At follow-up 6 months after discharge, the patient was still without psychopathological findings. Mesterolone (1-methyl-dihydrotestosterone) is a relatively weak androgen with only partial androgenicity. Thus, it is rarely used for oral testosterone replacement therapy in male patients with hypogonadism associated with androgen deficiency. Although its application in medicine is rather infrequent, mesterolone is
still used in professional as well as in amateur sports to enhance training success, particularly regarding muscle hypertrophy, and thus, it can require medical treatment for the development of adverse effects, as in the case presented. To our knowledge, this is the first case report that describes the development of a manic episode related to the use of mesterolone. Considering the absence of other conclusive etiologic factors and the chronological coherence between the onset of AAS use and the development of psychiatric symptoms, mesterolone is the most plausible causal agent in the development of the manic episode. Taking into account the short elimination half-life of mesterolone (drug levels in serum decrease with a terminal half-life of 12-13 hours and 50 % of the orally applied dose is excreted in urine within 24 hours), absent proof of metabolites in the urine 4 days after the last intake does not speak against the use of mesterolone, rather being in full agreement with the patient’s reports. He was also able to show the Proviron medicine box in detail. He maintained his statements after remission of mania and came from the bodybuilding scene, so we had no reasons to believe that the patient had not told the truth about taking mesterolone; although, unfortunately, we were not able to prove its use. Apart from that, the patient’s positive family history of mood disorders certainly has posed an additional individual risk factor for the development of mania related to mesterolone use. Another aspect is the comparatively low dosage of mesterolone (175 mg/wk) and its short duration of use that was associated with the development of mania in our case, especially when compared to clinical and field studies that described effects of AAS on mood after significantly higher dosages (up to 600 mg testosterone cypionate per week or even up to 1000 mg testosterone per week) and longer application intervals (up to 6 weeks) [12137].

**Effects on GABA**

Anabolic androgenic steroids are synthetic derivatives of testosterone designed for therapeutic purposes, but now taken predominantly as drugs of abuse. The most common behavioral effects associated with anabolic androgenic steroid use are changes in anxiety, aggression and reproductive behaviors, including the onset of puberty and sexual receptivity. GABAergic circuits in the forebrain underlie these behaviors and are regulated by gonadal steroids. Work from one laboratories has shown that the expression and function of GABA_A receptors in the rat and mouse forebrain varies between the sexes and across the estrous cycle. It was also shown that there are significant changes in GABA_A receptor expression that occur with the progression through puberty to adulthood. Because GABAergic systems are both steroid-sensitive and critical for the expression of behaviors altered with anabolic androgenic steroid use, forebrain GABA(A) receptors are an attractive candidate to assess how molecular actions of anabolic androgenic steroids may be translated to known behavioral outcomes. The studies demonstrate that anabolic androgenic steroids elicit both acute modulation of GABA_A receptor-mediated currents, as well as chronic regulation of GABA_A receptor expression and forebrain GABAergic transmission. Because anabolic androgenic steroid use has now become prevalent not only among adolescent boys, but in an increasing number of adolescent girls, we have also been particularly interested in determining age- and sex-specific effects of anabolic androgenic steroids. The data show that the effects of chronic anabolic androgenic steroid exposure can be greater for adolescent than adult animals and are more marked in females than in males. These data have particularly important implications with respect to studies we have done demonstrating that chronic anabolic androgenic steroid exposure alters the onset of puberty, estrous cyclicity and sexual receptivity [06076].

**GABAergic neuroactive steroids**

Neurosteroids are synthesized in the brain and modulate brain excitability. There is increasing evidence of their sedative, anesthetic and antiseizure properties, as well as their
influence on mood. Currently neurosteroids are classified as pregnane neurosteroids (allopregnanolone and allotetrahydrodeoxycorticosterone), androstane neurosteroids (androstanediol and etiocholanone) or sulfated neurosteroids (pregnenolone sulfate and dehydroepiandrosterone sulfate). Both preclinical and clinical findings indicate that progesterone derivative neurosteroids such as allopregnanolone and allotetrahydrodeoxycorticosterone play a role in mood disorders. Clozapine and olanzapine, which were shown to be effective in stabilizing bipolar disorder, elevate pregnenolone levels in rat hippocampus, cerebral cortex, and serum. In lithium-treated mice, the blood levels of allopregnanolone and pregnenolone were elevated compared to control levels. Women diagnosed with bipolar disorder typically show symptomatic exacerbation in relation to the menstrual cycle, and show vulnerability to the onset or recurrence of mood disorders immediately after giving birth, when the levels of neurosteroid derivatives of progesterone drop. Whereas in women who had recovered from bipolar disorder, the plasma concentration of allopregnanolone was elevated compared to either healthy controls or women with major depressive disorder during the premenstrual period. During depressive episodes, blood level of allopregnanolone is low. Treatment with fluoxetine tends to stabilize the levels of neurosteroids in depression. These findings converge to suggest that these steroids have significant mood-stabilizing effect. This hypothesis is consistent with the observation that a number of anticonvulsants are effective therapies for bipolar disorder, a finding also consistent with the antiseizure properties of neurosteroids. Further exploration of action of neuroactive steroids is likely to open new frontiers in the investigation of the etiology and treatment of mood disorders, particularly bipolar disorders [12140].

**Rewarding systems**

Research findings regarding androgen abuse in people and hedonic effects of androgens in laboratory rats are reviewed. Androgens, like other steroids, can have traditional actions via cognate intracellular steroid receptors, as well as other substrates. Results indicate that testosterone (T) metabolites may have actions in part via gamma-aminobutyric acid (GABA_A)/benzodiazepine receptor complexes (GBRs) and/or dopaminergic neurons in the nucleus accumbens, to mediate T's positive hedonic states. This may provide the basis for positive reinforcing effects of androgen seeking and use behavior. Following a comprehensive review of the background literature, findings are presented that have explored the extent to which metabolites of T mediate euphorogenic effects of androgens by acting in the nucleus accumbens. Then results regarding whether GBRs are necessary substrates for androgens' positive hedonic effects are discussed. Lastly, research that addresses if dopaminergic neurons in the nucleus accumbens are necessary for these effects of androgens are discussed. This review provides a comprehensive examination of the hedonic properties and abuse/addiction potential of androgens and the putative mechanisms underlying these effects [06077].

**Monoygotic twins**

Anabolic androgenic steroids (AAS) are derived by chemical manipulation of the testosterone molecule. The specified category of drugs produces anabolic, androgenic and psycho-active effects including elevated aggressive, hostile, violent and anti social behavior. The objective of this case report observational study was to evaluate the possible psychological consequences of AS use in the twin user of each pair, compared with the non-user twin. It was studied two pairs of male monозygotic twins: one pair 24 years old and the other 31 years old, with absolute genome and phenotype similarity. One of the twins of each pair used AAS while the other did not. Both pairs lived in Hellenic provincial towns and followed a common training and nutrition regime. The psychometric instruments used were the
Symptoms Check List-90 (SCL-90) and the Hostility and Direction of Hostility Questionnaire (HDHQ). The psychometric evaluations took place within a time interval of 6 months. The study found high levels of aggressiveness, hostility, anxiety and paranoid ideation in the twins who used AS. The non-user twins showed no deviation from their initial status. The use of AAS induced several important psychiatric changes in monozygotic twins which were not present in the twin who did not use AAS [06078].

**Sleeping pattern**

Anabolic androgenic steroid (AAS) abuse has become a public health problem in many countries, and is associated with many psychiatric disorders. Epidemiological studies have also found increasing numbers of sleep disorders reported by individuals using AASs. The purpose of this study was to evaluate sleep patterns and disorders in anabolic androgenic users who practice resistance exercise. The sample comprised 58 males divided into three groups: 20 current AAS users aged 26, and 21 controls with no history of AAS use, aged 26, and 17 sedentary men with no sleep disorders aged 27. The volunteers spent a night in the sleep laboratory for polysomnography. Comparing the three groups, the user group showed significantly reduced sleep efficiency and more wakings after sleep onset than the sedentary group. The sedentary group showed a higher percentage of stage 4 than the non-users group. It was suggested that using of anabolic steroids reduced sleep efficiency and alters sleep architecture [07075].

**Psychologic and psychiatric effects**

*Human*

The objective of one study was to evaluate the psychological consequences of real-world AAS use in athletes abusing such agents, in comparison with a placebo and control group of comparable athletes, while correlating the severity of abuse with the side effects observed. The hypothesis tested by the study was that the use of AAS induces a wide range of psychological side effects whose impact and emergence is dependent upon the severity of the abuse. The study includes a substantial group of AAS abusing athletes and two more groups demographically similar to the first, one composed of athletes not using any substance and a placebo group. All athletes were stratified according to the severity of AAS abuse. Psychometric instruments were applied to all athletes in specific time intervals, dependent to the AAS abusers’ regimens, providing us with a final psychological profile that was to be compared to the pre-study profile. All results were comparable (within and between groups) for statistically significant differences and correlated to the severity of the abuse. Homogeneity of all groups was safeguarded by random doping controls, monitoring of drug levels and analysis of all self obtained drugs by method of liquid chromatography/mass spectrometry. All athletes were provided with a common exercise and dietary regime, so common training and nutritional conditions were achieved. It was studied a cohort of 320 body-building, amateur and recreational athletes, of whom 160 were active users of AAS (group C), 80 users administering placebo drugs (group B) and 80 not abusing any substance (Group A). Group C athletes were stratified according to AAS abuse parameters, thus providing us with three subgroups of "light, medium and heavy abuse". Athletes of groups A and B were included in a "no abuse" subgroup. The psychometric instruments used were the Symptoms Check List-90 (SCL-90) and the Hostility and Direction of Hostility Questionnaire (HDHQ). The psychometric evaluations took place within a time interval of 13 months. Statistical analysis was performed by using the Mann-Whitney/Wilcoxon two-sample non-parametric test (Kruskal-Wallis test for two groups) for data that were not normally distributed and Linear regression analysis was used to ascertain the correlation between severity of use and escalation of side effects. The study showed a statistically significant
increase in all psychometric subscales recorded in group C, and no statistically significant difference in group C and A. There was a significant increase in the scorings of group C for all subscales of SCL-90 and HDHQ. Correlation of abuse severity and side effects showed that there was a statistical significant increase in Delta values of all SCL-90 and HDHQ subscales that escalated from light abuse to medium and heavy abuse/consumption patterns. The results of the study suggest that the wide range of psychiatric side effects induced by the use of AAS is correlated to the severity of abuse and the force of these side effects intensifies as the abuse escalates [06075].

Muscle dysmorphia has been described as a disorder in which individuals are pathologically preoccupied with their muscularity. One study was designed to further investigate the symptom characteristics and psychiatric conditions associated with the disorder. Weight lifting males meeting current criteria for muscle dysmorphia (n=15), past muscle dysmorphia (n=8), and no history of muscle dysmorphia (n=28) responded to advertisements placed in gymnasium and nutrition stores. Structured and semistructured interviews were administered, as well as survey measures. Relative to controls, males with current muscle dysmorphia experienced more aversive symptoms related to the appearance of their bodies, including more often thinking about their muscularity, dissatisfaction with appearance, appearance checking, bodybuilding dependence, and functional impairment. Higher rates of mood and anxiety disorders were found among individuals with a history of muscle dysmorphia relative to individuals with no history of muscle dysmorphia. The findings suggest that muscle dysmorphia can be distinguished from normal weight lifting on a number of clinical dimensions. Muscle dysmorphia appears to be comorbid with other psychiatric conditions [08127].

The risks from chronic administration of anabolic steroids may appear relatively low when compared with the use of socially acceptable drugs such as tobacco and alcohol. The literature tends to rely heavily on specific case reports, identifying psychiatric or psychological disorder, because of the private and personal nature of the abuse of this class of drug [08128, 08129]. Anabolic androgen steroids self-administration have increased over the last decade, in the wake of the demonstration that steroids increase muscle mass and strength in healthy adult males, over and above resistance training [08130]. However, there may be other reasons why individuals abuse anabolic substances. Thirteen percent of 75 female weightlifters, who admitted to abusing androgenic anabolic steroids to gain muscle mass, had increased their weightlifting activities to be better able to defend themselves against men and also reported that they were previously sexually abused [08131]. It is believed that compulsive weightlifting and steroid abuse may represent a form of response to the trauma of sexual assault and also may assist in raising self-esteem. The psychiatric evaluation of dedicated female athletes also demonstrated the exhibition of ergogenic polysubstance dependence, often with significant co-morbidity [08132]. Fifty six percent demonstrated hypomania during the “administration-phase” and 40 percent reported depressive symptoms during withdrawal. These athletes also displayed several psychiatric syndromes, previously ill-defined, such as obsessive compulsive disorder, rigid dietary practice, nontraditional gender roles, and chronic dissatisfaction and preoccupation with their physiques (muscle dysmorphia) [08133].

Pagonis et al [08134] has shown in a study of 160 steroid-abusing athletes compared with 160 placebo and controls that the wide range of psychiatric side effects induced by the abuse of anabolic steroids is correlated to the severity of abuse. The force of these side effects intensifies as the abuse escalates. They have a distorted body image and reported the condition of “reverse anorexia”, where they believed they were small and weak, despite being large and muscular [08135]. The reasons for use of these anabolic agents appear to be based not only on peer review, but also scientific research [08042]. Low self-esteem and
unrealistic, muscular male body ideals, puts individuals at risk for negative body images and unhealthy eating and exercise habits. These individuals resort to drug-taking to counteract their altered body images [08133].

The psychiatric effects of anabolic-androgenic steroids (i.e. testosterone and its derivatives) have been less well studied than their physical effects but are reported to include depression, mania, psychosis, and aggression. Dependence can also occur, with withdrawal involving psychiatric and physical symptoms [07031].

The psychiatric effects of anabolic-androgenic steroids are hard to study, for several reasons. Many of the available studies are, by necessity, observational. But because the substances are illicit, users have no way to verify their exact nature or amounts taken. Moreover, many steroid users concomitantly take a multitude of other performance-enhancing drugs and dietary supplements that also may have psychiatric effects. Prospective studies are hard to carry out because of the ethical issues inherent in testing a potentially dangerous substance. Because many users belong to a subculture of bodybuilders, weightlifters, or elite athletes, study results are hard to extrapolate to the general public. Most studies to date have evaluated dosages lower than most users report taking. Further, users of anabolic-androgenic steroids tend to use them for prolonged and repeated cycles over many years, which is hard to recreate in clinical trials. More studies are needed on a larger scale with dosing that is compatible with the supraphysiologic dosages used in the community. In addition, pre-existing personality traits that might predispose people to use steroids may significantly confound assessing any psychiatric effects of drug use. Suspected risk factors for men include antisocial personality traits, low self-esteem, and poor body image (body dysmorphia). It was found that weightlifters and bodybuilders who used anabolic steroids had significantly higher scores on dimensions of pathologic narcissism and lower scores on ratings of empathy. Another study found that up to 50 percent of steroid users had worked as bouncers and described themselves as aggressive regardless of their drug use [07031].

Uncontrolled, observational trials in the 1930s and 1940s found that men with refractory depression responded favorably to testosterone treatment. However, randomized, placebo-controlled studies conducted in the 1980s were equivocal. Observational studies show hypomania, mania, and depression. It was retrospectively studied 164 weightlifters and bodybuilders who used anabolic-androgenic steroids and found that about 10 percent had hypomania. Depression occurred when steroids were stopped in about 10 percent. In another study it was interviewed 41 bodybuilders and football players taking anabolic-androgenic steroids and found that 9 displayed full affective syndromes and 5 showed psychotic symptoms. In a later study, it was compared 88 athletes who were using anabolic-androgenic steroids with 68 nonusers and found that 23 percent of the steroid users reported major mood symptoms (including mania, hypomania, and depression) versus only 6 percent of the nonusers, and several users reported aggressive thoughts. The higher the steroid dosage, the more severe were the psychiatric symptoms. It was conducted a similar study of weightlifters and found more symptoms of depression and mania among users of anabolic-androgenic steroids, although formal diagnoses were not made. In controlled studies, high dosages led to mood changes in some users. Studies with supraphysiologic doses of anabolic-androgenic steroids found minimal or no changes in mood in most users, but a minority of users had significant mood changes. In another randomized, placebo-controlled crossover trial, it was given injections of testosterone cypionate (Depotest®) to 56 men, gradually increasing the dosage to 600 mg/week. Most of the men showed no significant manic symptoms, but 6 (12 %) had mild hypomania and 2 (4 %) had marked hypomania. In yet another placebo-controlled, crossover prospective trial, oral methyltestosterone (Virilon®) 40 or 240 mg/day was given to 20 normal men. Those on the high dose had increased
positive mood changes (euphoria, increased energy, and sexual arousal) as well as negative mood changes (irritability, violent feelings, hostility, and distractibility). One man developed mania at the high dosage, and another developed hypomania. Physiologic doses have minimal mood effects. Studies of the effects of low or near-physiologic doses of anabolic-androgenic steroids found minimal effects on mood [07031].

Studies in mice have found aggressive behavior correlating with increasing dosages and duration of anabolic-androgenic steroid treatment, culminating in females killing their offspring. Observational studies of aggressive behavior changes in people taking steroids have been equivocal. Placebo-controlled studies using supraphysiologic doses of anabolic-androgenic steroids have also been equivocal. Physiologic doses do not enhance aggression. However, serum testosterone level correlates with aggressiveness [07031].

AAS use has been associated with self-reported changes in mood and behavior. One study identified psychiatric syndromes in weightlifters using AASs. Twenty-three percent of AAS users experienced major mood changes of mania, hypomania, or major depression. Also common in AAS users was aggressive behavior resulting in fights, domestic disturbances, assaults, and arrests. Data from the National Household Survey on Drug Abuse have demonstrated a strong association between AAS use and self-acknowledged acts of violence against people and crimes against property. In general, the behavioral effects of AASs are variable, short-lived on discontinuation, and seem to be related to the type and dosage of AAS. The potential for physical dependence upon AASs does exist. In one study of AAS users, 50 percent of them met the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition criteria for dependence or abuse of steroids. Deeply entrenched body dissatisfaction and body dysmorphic disorder may underlie a psychologic dependence. Clearly, the addictive potential of AASs cannot be [07008].

AASs induce a state of euphoria and diminished fatigue that enables prolongation of training sessions by users. Recent data may explain how AASs exert these psychoactive effects on the brain. It has been proposed that AAS-mediated acute and chronic changes in the gamma-aminobutyric acid (GABA) receptor system cause many of the known behavioral effects. For instance, the immediate effects of decreased anxiety and enhanced sense of well-being that are experienced by AAS users likely arise from enhancement of forebrain GABAergic circuits. In contrast, anxiety and aggression are the result of a down-regulation of GABA receptor expression secondary to chronic AAS exposure. Further study may reveal that expression of these behaviors is influenced by the age and gender of the AAS user and the particular chemical composition of the AAS administered [07008].

In a study in middle and high school students, 5 percent of boys and 3 percent of girls had used steroids in the previous year. Use in boys was associated with higher rates of depressed mood, prior suicide attempts, greater substance abuse, and lower self-esteem. Another study of adolescents suggested that steroid use was associated with other high-risk behaviors and was less likely to be an isolated behavior. Many case reports describe psychiatric symptoms in patients using AASs. Reports of suicide include at least one patient who did not have a personal or family history of depression or suicidal behaviors. In one series of eight suicides in AAS users from Sweden, collateral information was sought, and when possible, “psychologic autopsies” were performed. Retrospectively, psychiatric symptoms, such as irritability, aggressiveness (“roid rage”), mood swings, decreased impulse control, and increased energy were noted during AAS use; however, the series included men who had prior psychiatric syndromes, personality disorders, and other substance abuse. Another report showed homicidal or near homicidal behavior in three men during AAS use. None had a history of psychiatric illness or violence before AAS use, and all met the Diagnostic and Statistical Manual of Mental Disorders, Revised Third Edition criteria for
manic episode during use. Two of the three men experienced depression and suicidal ideation upon the abrupt discontinuation of use. Although it is tempting to attribute this behavior to AAS use, this is a small set of case studies and as the investigators note, legal ramifications for the patients may have led to exaggeration in their reports. A series of 34 deaths of AAS users revealed nine victims of homicide, 11 suicides, 12 accidental deaths, and 2 deaths of indeterminate cause. The homicide victims showed high levels of aggression; in most of the suicide and accidental deaths, impulsive and violent behaviors had been noted by family or physicians. Most of the cases of accidental death were related to polysubstance overdose; four of the victims were heroin addicts who had histories of only sporadic or moderate AAS use. One study identified seven AAS users and evaluated them every 2 weeks for as long as 44 weeks. During clinic visits, subjects reported their AAS use, and assessments, including the Beck Depression Inventory (BDI), Profile of Mood States Questionnaire, and Buss-Durkee Hostility Scale, were administered. Scores fluctuated over time, but the fluctuations were not associated with AAS use. Additionally, most of the subjects had a history of major depression, and five reported abusing other substances. A larger study (n=160) comparing AAS-using athletes with nonusing athletes revealed that far more AAS users displayed mood disorders compared with nonusers and AAS users during periods of no use. Another study describing 41 AAS-using athletes reported that 22 percent displayed mood syndromes during use, which was significantly higher than the rate observed during periods of no exposure. Additionally, this study reported that 12 percent displayed psychotic symptoms during use compared with 0 percent during AAS-free periods. Yet another naturalistic study comparing weight-lifting AAS users with nonusers correlated supranormal testosterone levels with subjective and objective measures of aggression. Cluster B personality traits, including antisocial, borderline, and histrionic, were more prominent in AAS users [07058].

Attempts have been made to study the effects of AASs in humans in prospective laboratory-controlled settings. One double-blind study administered placebo followed by low-dose (40 mg/d) and then high-dose (240 mg/d) methyltestosterone to 20 normal healthy men without psychiatric disease or history of AAS use. During the high-dose period (3 days), distractibility, irritability, and energy level increased significantly, and there was a trend for an increase in anger and violent feelings. One subject developed acute mania, and another developed hypomania. Subtle, but significant, elevations in the BDI, Hamilton Depression Rating Scale, Brief Psychiatric Rating Scale, and hostility, anxiety and somatization on the Symptom Checklist (SCL-90) were observed. In follow-up studies, an increase in aggressiveness correlated with an increase in free T4, an increase in forgetfulness and distractibility correlated with total testosterone levels, and an increase in activation symptoms (energy, sexual arousal, and diminished sleep) correlated with cerebrospinal fluid 5-hydroxyindole acetic acid levels. In another placebo-controlled cross-over study of 50 men free of substance abuse or psychiatric illness, increasing levels of testosterone cypionate were administered over 6 weeks. Aggressive responses on the Point Subtraction Aggression Paradigm and increased manic scores on the Young Mania Rating Scale were demonstrated; 84 percent showed minimal psychiatric symptoms, 12 percent became mildly hypomanic, and 4 percent became markedly hypomanic. An additional study evaluated testosterone cypionate over 14 weeks at levels up to 500 mg/week in healthy men free of psychiatric illnesses and personality disorders; it found minimal psychologic effects in most men, but one adverse psychiatric effect resembled mania. Additionally, some studies showed no changes in psychometric measures in healthy men who were administered AASs. All of these studies used doses lower than those typical in AAS use, so they likely underestimated the psychiatric consequences of AAS [07058].

Several case reports and survey studies have indicated that abuse of anabolic androgenic steroids (AAS) often leads to increased aggressiveness and feelings of hostility that may
occasionally trigger violent behaviour. Other observations indicate that many users of AAS also abuse alcohol and/or various illegal substances. Since substance abuse is a well-known risk factor for violent behaviour, it could be that violence committed by AAS users might, at least in many cases, actually be caused by abuse of other drugs. In order to examine this possibility further here, the criminal histories (in terms of incidences of convictions) of deceased users of AAS with (AASpos-subst.pos) and without (AASpos-subst.neg) signs of abuse of other illegal substances were compared to the corresponding histories of deceased users of illicit substances testing negatively for AAS (subst.pos-AASneg) at the time of autopsy. The risk of being convicted for a crime against property was significantly higher in the subst.pos-AASneg group than in either the AASpos-subst.neg or AASpos-subst.pos groups (RR=0.048 versus 0.408). At the same time, the risk of being convicted for a crime of violence was at least as high for the two AAS-positive groups as for the AAS-negative group. Furthermore, when compared with the first 3 years after the first criminal conviction, a pronounced increase in the proportion of incidence of violent crimes and a marked reduction in the proportion of incidence of crime against property was observed during the 3-year period immediately preceding death only among the AASpos-subst.neg subjects. In conclusion, the incidence of violent crime among users of AAS without signs of other drug abuse was comparable to the corresponding incidences for drug addicts without AAS use. This observation suggests that the violent criminality observed among AAS users is not confounded in any systematic fashion by abuse of other drugs. The findings also indicate that use of AAS in certain predisposed individuals might cause a high rate of violent crimes, especially if the use of AAS is combined with the use of other illegal substances [07076].

**Long-term effects on mental health**

The knowledge concerning the long-term effect of former anabolic androgenic steroids (AAS)-use on mental health is sparse. One study aims to investigate whether previous AAS-use affects mental health, present sociodemographic data, sport activity and substance abuse in a retrospective 30-year follow-up study of former elite athletes. Swedish male-elite power sport athletes (n=683) on the top 10 national ranking lists during any of the years 1960-1979 in wrestling, Olympic lifting, powerlifting and the throwing events in track and field answered a questionnaire. At least 20 percent of the former athletes admitted previous AAS-use. They had more often sought professional expertise for mental problems and had used illicit drugs compared to those not having used AAS. The AAS-users also differed in former sport activity pattern compared to non AAS-users. It is clear that a relationship exists between use of AAS and mental-health problems. Further studies need to be done in order to clarify this relationship [13149].

**Rat**

It was previously showed that 14 days of daily intramuscular injections of the anabolic androgenic steroid nandrolone decanoate (15 mg/kg) reduced the extracellular levels of the dopaminergic metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the nucleus accumbens shell using microdialysis. The aim of one study was to investigate whether the same dose regimen of nandrolone decanoate may affect the activities of the dopamine-metabolizing enzymes monoamine oxidases A and B (MAO-A and MAO-B). A radiometric assay was used to determine the activities of MAO-A and MAO-B in rat brain tissues after 14 days of daily i.m. nandrolone decanoate injections at the doses 3 and 15 mg/kg. Gene transcript contents of MAO-A, MAO-B and cathecol-O-methyltransferase (COMT) were measured with quantitative real-time reverse transcription PCR. 3 mg/kg of nandrolone decanoate significantly reduced the activity of both MAO-A and -B in the caudate putamen. 15 mg/kg of nandrolone decanoate significantly reduced the activity of MAO-A in the amygdala and increased the gene transcript level of MAO-B in the substantia nigra. In conclusion, imbalanced MAO activities may contribute to explain the impulsive and aggressive behaviour often described in AAS abusers. The reduced MAO
activities observed are in line with previously presented findings of decreased extracellular levels of DOPAC and HVA in the rat brain, indicating decreased monoaminergic activity following repeated AAS administration [08136].

It has been shown that male rats pubertally exposed to anabolic androgenic steroids (AAS) displayed aggression towards females in response to physical provocation. This experiment examined two factors that may modulate AAS-induced behavior towards females: olfactory cues and frustration. Gonadally intact males began one of three AAS treatments at puberty (D40): testosterone propionate (T), stanozolol (S), T+S, or vehicle control. To test for the relevance of olfactory cues in the elicitation of behavior toward females, a hidden neighbor paradigm was used. The proximal stimulus was an ovariectomized (OVX) female, estrogen plus progesterone (E+P) female, or an E+P female with tape-obstructed vagina (OBS). Distal olfactory cues from a hidden neighbor were delivered from a separate cage connected to the testing arena. The vaginally obstructed, sexually receptive female (OBS) was used to determine the effects of frustration on behavior by AAS males. Both sexual and aggressive behaviors were measured. The presence of distal olfactory cues had no effect on either sexual or aggressive behavior. In the presence of E+P and OBS females, all males displayed sex behaviors, not aggression. However, AAS males displayed significantly more aggression towards proximal OVX females than controls. AAS males mounted OBS females significantly more than controls, indicating a persistence of once rewarded behavior. These results suggest that proximal cues of the conspecific female are more salient than distal olfactory cues in determining behavior and that AAS males display frustration-induced persistence in response to vaginally obstructed receptive females [06081].

Human studies suggest that anabolic androgenic steroid (AAS) users are aggressive towards women. This study used a rat model to evaluate whether AAS potentiated aggression towards females and the conditions under which this occurs. Gonadally intact pubertal male rats received one of the following AAS treatments (5 mg/kg s.c. 5 days/week for nine weeks): testosterone (T), stanozolol (S), testosterone + stanozolol (T + S), or vehicle control. Each rat was tested with 3 conspecific stimuli: ovariectomized females (OVX), estrogen only females (E), and estrogen + progesterone females (E + P). The response to physical provocation was tested under three conditions: without physical provocation, provocation of the experimental male, and provocation of the conspecific female. Provocation was a mild tail pinch. Both aggressive and sexual behaviors were measured during each test. In the absence of physical provocation, AAS males were not aggressive towards females. However, provocation significantly increased aggression in males treated with testosterone but only towards OVX females. In the presence of E or E + P females, all animals displayed sex behavior, not aggression. Thus, factors such as the nature of the AAS and the hormonal status of the females are important in determining whether male rats will be aggressive towards females. However, the most salient factor determining aggression towards females is the presence of provocation in combination with high levels of testosterone [06082].

Illicit use of anabolic androgenic steroids (AAS) has become a prevalent health concern not only among male professional athletes, but, disturbingly, among a growing number of women and adolescent girls. Despite the increasing use of AAS among women and adolescents, few studies have focused on the effects of these steroids in females, and female adolescent subjects are particularly underrepresented. Among the hallmarks of AAS abuse are changes in reproductive behaviors. Here, it was discussed work from laboratories on the actions of AAS on the onset of puberty and sexual behaviors in female rodents, AAS interactions and sex- and age-specific effects of these steroids on neural transmission mediated by gamma-aminobutyric acid receptors within forebrain neuroendocrine control regions that may underlie AAS-induced changes in these behaviors [06083].
Anabolic androgenic steroid (AAS) abuse is increasing in teenagers. It was examined the effects of stacked AAS in adolescent male rats. Stacking, in which multiple AAS are taken simultaneously, is commonly employed by humans. Beginning at puberty gonadally intact male rats received testosterone, nandrolone, or stanozolol. Additional groups received stacked AAS: testosterone + stanozolol, nandrolone + stanozolol, or nandrolone + testosterone. Injections continued during tests for sexual behavior, vocalizations, scent marking, partner preference, aggression and fertility. Body and reproductive tissue weights were taken. Sexual and aggressive behaviors were increased by testosterone yet inhibited by stanozolol; nandrolone had no effect. Stacking testosterone with stanozolol prevented the inhibitory effects of stanozolol. Body weight was decreased by testosterone and all stacked AAS. Cell nuclear androgen receptor binding in brain was significantly increased in nandrolone males and decreased in stanozolol males; testosterone males were slightly higher than controls. Androgen receptors in stacked groups were intermediate between individual AAS suggesting that stanozolol competed with other AAS for androgen receptors despite its low affinity. The results indicate that stacking AAS influences the effects of individual AAS on behavioral and endocrine measures, and levels of androgen receptor occupation are not directly correlated with AAS effects on behavior [06084].

**Mouse**

Anabolic androgenic steroid abuse triggers impulsive aggression, anxiety, and depression, which suggests a dysfunction of GABAergic neurotransmission. Socially isolated female mice that have received testosterone propionate (1.45 micromol/kg) treatment for 3 weeks during social isolation express aggression, neurosteroid downregulation, and changes in the cortical mRNA expression of several gamma-aminobutyric acid type A receptor subunits (alpha1, alpha2, gamma2 are decreased by 30-40 %, and alpha4 and alpha5 are increased by 50 %). Administration of allopregnanolone or the potent selective brain steroidogenic stimulant S-norfluoxetine, in doses (1.8-3.6 micromol/kg) that fail to inhibit 5-hydroxytryptamine reuptake, normalizes olfactory bulb neurosteroid level downregulation and abolishes aggression. This work underscores the role of neurosteroids in the regulation of aggression elicited by testosterone propionate in socially isolated female mice [06080].

Anabolic androgenic steroids effects vary according to chemical structure and metabolism, route of administration, and AAS regimen. In one study, male mice were systemically exposed to testosterone propionate, nandrolone or 17alpha-methyltestosterone, type I, type II and type III AAS, respectively, in order to determine the hedonic or aversive properties of each drug. For this purpose, the conditioned place preference test was employed at three different AAS doses (0.075, 0.75 and 7.5 mg/kg). Other behavioral domains monitored were light-dark transitions (side changes) and general activity. Testosterone propionate shifted place preference at all doses tested, and nandrolone shifted place preference at 0.75 and 7.5 mg/kg, but not at 0.075 mg/kg, the lower dose tested. Conversely, mice receiving 17alpha-methyltestosterone did not show alteration in the preference score. The lower dose of nandrolone did modify exploratory-based anxiety showing a decrease in light-dark transitions if compared to vehicle-treated animals, while mice treated with testosterone propionate 17alpha-methyltestosteronewere not affected. The data suggest that when studying hedonic and rewarding properties of synthetic androgens, distinction has to be made based on type of AAS and metabolism [08137].

**Hamster**

Repeated exposure to anabolic/androgenic steroids (AAS) during adolescence stimulates high levels of offensive aggression in Syrian hamsters. The current study investigated whether adolescent AAS exposure activated neurons in areas of hamster forebrain implicated in aggressive behavior by examining the expression of FOS, i.e., the protein product of the immediate early gene c-fos shown to be a reliably sensitive marker of neuronal
activation. Adolescent AAS-treated hamsters and sesame oil-treated littermates were scored for offensive aggression and then sacrificed 1 day later and examined for the number of FOS immunoreactive (FOS-ir) cells in regions of the hamster forebrain important for aggression control. When compared with non-aggressive, oil-treated controls, aggressive AAS-treated hamsters showed persistent increases in the number of FOS-ir cells in select aggression regions, namely the anterior hypothalamus and lateral septum. However, no differences in FOS-ir cells were found in other areas implicated in aggression such as the ventrolateral hypothalamus, bed nucleus of the stria terminals, central and/or medial amygdala or in non-aggression areas, such as the somatosensory cortex and the suprachiasmatic nucleus. These results suggest that adolescent AAS exposure may constitutively activate neurons in select forebrain areas critical for the regulation of aggression in hamsters. A model for how persistent activation of neurons in one of these brain regions (i.e., the anterior hypothalamus) may facilitate the development of the aggressive phenotype in adolescent-AAS exposed animals is presented [06079].

Chronic treatment with anabolic-androgenic steroids during adolescence alters the activity of various neurotransmitter systems in male Syrian hamsters (Mesocricetus auratus). One study was conducted to determine whether glutamatergic cells in the lateral anterior hypothalamus, a sub-region of the anterior hypothalamus, have lasting activation following adolescent AAS exposure, and to examine AAS-induced alterations in the connections between the lateral anterior hypothalamus and the ventrolateral hypothalamus governed by glutamate. Hamsters were administered AAS during adolescence and then examined for changes in FOS (protein product of the immediate early gene c-fos) and phosphate activated glutaminase (PAG; the rate-limiting enzyme in the synthesis of glutamate) immunoreactivity (FOS/PAG-IR) using double-label immunohistochemistry. In a second experiment, a retrograde tracing study was conducted using a red fluorescent tracer microinjected into the ventrolateral hypothalamus. Then brains were processed for PAG immunofluorescence and examined for AAS-induced changes in the number of PAG positive cells containing the tracer (PAG/Tracer) in the lateral anterior hypothalamus. When compared to oil-treated controls, AAS-treated hamsters showed significant increases in PAG-IR and FOS/PAG-IR in the lateral anterior hypothalamus, decreases in afferent innervation from the lateral anterior hypothalamus to the ventrolateral hypothalamus and decreases in the total number of glutamate cells in the lateral anterior hypothalamus projecting to the ventrolateral hypothalamus. Together with previous research from our lab showing increased AAS-induced expression of PAG in the AH and increased glutamate receptor expression in the ventrolateral hypothalamus, the current results suggest that adolescent AAS exposure leads to alterations in the function and expression of the glutamatergic system as well as changes in hypothalamic neural connections. In addition, the current results further strengthen the notion that a specific nucleus in the lateral anterior hypothalamus is a critical hypothalamic sub-region particularly sensitive to AAS-induced neurodevelopmental effects [08138].

Influence on serotonin in the brain
The goal of this study was to assess the interactive effects of chronic anabolic androgenic steroid (AAS) exposure and brain serotonin (5-hydroxytryptamine, 5-HT) depletion on behavior of pubertal male rats. Serotonin was depleted beginning on postnatal day 26 with parachlorophenylalanine (PCPA 100 mg/kg, every other day); controls received saline. At puberty (P40), half the PCPA-treated rats and half the saline-treated rats began treatment with testosterone (T, 5 mg/kg, 5 days/week). Behavioral measures included locomotion, irritability, copulation, partner preference, and aggression. Animals were tested for aggression in their home cage, both with and without physical provocation (mild tail pinch). Brain levels of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), were determined using HPLC. PCPA significantly and substantially depleted 5-HT and 5-HIAA in all brain regions examined. Chronic T treatment significantly decreased 5-HT and 5-HIAA in
certain brain areas, but to a much lesser extent than PCPA. Chronic exposure to PCPA alone significantly decreased locomotor activity and increased irritability but had no effect on sexual behavior, partner preference, or aggression. T alone had no effect on locomotion, irritability, or sexual behavior but increased partner preference and aggression. The most striking effect of combining T+PCPA was a significant increase in attack frequency as well as a significant decrease in the latency to attack, particularly following physical provocation. Based on these data, it can be speculated that pubertal AAS users with low central 5-HT may be especially prone to exhibit aggressive behavior [06085].

Agression and violence

It was examined the effects of anabolic-androgenic steroid use on serious violent behavior. Multivariate models based on data from the National Longitudinal Study of Adolescent Health in the US (n=6823) were used to examine the association between lifetime and past-year self-reported anabolic-androgenic steroid use and involvement in violent acts. Compared with individuals who did not use steroids, young adult males who used anabolic-androgenic steroids reported greater involvement in violent behaviors after it was controlled for the effects of key demographic variables, previous violent behavior, and polydrug use [08147].

In humans and animals, anabolic-androgenic steroids (AAS) increase aggression, but the underlying behavioral mechanisms are unclear. AAS may increase the motivation to fight. Alternatively, AAS may increase impulsive behavior, consistent with the popular image of ‘roid rage. To test this, adolescent male rats were treated chronically with testosterone (7.5 mg/kg) or vehicle and tested for aggressive motivation and impulsivity. Rats were trained to respond on a nose-poke on a 10min fixed-interval schedule for the opportunity to fight in their home cage with an unfamiliar rat. Although testosterone increased aggression (6.3 ± 1.3 fights/5 min vs 2.4 ± 0.8 for controls), there was no difference in operant responding (28.4 ± 1.6 nose-pokes/10 min for testosterone, 32.4 ± 7.0 for vehicle). This suggests that testosterone does not enhance motivation for aggression. To test for impulsivity, rats were trained to respond for food in a delay-discounting procedure. In an operant chamber, one lever delivered one food pellet immediately, the other lever gave 4 pellets after a delay (0, 15, 30 or 45s). In testosterone- and vehicle-treated rats, body weights and food intake did not differ. However, testosterone-treated rats chose the larger, delayed reward more often (4.5 ± 0.7 times in 10 trials with 45s delay) than vehicle controls (2.5 ± 0.5 times), consistent with a reduction in impulsive choice. Thus, although chronic high-dose testosterone enhances aggression, this does not include an increase in impulsive behavior or motivation to fight. This is further supported by measurement of tyrosine hydroxylase (TH) by Western immunoblot analysis in brain regions important for motivation (nucleus accumbens, Acb) and executive function (medial prefrontal cortex, PFC). There were no differences in TH between testosterone- and vehicle-treated rats in Acb or PFC. However, testosterone significantly reduced TH (to 76.9 ± 3.1 % of controls) in the caudate-putamen, a brain area important for behavioral inhibition, motor control and habit learning [12138].

Millions of individuals worldwide have used anabolic-androgenic steroids (AAS) to gain muscle or improve athletic performance. Recently, in vitro investigations have suggested that supraphysiologic AAS doses cause apoptosis of neuronal cells. These findings raise the possibility, apparently still untested, that humans using high-dose AAS might eventually develop cognitive deficits. It was administered five cognitive tests from the computerized CANTAB battery (Pattern Recognition Memory, Verbal Recognition Memory, Paired Associates Learning, Choice Reaction Time, and Rapid Visual Information Processing) to 31 male AAS users and 13 non-AAS-using weightlifters age 29-55, recruited and studied in 2012 in Middlesbrough, UK. Testers were blinded to participants' AAS status and other historical data. Long-term AAS users showed no significant differences from nonusers on
measures of response speed, sustained attention, and verbal memory. On visuospatial memory, however, AAS users performed significantly more poorly than nonusers, and within the user group, visuospatial performance showed a significant negative correlation with total lifetime AAS dose. These were large effects: on Pattern Recognition Memory, long-term AAS users underperformed nonusers by almost one standard deviation, based on normative population scores (adjusted mean difference in z-scores=0.89; p=0.036), and performance on this test declined markedly with increasing lifetime AAS dose. These results remained stable in sensitivity analyses addressing potential confounding factors. These preliminary findings raise the ominous possibility that long-term high-dose AAS exposure may cause cognitive deficits, notably in visuospatial memory [12139].

The association between substance abuse, particularly alcohol abuse, and violence has been well established. However, since substance abuse co-occurs with several other risk factors for violence, the causal link between substance abuse and violence and the extent to which the acute influence of alcohol, illicit drugs, benzodiazepines, and anabolic androgenic steroids have a triggering effect on violent behavior are more uncertain. Case-crossover design was used based on data from structured face to face interviews with remand prisoners (n=194; 172 men, 22 women) suspected of violent crimes. Main outcome measure: odds ratio (OR) for a violent crime, 24h after exposure to different substances, compared to periods of no exposure was calculated using conditional logistic regression and a Mantel-Haenszel estimator with confidence intervals for sparse data. Intake of alcohol (OR 6) and large doses of benzodiazepines (OR 36) triggered interpersonal violence. Stratified analyses of possible effect modifiers were sex, conduct/behavioral problems, trauma experiences; psychiatric vulnerability did not reveal any substantial differences. Influences of alcohol and unusually high doses of benzodiazepines are proximal risk factors for violent crime. Improved knowledge of short-term (and dose-related) risk factors may contribute to treatment planning and risk assessment of violence [13160].

In humans and animals, anabolic-androgenic steroids (AAS) increase aggression, but the underlying behavioral mechanisms are unclear. AAS may increase the motivation to fight. Alternatively, AAS may increase impulsive behavior, consistent with the popular image of 'roid rage. To test this, adolescent male rats were treated chronically with testosterone (7.5 mg/kg) or vehicle and tested for aggressive motivation and impulsivity. Rats were trained to respond on a nose-poke on a 10 min fixed-interval schedule for the opportunity to fight in their home cage with an unfamiliar rat. Although testosterone increased aggression (6.3 ± 1.3 fights/5 min vs 2.4 ± 0.8 for controls), there was no difference in operant responding (28.4 ± 1.6 nose-pokes/10 min for testosterone, 32.4 ± 7.0 for vehicle). This suggests that testosterone does not increase motivation for aggression. To test for impulsivity, rats were trained to respond for food in a delay-discounting procedure. In an operant chamber, one lever delivered one food pellet immediately, the other lever gave 4 pellets after a delay (0, 15, 30 or 45 s). In testosterone- and vehicle-treated rats, body weights and food intake did not differ. However, testosterone-treated rats chose the larger, delayed reward more often (4.5 ± 0.7 times in 10 trials with 45 s delay) than vehicle controls (2.5 ± 0.5 times), consistent with a reduction in impulsive choice. Thus, although chronic high-dose testosterone enhances aggression, this does not include an increase in impulsive behavior or motivation to fight. This is further supported by measurement of tyrosine hydroxylase (TH) by Western immunoblot analysis in brain regions important for motivation (nucleus accumbens, Acb) and executive function (medial prefrontal cortex, PFC). There were no differences in TH between testosterone- and vehicle-treated rats in Acb or PFC. However, testosterone significantly reduced TH (to 76.9 ± 3.1 % of controls) in the caudate-putamen, a brain area important for behavioral inhibition, motor control and habit learning [13206].

Twin studies

598
Individual behavioral and psychiatric responses to chronic AAS abuse are extremely variable, depending on pre-existing psychiatric conditions, personality, and type and dose of AAS. Case reports and some epidemiology studies associate chronic AAS use with changes in behavior, mood, and somatic perceptions in a small subset of chronic AAS abusers. Psychiatric complications associated with chronic AAS use include mania, aggression, and agitation, but no large-scale epidemiologic studies have confirmed a causal relationship between chronic AAS abuse and severe psychiatric complications (e.g. psychosis). Depression may occur, particularly in the immediate period after cessation of use, along with fatigue, decreased libido, insomnia, anorexia, and headaches. Studies suggest that male AAS users have more frequent and prolonged periods of anger, aggression, irritability, and hostility than nonusers. Multivariate models based on data from the National Longitudinal Study of Adolescent Health (n=6823) indicate that young men using anabolic-androgenic steroids report greater involvement in violent behaviors than non-users after control for the effects of key demographic variables, previous violent behavior, and polydrug use. In a study of 2 sets of male monozygotic twins with only 1 twin using AASs, the use of AASs was associated with anxiety, hostility, aggressiveness, and paranoid ideations as determined by comparing responses to a Symptoms Checklist-90 and the Hostility and Direction of Hostility Questionnaire. Behavioral abnormalities included are tractibility, delirium, delusions, irritability, paranoia, impulsivity, insomnia, hostility, anxiety, agitation, aggression, violence, and mood lability. These behavioral effects are dose dependent with effects ranging from mild effects (increased confidence, enhanced self-esteem, and euphoria) to serious behavioral abnormalities (mood swings, grandiose thinking, paranoia, impulsivity, hostility, violence, and antisocial behavior) [13003].

Experimental
In the US and worldwide anabolic/androgenic steroid use remains high in the adolescent population. This is concerning given that anabolic/androgenic steroid use is associated with a higher incidence of aggressive behavior during exposure and anxiety during withdrawal. This study uses pubertal Syrian hamsters (Mesocricetus auratus) to investigate the hypothesis that an inverse behavioral relationship exists between anabolic/androgenic steroid-induced aggression and anxiety across adolescent exposure and withdrawal. In the first experiment, it was examined aggression and anxiety during adolescent anabolic/androgenic steroid exposure and withdrawal. Adolescent anabolic/androgenic steroid administration produced significant increases in aggression and decreases in anxiety during the exposure period followed by significant decreases in aggression and increases in anxiety during anabolic/androgenic steroid withdrawal. In a second experiment, anabolic/androgenic steroid exposed animals were separated into groups based on their aggressive response during the exposure period and then tested for anxiety during exposure and then for both aggression and anxiety during withdrawal. Data were analyzed using a within-subjects repeated measures predictive analysis. Linear regression analysis revealed that the difference in aggressive responding between the anabolic/androgenic steroid exposure and withdrawal periods was a significant predictor of differences in anxiety for both days of testing. Moreover, the combined data suggest that the decrease in aggressive behavior from exposure to withdrawal predicts an increase in anxiety-like responding within these same animals during this time span. Together these findings indicate that early anabolic/androgenic steroid exposure has potent aggression- and anxiety-eliciting effects and that these behavioral changes occur alongside a predictive relationship that exists between these two behaviors over time [13150].

Association with criminality

Sweden
Observations suggest that the use of anabolic androgenic steroids (AAS) may trigger uncontrolled, violent rage. Other observations indicate that certain groups of criminals may
use AAS with the intention of being capable of committing crime more efficiently. To examine the proposed association between the use of AAS and criminality a controlled retrospective cohort study of registered criminal activity among individuals tested for AAS use during the period of 1995 to 2001 was performed. All individuals in Sweden who were tested for AAS use during this period were included. These individuals were referred for testing from both inpatient and outpatient clinics as well as from centers for treatment of substance abuse: individuals testing positive for AAS (n=241), with those testing negative for AAS during the same period (n=1199) serving as the control group. The risk of having been convicted for a weapons offense or fraud was higher among individuals testing positive for AAS than among those testing negative (RR, 2.090 and 1.511, respectively) whereas there were no significant differences with respect to violent crimes (RR, 1.116) or crimes against property (RR, 0.942). When patients referred from substance abuse centers were excluded, a lower risk for crimes against property was observed for the individuals who tested positive for AAS (RR, 0.761) and the risk for fraud in the 2 groups was equalized (RR, 1.117). The increased risk for a weapons offense among the individuals testing positive for AAS remained virtually unchanged. It was concluded that in addition to the impulsive violent behavior previously shown to be related to AAS use, such use might also be associated with an antisocial lifestyle involving various types of criminality. However, the existence and nature of this possible association remain unclear and call for further investigation [06086].

**Suicide risk**

It was studied 62 professional weightlifters in Finland who were strongly suspected of using anabolic-androgenic steroids. They were compared them with 1,094 population controls. Over a 12-year period, 8 (13 %) of the weightlifters died versus 34 (3 %) of the controls, which was a statistical significant difference. The causes of death in the weightlifters were suicide (3 subjects), acute myocardial infarction (3), hepatic coma (1), and non-Hodgkin lymphoma (1) [07031].

**Addiction**

An estimated 14 to 57 percent of anabolic-androgenic steroid users develop dependence. How addiction develops is unknown, but psychological dependence is believed to play a large role. Different substance abuse patterns exist in different populations that use anabolic-androgenic steroids. In one study it was found that steroid-using weightlifters almost always previously tried other illicit substances. On the other hand, others found that elite athletes, weightlifters, and bodybuilders rarely abuse illicit drugs, reflecting their interest in optimizing their physique and performance. Adolescents who abuse steroids are more likely to smoke and use other illicit substances than are older users [07031]

**Withdrawal effects**

Users of high-dose AAS regimens report a withdrawal syndrome, including steroid craving, depression, suicidality, irritability, muscle aches, and autonomic instability including hot flashes, nausea and vomiting, tachycardia, and hypertension [07058].

Physical symptoms of withdrawal are similar to those seen during alcohol and opioid withdrawal, including diaphoresis, myalgias, nausea, and increases in blood pressure and heart rate. Withdrawal may also be characterized by depressive symptoms [07008].

**Treating psychiatric effects of steroid use**
Steroid abusers rarely seek help, and many regard the psychiatric effects as beneficial, especially for athletes in certain sports. Illicit use is compounded by mistrust of doctors, a perception that medical people lack knowledge about these drugs, and fear of stigma or negative consequences that may result from drug use being exposed. Adverse effects of steroid abuse should be managed by discontinuing the drugs by tapering if necessary and by treating the symptoms. Steroid abusers typically take doses 10 to 100 times higher than physiologic doses, in cycles lasting 6 to 14 weeks, consisting of daily oral doses plus weekly or monthly intramuscular injections. Treatment of psychiatric effects starts with stopping the steroids. It is reasonable to substitute testosterone enanthate (Andro-Estro) and gradually taper the dose. The short-term use of antipsychotic medications may help treat steroid-induced mania and psychosis. Benzodiazepines may help control panic or anxiety in the short term. Selective serotonin reuptake inhibitors or tricyclic antidepressants should be used if long-term treatment is needed. Depression sometimes occurs when use is stopped. Fluoxetine (Prozac®) can be used in this situation. Anabolic-androgenic steroid abuse is no longer confined to professional athletes; therefore physicians should be aware of its signs and symptoms in order to address adverse effects and provide treatment [07031].

**Cognitive deficits**

Millions of individuals worldwide have used anabolic-androgenic steroids (AAS) to gain muscle or improve athletic performance. Recently, in vitro investigations have suggested that supraphysiologic AAS doses cause apoptosis of neuronal cells. These findings raise the possibility, apparently still untested, that humans using high-dose AAS might eventually develop cognitive deficits. It was administered five cognitive tests from the computerized CANTAB battery (Pattern Recognition Memory, Verbal Recognition Memory, Paired Associates Learning, Choice Reaction Time, and Rapid Visual Information Processing) to 31 male AAS users and 13 non-AAS-using weightlifters age 29-55, recruited and studied in May 2012 in the UK. Testers were blinded to participants’ AAS status and other historical data. Long-term AAS users showed no significant differences from nonusers on measures of response speed, sustained attention, and verbal memory. On visuospatial memory, however, AAS users performed significantly more poorly than nonusers, and within the user group, visuospatial performance showed a significant negative correlation with total lifetime AAS dose. These were large effects: on Pattern Recognition Memory, long-term AAS users underperformed nonusers by almost one standard deviation, based on normative population scores, and performance on this test declined markedly with increasing lifetime AAS dose. These results remained stable in sensitivity analyses addressing potential confounding factors. These preliminary findings raise the ominous possibility that long-term high-dose AAS exposure may cause cognitive deficits, notably in visuospatial memory [13144].

**Effects on learning**

The illicit use of anabolic androgenic steroids (AAS) has gained popularity among adolescents in the last decade. However, although it is known that exposure to AAS impairs cognition in adult animal models, the cognitive effects during adolescence remain undetermined. An inhibitory avoidance task (IAT) was used to assess the effect of AAS (17alpha-methyltestosterone; 17alpha-meT-7.5 mg/kg) in male and female periadolescent rats. A single injection of 17alpha-meT immediately before the footshock produced significant impairment of inhibitory avoidance learning in males but not females. Generalized anxiety, locomotion, and risk assessment behaviors (RAB) were not affected. The results show that exposure to a single pharmacological dose of 17alpha-meT during periadolescence exerts sex-specific cognitive effects without affecting anxiety. Thus, disruption of the hormonal milieu during this early developmental period might have negative impact on learning and memory [13145].
Mood disorders
The knowledge concerning the long-term effect of former anabolic androgenic steroids (AAS)-use on mental health is sparse. One study aimed to investigate whether previous AAS-use affects mental health, present sociodemographic data, sport activity and substance abuse in a retrospective 30-year follow-up study of former elite athletes. Swedish male-elite power sport athletes (n=683) on the top 10 national ranking lists during any of the years 1960-1979 in wrestling, Olympic lifting, powerlifting and the throwing events in track and field answered a questionnaire. At least 20 percent of the former athletes admitted previous AAS-use. They had more often sought professional expertise for mental problems and had used illicit drugs compared to those not having used AAS. The AAS-users also differed in former sport activity pattern compared to non AAS-users. It is clear that a relationship exists between use of AAS and mental-health problems. Further studies need to be done in order to clarify this relationship [13146].

The true prevalence of these neuropsychiatric disorders is difficult to determine, but relatively few individuals develop these adverse effects; they are found primarily in high-dose chronic AAS abusers. In a study of 88 AAS-using athletes and 68 non-users, the incidence of major mood disorders (mania, hypomania, and major depression) was significantly more frequent in steroid users compared with non-users. Additionally, these mood disorders were more frequent in current AAS users than in abstinent AAS users [13003].

Effects on the brain's neurotrophyanabolic androgenic steroids (AAS) are synthetic androgen-like compounds that are abused in sport communities despite their adverse effects. Nerve growth factor (NGF) influences neuronal differentiation and survival, and it also mediates higher brain functions such as learning and memory. Changes in NGF expression have been implicated in neurodegenerative disorders, including Alzheimer disease. Hence, we decided to study the effect of chronic AAS exposure on brain NGF profile, NGF-dependent cholinergic function, and related behavioral performance. Male Wistar rats were injected for 4 weeks with either nandrolone or stanozolol at daily doses (5.0 mg/kg, s.c.) that are considered equivalent to those abused by humans. NGF levels and NGF receptor (TrkA and p75NTR) expression were measured in the hippocampus and in the basal forebrain. Choline acetyltransferase expression was evaluated in basal forebrain. Spatial learning and memory were assessed using the Morris water maze. AAS treatment caused region-specific changes in the expression of NGF and its receptors. Both nandrolone and stanozolol increased NGF levels in the hippocampus and reduced NGF levels in the basal forebrain, reduced p75NTR expression in the hippocampus, and failed to affect TrkA expression in the basal forebrain. Finally, AAS treatment reduced the expression of choline acetyltransferase in the basal forebrain and impaired the behavioral performance in the Morris water maze. It was concluded that the evidence that supraphysiological doses of AAS cause neurotrophic unbalance and related behavioral disturbances raises the concern that AAS abuse in humans may affect mechanisms that lie at the core of neuronal plasticity [13147].

Liver changes due to sex hormones (anabolic steroids and oral contraceptives)

Overview
The liver is a hormone-sensitive organ, and in fact both normal liver and hepatocellular carcinoma (HCC) tissues from male and female mammals have been shown to express specific estrogen receptors (ERs). Experimentally, estrogens may act as liver tumor inducers or promoters in vivo, and are involved in stimulating hepatocyte proliferation in vitro.
Moreover, anti-estrogens like tamoxifen have been shown to reduce levels of ERs and to inhibit hepatocyte proliferation following partial hepatectomy. As regards the role of androgens, it has also been observed that androgen receptors (ARs), specifically activated by testosterone, are present in normal liver tissue from both males and females and that their expression is increased in tumor tissue and in the surrounding liver of individuals with HCC. In addition, observations from clinical and epidemiological studies have highlighted that the long-term use of OCs and anabolic androgenic steroids (AASs) can induce benign and malignant hepatocellular tumors. One study provided definite and quantitative evidence that OC use was significantly, although modestly associated with FNH. The time-risk relation gave convincing support to the existence of a real association, given that there was a direct trend in risk with duration and an inverse trend with age at first use. Benign tumors of the liver are often discovered incidentally in asymptomatic individuals during diagnostic imaging or exploratory laparotomy performed for other reasons. Hemangiomas are the most common benign liver tumors, followed in prevalence by focal nodular hyperplasia (FNH) and the rarer condition of adenoma; their growth and development have been linked to hormonal stimulation. Long-term use of oral contraceptives (OCs) and anabolic androgenic steroids (AASs) can induce both benign (hemangioma, adenoma, and focal nodular hyperplasia, FNH) and malignant (hepatocellular carcinoma, HCC) hepatocellular tumors. Hepatic adenomas (HAs) are rare, benign neoplasms usually occurring in young women, the development and the complications of which have been related to the strength of OCs and the duration of their use. HA incidence has fallen since the introduction of pills containing smaller amounts of estrogens. In recent times AASs have also been proven to be involved in the development of hepatic adenoma. Apparently, androgen-induced HAs are relatively rare. However, the possibility that an oral AAS can induce liver cell proliferation must be taken into account and sportsmen taking AASs over a long period should be considered a group at risk for developing hepatic sex tumors. FNH is a benign lesion, most commonly seen in young women, which is thought to represent a local hyperplastic response of hepatocytes to a vascular abnormality. Because of the female predominance and the young age at onset, a role of female hormones has been suggested. Furthermore, a large proportion of women with FNH (50-75%) are OC users. Liver hemangiomas (LHs) are the most common benign liver tumors and are seen more commonly in young adult females. The female predilection and clinical observations of LH growth under conditions of estrogenic exposure suggest a possible role for estrogen in the pathogenesis of LHs. HA has been strongly associated with the use of OCs; in fact, it has been calculated that about 320 new cases are diagnosed each year, mostly attributable to OC use. Consequently, in contrast with what happens for LH and FNH, at least for HA there is an agreement among authors about the fact that the association between OCs and HA is strong and depends on the duration of use. Furthermore, unresected lesions may decrease in size in young women once they stop OC use. All these data taken together suggest that the association between HA and OC use is one of cause and effect. HCC has become one of the most widespread tumors in the world in recent years, representing the sixth leading cancer and the third most common cause of death from cancer. Apart from liver cirrhosis, numerous other factors responsible for its onset have been proposed: hepatitis infections from virus B (HBV) and C (HCV), alcohol, smoking, and aflatoxin. However, regardless of etiology, chronic liver diseases progress at unequal rates in the two sexes, with the major sequelae, such as cirrhosis and HCC, being more frequent in men than in women. These epidemiological data have prompted researchers to investigate the relationship between sex hormones and liver tumors. The human liver expresses estrogen and androgen receptors and experimentally both androgens and estrogens have been implicated in stimulating hepatocyte proliferation and may act as liver tumor inducers or promoters. As regards the role of estrogens in HCC, it seems that in the physiological status of premenopausal women, in the absence of other risk factors for liver disease, they have a somewhat protective role against the development of HCC [06074].
Athletes and nonathletes have been using anabolic-androgenic steroids (AAS) for a long time, in an inadequate and unsupervised manner, with the aim of improving sports performance or for cosmetic purposes. AAS consumption is becoming more widespread and involving younger people, and there is a trend for self-administration of higher doses and for combining AAS with other potentially harmful drugs. Almost any subject abusing AAS will experience adverse effects. Therefore, adverse effects from these exposures, including liver toxicity, are expected to increase in the years to come. It was described a representative case of intrahepatic cholestasis with the intention to discuss AAS-related liver toxicity (including the potential therapeutic role of ursodeoxycholic acid) and to comment on several aspects of the clinical scenario the gastroenterologist should be aware of [07061].

Cholestatic jaundice with intrahepatic cholestasis and variable degrees of hepatocellular necrosis on liver biopsy is the most commonly reported serious pathologic abnormality of the liver associated with AASs abuse. Rarely, case reports associate the presence of multiple, dilated liver cysts filled with blood in the liver (peliosis hepatitis) with chronic use of AASs. The pathogenesis of peliosis hepatitis is unknown. Other pathologic abnormalities of the liver detected in the autopsy of AAS abusers include focal nodular hyperplasia and adenomas. Rarely, the abuse of androgenic-anabolic steroids are associated with the development of blood-filled cysts (peliosis) involving the liver, spleen, bone marrow, lymph nodes, and lung. A 9-year-old bodybuilder was found dead at home; an autopsy demonstrated peliosis of the lung with the left pleural cavity filled with blood [13003].

**Metabolism of anabolic steroids in the liver**

Anabolic androgenic steroids are the xenobiotic substrates that are metabolized in the body by the protective enzyme systems. Mixed function oxygenase enzymes include a group of enzymes which play an essential role in the metabolism of a broad range of xenobiotics including endogenous and exogenous substrates. Cytochrome P-450, a member of mixed function oxygenase enzymes, plays an important role in oxidative metabolism of drugs and xenobiotics entering human body. Various anabolic steroids are found either to increase or decrease the activity of cytochrome P-450. However, effect of nandrolone decanoate, most commonly abused anabolic steroid, on cytochrome P-450 activity is still fragmentary. In one study, albino mice were administered intramuscular 2.5 mg of nandrolone decanoate injection at 15 days interval. Investigation shows a significant increase of cytochrome P-450 (nmol/mg) activity in liver tissue as compared to that of kidney tissues. A tissue specific and dose specific increase of cytochrome P-450 activity is observed. Mean cytochrome P-450 is found highest in liver tissue on 45th day whereas the activity in kidney tissue is noticed on 90th day of treatment. From the above observation, nandrolone decanoate can be suggested as a potent inducer of cytochrome P-450 activity like other anabolic steroids [09061].

**Enzyme elevations**

Various studies have demonstrated transient elevations of liver function tests (elevated plasma alkaline phosphates, aminotransferases, conjugated bilirubin, and plasma proteins) with and without significant hepatic injury. The orally 17-alpha alkylated steroids have a higher incidence of hepatotoxicity than other preparations. The mechanism of action is most likely from a direct toxic effect due to the brief period of time between exposure and liver damage and a dose-related effect. The most common used measures of hepatotoxicity are aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH). Values are usually in the range of two to three times normal. These changes often mimic the effects seen with oral contraceptives. Elevations in AST, ALT, and gamma-glutamyl-transferase (GGT) tend to peak within 2 to 3 weeks of consumption even at
relatively low doses, and will usually return to baseline within several weeks upon discontinuation. GGT was the most sensitive enzyme to detect hepatic dysfunction. However, physicians must be careful in evaluating serum elevations of these enzymes, because strenuous exercise alone can cause muscle breakdown, leading to transaminase elevations. In addition, with the exception of LDH, the enzymes can be found in other body tissues confounding the picture even more [06031].

**Cholestatic liver disease**

In the face of increasing societal pressure to achieve bodily perfection, young men in particular sometimes turn to anabolic steroids to help them achieve the body they want. The health consequences of this choice are often overlooked. It was described two cases of severe cholestatic liver disease in young men who had taken anabolic steroids with the aim of enhancing their body image. Both patients needed a prolonged stay in hospital for treatment resistant pruritus. The second case was associated with considerable psychological morbidity, so much so that the patient felt he had to leave school. The agent implicated in both the cases of severe cholestatic liver injury was methandrostenolone. This is a weak androgen receptor agonist and has long been recognised as a cause of liver damage. This fact seems to be well known among users of anabolic steroids as many internet steroid forums recommend taking the drugs for no more than four weeks to avoid going “yellow” [12025].

Many different formulations and types of anabolic steroids are available to users. However, it is the 17alpha alkylated steroids, such as methandrostenolone and methyltestosterone that have the most capacity to be hepatotoxic – 17alpha alkylation slows down metabolism of the steroids in the liver, thereby exposing hepatocytes and cholangiocytes to the drug for longer. Fewer of the injectable anabolic steroids are 17alpha alkylated, so use of oral anabolic steroids is more commonly associated with abnormal liver function. Anabolic steroids vary in their androgenic and anabolic properties, and body builders often use several steroids with the intent of producing differing results. Most of these drugs are sourced either illegally or via the internet. The actual anabolic steroids used, and the true dosage, are often unknown to the user. Anabolic steroids are freely available online and there seems to be no regulation of the quality or quantity of drugs dispensed in the various formulations. Several “dietary supplements” have been found to contain substantial amounts of anabolic steroids. A report from Portugal described a case in which cardiomyopathy induced by anabolic steroids had caused fulminant liver failure in a bodybuilder who took large doses of anabolic steroids. A further, Canadian case report has described the simultaneous occurrence of cholestatic jaundice, acute kidney injury, and acute pancreatitis [12025].

Steroids induce a wide range of hepatic disorders ranging from impaired excretion, cellular hepatocyte changes, cholestasis, peliosis hepatitis, and hepatocellular hyperplasia to carcinomas. Androgen-related cholestasis has been observed in varying frequency from a few cases to 17 percent in some studies. The cholestasis results from the reduced bile transport and disruption of intrahepatic microfilaments. This jaundice appears to be transient in nature and is secondary to biliary stasis in the biliary canaliculi without any structural hepatic injury. This is in contrast to the associated inflammation and necrosis seen with other forms of hepatitis. There may also be a relationship between cholestasis and hypercholesteremia [06031].

Cholestasis is common, but often is asymptomatic or associated with subclinical elevation of hepatic transaminases. Hepatocellular hyperplasia and elevations of transaminases, conjugated bilirubin, alkaline phosphatase, and lactate dehydrogenase occur. Early elevation
of transaminases without increasing gamma-glutamyl transpeptidase may represent muscle damage; serum creatine phosphokinase should be measured. Dose-dependent jaundice is common after several months of AAS use and usually resolves after discontinuing AAS use. Steroid “cycling” is believed to reduce cholestasis and jaundice. Benign hepatic adenomas and rarer hepatocellular carcinoma have been reported in association with AAS use. Regression of adenomas after avoidance of AAS has occurred; death from carcinoma also has occurred. Peliosis hepatis is the presence of blood-filled cavities in the liver; this has occurred with iatrogenic AAS use and with AAS abuse. Sometimes reversible, peliosis hepatitis can cause liver failure, and the rupture of these cysts can cause fatal internal hemorrhage [07058].

Though possession of androgenic anabolic steroids (AAS) is illegal, non-prescription use of AAS persists. It was described two Caucasian males (aged 25 and 45 years) with cholestatic hepatitis following ingestion of the dietary supplement Mass-Drol (“Celtic Dragon”) containing the AAS 2alpha-17alpha-dimethyl-etochohan-3-one,17beta-ol. Despite substantial hyperbilirubinaemia peak gamma-glutamyl transferase (GGT) remained normal. Besides bland intralobular cholestasis, liver biopsy in both found deficiency of canalicular expression of ectoenzymes as seen in ATP8B1 disease. In the older patient, bile salt export pump marking (encoded by ABCB11) was focally diminished. It was hypothesized that AAS had either induced inhibition of normal ATP8B1/ABCB11 expression or triggered initial episodes of benign recurrent intrahepatic cholestasis (BRIC) type 1/or 2. On sequencing, ATP8B1 was normal in both patients although the younger was heterozygous for the c.2093G>A mutation in ABCB11, a polymorphism previously encountered in drug-induced liver injury. Thus, AAS marketed as dietary supplements continue to cause hepatotoxicity in the UK; underlying mechanisms may include unmasking of genetic cholestatic syndromes [13140].

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**Combination herbal and designer drugs**

It was reported a case of cholestatic jaundice as a result of combination herbal and designer supplement use. A 50-year-old Hispanic male presented to the hospital with a 1-week history of significant painless jaundice; total bilirubin on admission was 29.4 mg/dL. He reported use of both herbal (creatine and whey protein) and designer (Incredible Bulk and Spartan 45) supplements concurrently for approximately 2 months. Upon admission, all supplements were discontinued and multiple laboratory and diagnostic tests were ordered. On day 6 of his hospital admission, a liver biopsy was performed, the results of which indicated drug-induced hepato toxicity. On day 9 he was discharged with prescriptions for ursodeoxycholic acid and hydroxyzine. Three months post hospital discharge, the patient continued to be supplement-free and bilirubin had decreased substantially. Anabolic-androgenic steroids are capable of causing hepatotoxicity, and multiple cases reported in the literature support this. A case report described hepatotoxicity secondary to both creatine and whey protein consumption, and several reports have described liver damage secondary to designer supplement use. To
our knowledge, this is the first case to describe hepatotoxicity as a result of combination herbal and designer supplement use. The Roussel Uclaf Causality Assessment Method (RUCAM) score for drug-induced hepatotoxicity indicated a highly probable correlation between the use of combination supplements and cholestatic jaundice [13141].

Non-alcoholic fatty liver disease (NAFLD)

Industrial toxin and drugs have been associated with non-alcoholic fatty liver disease (NAFLD); in these cases, the disease has been termed toxicant-associated steatohepatitis (TASH). One study hypothesized that the use of anabolic-androgenic steroids (AAS) could also be a risk factor to TASH or better toxicant-associated fatty liver disease (TAFLD) development. A case-control study including 180 non-competitive recreational male bodybuilders from 2007 to 2009 was performed. Ninety-five had a history of intramuscular AAS use (cases; G1) and 85 were non-users (controls; G2). They underwent a clinical evaluation and abdominal ultrasound, and their blood levels of aminotransferases, creatine phosphokinase (CPK), lipids, glucose and insulin were measured. TAFLD criteria: history of AAS use >2 years; presence of hepatic steatosis on ultrasound and/or aminotransferase alterations with normal CPK levels; exclusion of ethanol intake ≥20 g/day or use of other drugs; and exclusion of obesity, dyslipidaemia, diabetes and other liver diseases. Homeostasis model assessment for insulin resistance ≥3 was considered insulin resistant. All cases were asymptomatic. Clinical and laboratorial data were similar in G1 and G2. TAFLD criteria were observed in 13 percent of the G1 cases and 2 percent of controls had criteria compliant with non-alcoholic fatty liver related to metabolic conditions. Odds ratio was 6.0 (95 % confidence interval 1.3 to 27.6). These results suggest that AAS could be a possible new risk factor for TAFLD. In this type of fatty liver disease, the individuals had a low body fat mass and they did not present insulin resistance [11066].

Peliosis

Other rare hepatic lesions include some potentially life-threatening lesions. Peliosis hepatis is a blood-filled cyst seen in many case reports in patients taking oral androgens, and is often correlated with more prolonged use. In the majority of these cases, the lesions were identified incidentally, most on autopsy, and the patients were completely symptom free. Several case reports of patients show direct mortality from internal hemorrhage or hepatic failure secondary to these lesions. Both cholestasis and peliosis hepatis are believed to be explained by similar processes. Oral 17-alpha alkylated androgens produce hepatocyte hyperplasia, with enlarged hepatocytes occluding both hepatic venous return and sinusoids. Sinusoidal dilatation at the peripheral zone of the hepatic lobule is a common finding with anabolic steroid use [06031].

Hepatocellular adenoma and carcinoma

Medical literature documents isolated cases of androgen- associated hepatic tumors. Many of these reports were in patients with known hereditary anemias being treated with long-term androgens. Most of these tumors are benign adenomas, but early detection may lead to prevention of life-threatening complications associated with these tumors. Hepatic adenomas are usually found in young women taking oral contraceptives, and are still relatively uncommon. These adenomas are hypervascular tumors with relatively thin-walled capsules [. They rarely transform to malignant tumors, but have risks of sudden rupture and bleeding, leading to hemoperitoneum which is a life-threatening condition. A particular problem is differentiating adenomas from hepatocellular carcinomas by ultrasound. If the differentiation is not obvious by clinical and histologic findings, a surgical resection is recommended.
Treatment protocols for these lesions are difficult to formulate secondary to their varied prognosis, their difficult resection, and their unknown potential for malignancy. However, regression of the majority of these lesions after discontinuation of the steroids raises the question if there is truly malignant potential attributable to AAS use. Most of these lesions are linked to usage of the orally administered 17-alpha alkylated AAS. Sources recommend repeat ultrasound every 6 months with consideration of excision if late diagnosis is made. Nonsurgical options should be the optimal approach because many of the tumors will regress after discontinuation of the AAS, especially if detected early. Prompt detection of these lesions prevents important, potentially life-threatening sequelae and possible malignant deterioration [06031].

In a case report a 35-year-old male bodybuilder was found to have a hepatocellular carcinoma (HCC) arising in a pre-existing hepatic adenoma following recreational anabolic steroid use. Malignant transformation to HCC from a pre-existing hepatic adenoma confirmed by immunohistochemical study has previously not been reported in athletes taking anabolic steroids [08146].

**Hepatocellular necrosis.**

Case reports associate AAS abuse with hepatic enlargement, peliosis hepatitis, serious cholestatic jaundice [2,17-dimethylidihydrotestosterone(methasterone)], and hepatorenal failure, particularly following the use of oral 17alpha-alkylated AAS (e.g. methandrostenolone, methyltestosterone, oxymetholone, oxandrolone, and stanozolol). Typically, non-17-alkylated anabolic steroids are not associated with hepatocellular damage. Jaundice typically resolves several months after cessation of AAS use, and liver transplantation is not usually necessary. Case reports also associate the use of high doses of AAS with the development of hepatocellular necrosis. A 26-year old male bodybuilder reportedly used testosterone enanthate (500 mg intramuscularly twice weekly), stanozolol (40 mg orally/day), and methylandrostenediol (30 mg/day orally for 5 weeks). He subsequently developed evidence of severe hepatitis with hepatic dysfunction that required a prolonged hospitalization. Peliosis hepatitis is a rare illness characterized by blood-filled spaces within the hepatic parenchyma that typically occurs in association with a variety of chronic wasting diseases (e.g. tuberculosis and malignancy). This condition has been associated with both the chronic use of pharmacologic doses of AAS and the chronic, intermittent abuse of AAS by bodybuilders. The appearance of peliosis hepatitis is not related to the dose of AAS or duration of use. Some athletes using AAS take polyunsaturated phospholipids and vitamin complex to reduce the elevation of hepatic aminotransferases often associated with the use of AAS; however, there are inadequate data to determine the efficacy of this combination supplement [13003].

**Spontaneous hepatic rupture**

Anabolic androgenic steroids are commonly used at high doses by bodybuilders and athletes to enhance physique and improve performance levels. It was reported a case of spontaneous hepatic rupture with life-threatening haemorrhage associated with a past history of anabolic steroid use [07062].

**Anabolic steroids and reproductive system and male infertility**

There is no doubt that lifestyle factors can be detrimental to fertility. The aim of one pilot study was to identify initial prevalence rates for behaviour-related fertility disorders in a clinical sample of couples wanting a child. Between February and August 2010, all patients
coming for the first time to Heidelberg University's Women's Hospital for consultation on involuntary childlessness were asked to fill out a questionnaire designed by the authors of this article. The questionnaire was based on a review of the relevant literature, with special reference to the latest research findings on behaviour detrimental to fertility. Of the 156 couples addressed, 110 women and 100 men took part in the study. For behaviour-related infertility, 9 percent of the women and 3 percent of the men in our sample were classified on the basis of BMI <18.5, sexual disorders, or abuse of anabolic steroids. If it was included smokers, these figures increase: 11 percent female smokers and 18 percent male smokers. A further 19 percent of the women practised sport to an excessive degree; and 26 percent of the women and 53 percent of the men had a BMI ≥25. The prevalence of behaviour-related fertility disorders should not be underestimated. For the prevention of behaviour-related fertility disorders, it is important to inform the population about lifestyle-mediated fertility risks [12144].

One-third of infertile couples may have a male factor present. Illicit drug use can be an important cause of male factor infertility and includes use of anabolic-androgenic steroids, marijuana, opioid narcotics, cocaine, and methamphetamine. The use of these illicit drugs is common in the United States, with a yearly prevalence rate for any drug consistently higher in males compared with females. The aim of one study was to provide a review of recent literature on the prevalence and effects of illicit drug use on male fertility and to aid health professionals when counseling infertile men whose social history suggests illicit drug use. Anabolic-androgenic steroids, marijuana, cocaine, methamphetamine, and opioid narcotics all negatively impact male fertility, and adverse effects have been reported on the hypothalamic-pituitary-testicular axis, sperm function, and testicular structure. The use of illicit drugs is prevalent in our society and likely adversely impacting the fertility of men who abuse drugs [12145].

AAS abuse inhibits gonadotropins secretion, endogenous testosterone production and spermatogenesis. AAS-induced sperm alterations include oligozoospermia, azoospermia, decreased sperm motility, and abnormal sperm morphology. These often result in decreased fertility in males. After AAS withdrawal the inhibited HPT axis functions will restore, but not always, within several months. In bodybuilders with a history of long-term AAS abuse, at least 6/12 months are needed for a full recovery of testicular functions. Clomiphene may successfully restore AAS-induced male HPG dysfunction and gonadotropins have been used to recover from AAS-linked azoospermia [12094].

The negative impact of AAS abuse on male fertility is well known by urologists. The secondary hypogonadotrophic hypogonadism is often highlighted when AAS and fertility are being discussed. On the other hand, the patterns of use, mechanisms of action and direct effects over the testicle are usually overseen. One study reviews the vast formal and "underground" culture of AAS, as well as their overall implications. Specific considerations about their impact on the male reproductive system are made, with special attention to the recent data on direct damage to the testicle. This kind of overview is absolutely unique, offering a distinguished set of information to the day-by-day urologists. For several decades, testosterone and its synthetic derivatives have been used with anabolic and androgenic purposes. Initially, these substances were restricted to professional bodybuilders, becoming gradually more popular among recreational power athletes. Currently, as many as 3 million anabolic-androgenic steroids (AAS) users have been reported in the United States, and considering its increasing prevalence, it has become an issue of major concern. Infertility is defined as the failure to achieve a successful pregnancy after 12 months or more of regular unprotected intercourse, with male factor being present in up to 50 percent of all infertile couples. Several conditions may be related to male infertility. Substance abuse, including AAS, is commonly associated to transient or persistent impairment on male reproductive
function, through different pathways. Herein, a brief overview on AAS, specially oriented to urologists, is offered. Steroids biochemistry, patterns of use, physiological and clinical issues are enlightened. A further review about fertility outcomes among male AAS abusers was also presented, including the classic reports on transient axial inhibition, and the more recent experimental reports on structural and genetic sperm damage [11571].

Infertility is generally defined as the inability to conceive a pregnancy or the failure to do so within a reasonable period (typically 12 months). Approximately 85 percent of couples conceive a first pregnancy within 12 months. The prevalence of infertility has increased over the past 10 years, with approximately 10 million affected couples in the United States. Roughly 40-50 percent of infertility is either due to, or is contributed by, a male factor. Given how common the condition is, men and their partners are understandably concerned and interested in identifying and eliminating risk factors for male infertility. One article reviewed the available literature on various aspects of male infertility related to athletic pursuits. These include the effects of exercise on semen parameters, hormonal axes, and testicular health. Due to the prevalence and particular relevance of anabolic steroid use by athletes and the impact of steroid use on fertility, this topic was also reviewed [10448].

In a case report of primary gonadal failure due to the chronic abuse of anabolic steroids used for bodybuilding the clinical symptoms, levels of serum T, FSH, and LH were given. It was a case of initially secondary gonadal failure resulting from anabolic steroid use with subsequent primary gonadal failure and infertility. It adds to the current literature and illustrates that the side effects of anabolic steroids can be prolonged and irreversible [11324].

Long-term side effects of high doses of anabolic androgenic steroids self-administration were evaluated in this study. Twenty male bodybuilders, voluntarily starting steroid self-administration, were followed every 6 months over 2 years. Physical examination, haematological, metabolic and endocrine variables, semen analysis, hepatic and prostate ultrasound and echocardiographic evaluations were performed. LH values were suppressed at 18 and 24 months and FSH at 6, 12, 18, and 24 months and SHBG values significantly lowered at 12, 18, and 24 months. A significant decrease in spermatozoa count and fertility index occurred. HDL-cholesterol was reduced at 18 and 24 months and Apo A-1 at 12, 18, and 24 months. The most important long-term adverse effects were lower fertility and the impairment of lipid profile associated with an increased cardiovascular risk [07071].

Although the same androgen receptor mediates both the anabolic and androgenic effects of steroids, the specific structural characteristics of individual AASs determine the balance between the anabolic and androgenic effects. Masculinization results from the presence of high doses of AASs in women. The aromatase cytochrome P450 (CYP19) enzyme complex converts testosterone to estradiol, which binds to the estrogen receptor. Although this conversion usually accounts for a small percentage of testosterone biotransformation at physiologic doses, excess amounts of testosterone increase the formation of estradiol. Gynecomastia is a consequence of excessive estradiol concentrations in chronic male AAS abusers that result from the peripheral conversion of excess testosterone to estradiol. Drug used by male AAS abusers to prevent the feminizing effects of estradiol include the use of aromatase inhibitors (e.g. anastrozole, and aminoglutethimide) and drugs that block estrogen receptor (clomiphene and tamoxifen) [13003].

All AASs suppress gonadotropin secretion and therefore suppress endogenous testicular function. Adverse effects of AASs on the male reproductive system include reduced hormone levels (gonadotrophic hormones, endogenous testosterone, and sex-hormone-binding globulin), impotence (erectile dysfunction), alterations in sperm morphology, and reductions in sperm count, sperm motility, and the size of the testicles. These changes are not
responsive to the administration of human chorionic gonadotropin, and resolution of the alterations in sperm count occurs several months after cessation of AAS use with some individuals requiring up to 30 weeks of abstinence. Although chronic AAS abuse produces hypogonadism, decreased serum testosterone, and impaired spermatogenesis, these changes are reversible within a few months to 1 year following cessation of AAS use. Abnormalities of the female reproductive system following chronic AAS abuse include menstrual irregularity, deep voice, increased libido, and increased clitoris size along with elevated sex-hormone-binding globulin. Anabolic-androgenic steroids are contraindicated in pregnant women with potential effects including masculinization of the female fetus, clitoral hypertrophy of the female fetus, decreased birth weight, and premature bone maturation [13003].

**Physiology of reproduction-endocrinology**

Exogenous steroids exert their affect on adrenal hormones by negative feedback on the hypothalamic-pituitary-gonadal axis. Large doses of exogenous AAS lead to a decrease in both follicle-stimulating hormone and luteinizing hormone serum concentrations. There is some controversial evidence to show that high doses of exogenous steroids lead to spontaneous increases in growth hormone (GH) production. In one study of men with hypogonadotropic hypogonadism, testosterone enanthate administration resulted in marked increases in spontaneous GH secretion. This elevation of GH provides the negative feedback that leads to decreased endogenous testosterone and gonadotropin release. Suppression of gonadotropin release leads to oligospermia and hypogonadism. This is reflected by decreased levels of testicular precursors of testosterone, namely serum pregnenolone, progesterone, 17-hydroxyprogesterone, and 17-hydroxypregnenolone. Supraphysiologic doses of AAS may lead to infertility due to decrease in both quantity and quality of sperm after several months of use. The return of normal levels of FSH and LH concentrations after discontinuation of AAS can take 6 to 12 weeks, whereas normalization of endogenous serum testosterone levels requires several additional weeks even after the return of normal gonadotropin levels. The amount of time needed for full recovery of normal reproductive function varies both on dose and duration of steroid use and can range from 4 to 5 months to several years. Many athletes turn to human chorionic gonadotrophin or clomiphene to reverse or even maintain spermatogenesis following or during courses of AAS [06031].

Since its isolation and characterization in 1935, there have been further studies on testosterone which have led to the synthesis of numerous derivatives with properties different from the original molecule. It is in 2011 estimated that there are as many as three million AAS users in the USA. Interestingly, two thirds of US users are non-competitive bodybuilders, or even non-athletes, who use these substances for aesthetic purposes only. The negative impact of AAS abuse on male fertility is well known by urologists. Infertility is defined as the failure to achieve a successful pregnancy after 12 months or more of regular unprotected intercourse, and male-factor infertility is the cause in about 50 percent of all infertile couples. Several conditions may explain male-factor infertility. Some are identifiable, but not reversible; others may be identified and also reversed. Hypogonadotrophic hypogonadism is a typical example of a reversible condition, whereas primary testicular impairment is often related to a less reversible one. The secondary hypogonadotropic hypogonadism is often highlighted when AAS and fertility are being discussed. On the other hand, the patterns of use, mechanisms of action and direct effects over the testicle are usually overseen. One review studied the vast formal and "underground" culture of AAS, as well as their overall implications. Specific considerations about their impact on the male reproductive system are made, with special attention to the recent data on direct damage to the testicle. For several decades, testosterone and its synthetic derivatives have been used with anabolic and androgenic purposes. Initially, these substances were restricted to
professional bodybuilders, becoming gradually more popular among recreational power athletes. Currently, as many as 3 million anabolic-androgenic steroids (AAS) users have been reported in the United States, and considering its increasing prevalence, it has become an issue of major concern. Infertility is defined as the failure to achieve a successful pregnancy after 12 months or more of regular unprotected intercourse, with male factor being present in up to 50 percent of all infertile couples. Several conditions may be related to male infertility. Substance abuse, including AAS, is commonly associated with transient or persistent impairment on male reproductive function, through different pathways. Infertility after AAS abuse commonly presents as oligozoospermia or azoospermia, associated with abnormalities in sperm motility and morphology. According to most reports, sperm quality tends to recover spontaneously within 4-12 months after discontinuation. However, the negative effect on semen quality may persist for longer periods. A hypogonadotrophic hypogonadism state is induced, characterized by decreased serum testosterone concentrations, testicular atrophy and impaired spermatogenesis. These effects result from the negative feedback of androgens on the hypothalamic-pituitary axis, and possibly from local suppressive effects of exogenous androgens on the testes. FSH and luteinizing hormone (LH) concentrations are typically low. In addition, during AAS use, serum androgen concentrations may be supraphysiological high, but the hypogonadotrophic state lowers the intratesticular testosterone concentrations required to maintain normal spermatogenesis. The management of AAS-induced male infertility has also been extensively reported. Simple discontinuation of AAS use may lead to fertility recovery in a certain proportion of male users, but there is little literature and considerable disagreement regarding the management of such cases. Patients may also be actively treated, in a manner similar to that used for other forms of hypogonadotrophic hypogonadism infertility, requiring the induction of spermatogenesis with gonadotropins or gonadotropin analogues, including i.m. injections of hCG, human menopausal gonadotropin (hMG) or even recombinant FSH. The use of hCG alone, or in combination with hMG, has been reported to be a successful treatment for this group of patients. Fertility restoration has been reported, even in situations of persistent azoospermia up to 5 years after AAS discontinuation so AAS-associated male infertility may be treatable because of its endocrine nature. Considering the prevalence of AAS abuse and the favourable results after treatment, it is reasonable to consider it during the infertility consultation [11028].

In men, chronic AAS use can lead to decreased endogenous testosterone production and hypogonadotrophic hypogonadism associated with testicular atrophy. Chronic AAS abuse causes a decrease in gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) as part of the negative feedback system of the hypothalamic-pituitary-gonadal axis. LH and FSH are needed for spermatogenesis so when these hormones are decreased, there is a decrease in sperm count and mobility as well as an increase in the number of morphologically abnormal sperm. One study found a 73 percent overall decrease in sperm count; three individuals had azoospermia with chronic use of high-dose AASs. In individuals who did not experience azoospermia, there was a 10 percent increase in the number of immotile sperm and a 30 percent decrease in the number of motile sperm. Overall, fertility was severely reduced. Decreases in gonadotropins can be seen within 24 hours of beginning AASs. Infertility may result within months. After cessation of use, gonadotropin and testosterone secretion are suppressed for months to years. Usually, the infertility is spontaneously reversible, typically within 1 year of cessation of AAS abuse, but it may take longer in long-term users. At least one user of multiple AASs did not recover fertility spontaneously and required treatment with LH-releasing hormone to regain normal levels of testosterone and fertility. Men also may experience priapism, impotence, prostatic hypertrophy, difficulty/pain with urination, and a possible increased risk for prostate cancer. The risk for these consequences increases with dose and duration of use [07058].
Exogenous steroid administration thus provides feedback inhibition of luteinizing and follicle-stimulating hormones, which leads to testicular atrophy and decreased spermatogenesis. This testicular impairment is reversed upon cessation of AAS use. Excess steroids undergo peripheral aromatization to estrogens, which results in feminizing changes of high voice pitch and male gynecomastia. In long-term AAS abuse, this gynecomastia is irreversible, leaving surgical correction as the only solution. In addition to the female side effects of decreased menstruation and breast tissue atrophy, virilizing effects also occur and include deepened voice, clitoromegaly, and hirsutism. Sometimes these effects are irreversible, even after discontinuation of AAS use [07008].

**Anabolic steroid-induced hypogonadism in young men**

Use of anabolic androgenic steroids (AAS) has not been traditionally discussed in mainstream medicine. With the increased diagnosis of hypogonadism, a very heterogeneous population of men is now being evaluated. Within this larger population of patients, the existence of anabolic steroid-induced hypogonadism (ASIH), whether transient or permanent, should now be considered. An initial retrospective database analysis of all patients (2005-2010, n=6033) seeking treatment for hypogonadism was conducted. Subsequently, an anonymous survey was distributed in 2012 to established patients undergoing testosterone replacement therapy (TRT). Profound hypogonadism, defined as a testosterone ≤50 ng/dL, was identified in 1.6 percent (n=97) of the large retrospective cohort initially reviewed. The most common etiology was prior AAS exposure, identified in 43 percent (42/97) of men. Because of this surprising data, a follow-up anonymous survey of our current hypogonadal patient population (n=382; mean age 49 ± 13 years) was then performed which identified 21 percent of patients (n=80; mean age 40 ± 8 years) with prior AAS exposure. Hypogonadal men <50 years old were greater than 10 times more likely to have prior AAS exposure than men >50 (OR 10). Prior AAS use was significantly negatively correlated with education level and number of children. Prior AAS use is common in young men seeking treatment for symptomatic hypogonadism, and ASIH is the most common etiology of profound hypogonadism. These findings suggest a necessary refocused approach in the evaluation and treatment paradigms of young hypogonadal men [13152].

The use of anabolic androgenic steroids has not been traditionally discussed in mainstream medicine. With the increased diagnosis of hypogonadism a heterogeneous population of men is now being evaluated. In this larger patient population the existence of anabolic steroid induced hypogonadism, whether transient or permanent, should now be considered. It was performed an initial retrospective database analysis of all 6,033 patients who sought treatment for hypogonadism from 2005 to 2010. An anonymous survey was subsequently distributed in 2012 to established patients undergoing testosterone replacement therapy. Profound hypogonadism, defined as testosterone 50 ng/dL or less, was identified in 97 men (1.6 %) in the large retrospective cohort initially reviewed. The most common etiology was prior anabolic androgenic steroid exposure, which was identified in 42 men (43 %). Because of this surprising data, it was performed an anonymous follow-up survey of our current hypogonadal population of 382 men with a mean ± SD age of 49 ± 13 years. This identified 80 patients (21 %) with a mean age of 40 ± 8 years who had prior anabolic androgenic steroid exposure. Hypogonadal men younger than 50 years were greater than 10 times more likely to have prior anabolic androgenic steroid exposure than men older than 50 years (OR 10). Prior anabolic androgenic steroid use significantly correlated negatively with education level and number of children. It was concluded that prior anabolic androgenic steroid use is common in young men who seek treatment for symptomatic hypogonadism and anabolic steroid induced hypogonadism is the most common etiology of profound hypogonadism. These findings suggest that it is necessary to refocus the approach to evaluation and treatment paradigms in young hypogonadal men [13153].


**Azospermi**

Azoospermia may sometimes be related to the use of androgenic anabolic steroids. It was reported a case of an azoospermic man who had abused androgenic anabolic steroids and who recovered spermatogenesis six months after cessation of abuse and the administration of hormonal therapy. An azoospermic 34-year-old man came to Regional Referral Center for Male Infertility. The recovery of spermatogenesis was observed after the cessation of abuse of steroids and the administration of hormonal therapy. Ultrastructural analysis of sperm was carried out by transmission electron microscopy, and the meiotic segregation of chromosomes 1, 9, 18, X, Y was investigated. Mathematically elaborated transmission electron microscopy data highlighted seminal features close to normal fertility. Fluorescence in situ hybridisation showed a high frequency of XY disomy in sperm. The findings confirm the recovery of spermatogenesis but suggest a possible relationship between altered meiotic segregation and the abuse of androgenic anabolic steroids [07072].

**Spermatogenesis**

It was reviewed the current literature for the effect of hormones used in rejuvenation clinics on the maintenance of spermatogenesis. Exogenous testosterone and anabolic androgenic steroids suppress intratesticular testosterone production, which may lead to azoospermia or severe oligozoospermia. Therapies that protect spermatogenesis involve human chorionic gonadotropin (hCG) therapy and selective estrogen receptor modulators (SERMs). The studies examining the effect of human growth hormone (HGH) on infertile men are uncontrolled and unconvincing, but they do not appear to negatively impact spermatogenesis. At present, routine use of aromatase inhibitors is not recommended based on a lack of long-term data. The use of hormones for rejuvenation is increasing with the aging of the Baby Boomer population. Men desiring children at a later age may be unaware of the side-effect profile of hormones used at rejuvenation centers. Testosterone and anabolic androgenic steroids have well-established detrimental effects on spermatogenesis, but recovery may be possible with cessation. Clomiphene citrate, human growth hormone (HGH)/insulin-like growth factor-1 (IGF-1), human chorionic gonadotropin (hCG), and aromatase inhibitors do not appear to have significant negative effects on sperm production, but quality data are lacking [13151].

**Histopathology**

Experiments in animal models mainly report AAS-induced Leydig cell alterations, but cellular morphology anomalies have also been reported. The decrease in this population of cells is accompanied by low testosterone and LH levels in all papers reviewed, especially in those papers reporting on adult animal models. Immunohistochemical findings have suggested decreased steroidogenesis in testicular tissue, hence spermatogenesis was considered unchanged by some other authors. Nethertheless, specific end-stage spermatogenesis impairment, with a lack of advanced forms of spermatids, has been described. After AAS discontinuation, Leydig cells tend to proliferate but remain below the regular counts, even after longer periods. Clearly, long-lasting, or possibly persistent effects of AAS use cannot be ruled out [11028].

**Impact on semen quality**

The use of a combination of hCG and steroids is a common practice among AAS users. The goal is to avoid the impact of LH suppression after long-term AAS administration, which may lead to a persistent state of hypogonadism and low-quality semen. Restoration of
Spermatogenesis has been described; however, more abnormal and hypokinetic spermatozoa are found, even after hCG “post-cycle” use, showing a potential for persistent alterations after the discontinuation of AAS use [11028].

Aneuploidies and ultrastructural changes in spermatozoa

The innovative use of both transmission electron microscopy and fluorescence in situ hybridization (FISH) has recently been reported in an AAS user sperm sample, searching for genetic and ultrastructural consequences of steroid abuse. Immaturity, necrosis and apoptosis were assessed, and a high percentage of structurally normal spermatozoa were found, which showed the absence of a correlation between AAS and ultrastructural sperm changes. In contrast to these findings, FISH sperm analysis revealed XY and chromosomes 1 and 9 disomies, suggesting anomalies in the meiotic process and genetic damage among AAS users [01128].

Case report

A 32-year-old man complained about a reduction of testicular volume and loss of libido. He had been abusing androgenic anabolic steroids for 7 years. Genital examination revealed that both testicular volumes were reduced to 13 ml. Endocrinological investigations showed luteinizing hormone, follicle-stimulating hormone and total testosterone levels to be low. The level of free testosterone was documented to be high. Later, sex hormone-binding globulin (SHBG) and calculated bioavailable testosterone (cBAT) levels were found to be low. Based on these features, it was diagnosed hypogonadotrophic hypogonadism caused by AAS abuse. Treatment with injections of human chorionic gonadotropin (hCG) was started. About one month after treatment with hCG started, his symptoms and endocrinological features were not improved. It is also reported that normal hormonal function usually recovers after AAS are discontinued, but sometimes the condition is not reversible [08144].

Reproductive-endocrine effects in women

The sexual and reproductive effects of AAS are more dramatic in women. Although AASs have been developed to try to minimize androgen effects, all AASs exert some degree of virilizing effects if given for long enough and in sufficiently large doses. Virilization occurs with AAS use by women, regardless of the type used. Early effects include acne, deepening of the voice, and changes in libido. Deepening of the voice occurs as a result of laryngeal hypertrophy. Long-term use can lead to clitoral enlargement, male-pattern baldness, and alterations in pubic hair. Other virilizing effects include decreased body fat, breast atrophy, amenorrhea or oligomenorrhea, uterine atrophy, and hirsutism. The changes in menses are due to suppression of the hypothalamic-pituitary-gonadal axis. Some of these effects may be irreversible with chronic use. AASs may act as a teratogen [07058].

Skin

Cutaneous manifestations develop early in the use of anabolic-androgenic steroids, placing dermatologists in a unique position to make an early diagnosis of AAS abuse in patients who engage in competitive sports. One review of the literature focuses on dermatologic presentations of anabolic-androgenic steroids use [09055].

The most common dermatologic effects of AAS abuse are alopecia, male pattern baldness,
and hirsutism, particularly in women. Other adverse dermatologic effects include keloid formation, sebaceous cysts, comedones, seborrheic furunculosis, folliculitis, striae, and acne. A common triad of chronic AAS abuse is the combination of acne, striae, and gynecomastia. Hyperplasia of the pilosebaceous glands and increased sebum production cause a high incidence of acne in chronic AAS abusers. These effects become prominent about 1 month after initiation of AAS abuse, depending on the dose and frequency of use [13003].

**Acne**

A 22-year-old male amateur bodybuilder presented with a 3-month history of severe acne lesions on his upper trunk and face, accompanied by arthralgia of several joints. He reported the use of anabolic androgenic steroids (AAS) (testosterone enanthate, trenbolone acetate, drostanolone propionate, and methandrostenolone) for 3 months to increase his muscle mass. Shortly after he discontinued AAS intake, he developed severe inflammatory acne with painful rupturing and draining inflammatory nodules, pustules, and hemorrhagic ulcerations on his upper trunk and face. Moreover, he described an immobilizing arthralgia of his right ankle and both shoulder joints, as well as general symptoms including fatigue and a 15-kg weight loss over the 6 weeks prior to presentation. As derivatives of the hormone testosterone, AAS lead to hypertrophy of the sebaceous glands, increased sebum production, and increased density of the *Propionibacterium acnes* population. The patient developed AAS-induced acne fulminans with the typical unresponsiveness to systemic antibiotics. After initial therapy with oral prednisolone, 0.5 mg/kg, and debridements, a clinical response was achieved with isotretinoin, 0.75 mg/kg. In conclusion, it is important for health care providers to keep in mind that androgen-induced acne is one of the most frequent symptoms of AAS abuse. The most important measure is the immediate termination of AAS administration [12141].

Severe cases of acne, especially on the face and back of AAS users, are common dermatologic findings. Premature baldness is noted as well. It has been reported multiple cases of serious muscular abscesses resulting from the common practice of shared needles and shared steroid vials among adolescent AAS users. A limited knowledge of sterile injection technique, as well as limited access to sterile needles and syringes are likely additional causative factors in these infections [07008].

Abuse of anabolic-androgenic steroids (AAS) by members of fitness centers and others in Germany has reached alarming dimensions. Besides health-threatening cardiovascular, hepatotoxic and psychiatric long-term side effects of AAS, acne occurs in about 50 percent of AAS abusers and is an important clinical indicator of AAS abuse, especially in young men 18-26 years of age. Both acne conglobata and acne fulminans can be induced by AAS abuse. The dermatologist should recognize bodybuilding acne, address the AAS abuse, and warn the patient about other potential hazards [07074].

Men experience male-pattern baldness, acne (mostly on the trunk), which likely is due to the effects of DHT. Acne is the result of androgenic stimulation of sebaceous glands [07058].

**Hirsutism**

Hirsutism is a symptom or sign, which may have more serious associations than cosmetic and psychological concern alone, such as adrenal hyperplasia and ovarian tumor, particularly if it develops well after puberty. Some medicines having androgenic activity may also cause this problem. It was presented a case of a young unmarried girl who was given anabolic steroid for the treatment of dysmenorrhoea which resulted in hirsutism [06092].
Androgenic alopecia (baldness)

Adolescent androgenic alopecia is pattern hair loss occurring in boys and girls younger than 18 years, whereas early-onset androgenic alopecia refers to pattern hair loss before 35 years of age. A number of studies published in the last decade have helped to elucidate the prevalence of adolescent androgenic alopecia, have clarified the genetic as well as physiologic mechanisms underlying hair loss, and have revealed the associated psychologic and systemic morbidities. One article provides an overview of the pathophysiology, diagnosis, and treatment of adolescent androgenic alopecia [11448].

Effects on the immune system

Anabolic-androgenic steroids are potentially immunosuppressive based on a study suggesting that chronic AAS abuse reduces immunoglobulins (i.e. IgA), increases natural killer cell activity, and increases mitogen response to staphylococca lantigens. There were no significant differences in T-cell subsets among steroid users and non-users in this study. However, the clinical relevance of these potential immunosuppressive effects is unclear. Sporadic case reports associated infectious complications from the parenteral use of AASs including local abscess at the site of injection, septic arthritis, HIV, and hepatitis, usually as a result of sharing contaminated needles [13003].

Infection risks after injections of anabolic steroids

To describe drug use, sexual risks and the prevalence of blood-borne viral infections among men who inject image and performance enhancing drugs (IPEDs), 19 needle and syringe programmes with 395 men who had injected PEDs were investigated Of the participants (median age 28 years), 36 percent had used IPEDs for <5 years. Anabolic steroids (86 %), growth hormone (32 %) and human chorionic gonadotropin (16 %) were most frequently injected, with 88 percent injecting intramuscularly and 39 percent subcutaneously. Two-thirds also used IPEDs orally. Recent psychoactive drug use was common (46 % cocaine, 12 % amphetamine), 5 percent had ever injected a psychoactive drug and 9 percent had shared injecting equipment. Viagra/Cialis was used by 7 percent, with 89 percent reporting anal/vaginal sex in the preceding year (20 % had 5+ female-partners, 3 % male-partners) and 13 percent always using condoms. Overall, 1.5 percent had HIV, 9 percent had antibodies to the hepatitis B core antigen (anti-HBc) and 5 percent to hepatitis C (anti-HCV). In multivariate analysis, having HIV was associated with: seeking advice from a sexual health clinic; having had an injection site abscess/wound; and having male partners. After excluding those reporting male partners or injecting psychoactive drugs, 0.8 percent had HIV, 8 percent anti-HBc and 5 percent anti-HCV. Only 23 percent reported uptake of the hepatitis B vaccine, and diagnostic testing uptake was poor (31 % for HIV, 22 % for hepatitis C) [13026].

Despite intensive information on possible side effects and complications of performance-enhancing substances in sports, the use of AAS (anabolic androgenic steroids) is far common. Particularly in sports like bodybuilding or weight lifting AAS are used for setting up muscle mass and increasing muscle power. It was presented a case of a 27 year old bodybuilder, who was transferred due to suspected malignant expansion of the upper limb to a department of orthopaedic surgery, not knowing that the patient had injected AAS. At biopsy the tumor was found to be an abscess formation, that had to be treated surgically with curettage. The microbiological analysis detected an infection with Pseudomonas fluorescens
and Erwinia species. Erwinia species are associated with plants. Pseudomonas fluorescens is found in feces, sewage and soil. It is obvious, that the infection is caused by an inappropriate injection of AAS or by the contamination of the injected substances [07077].

Detailed data on performance- and image-enhancing drug injections are difficult to obtain because of the illicit and unsupervised way in which many these drugs are used, and the hidden nature of the group. One study examined the patterns of use, risk behaviours and related harm associated with injections. Data were obtained via a structured questionnaire administered in face-to-face interviews with 60 men who used performance- and image-enhancing drugs (primarily anabolic androgenic steroids) for non-medical purposes. Although the rates of needle sharing were low (5 %), the men more frequently reported re-use of needles/equipment, injecting from a shared container (bladders, vials, etc.), injecting other illicit drugs, injecting insulin and targeting small muscle groups. Self-reports of being hepatitis C antibody positive were associated with lifetime use of heroin and injection of other illicit drugs. All HIV positive participants were gay or bisexual men. Participants reported a range of other injection-related injuries and diseases such as fevers, scarring and abscesses. “Risky injectors” (38 % of participants) were more likely to initiate performance- and image-enhancing drugs use at a younger age, use these drugs in a larger number of cycles per year and report involvement in a violent/aggressive incident than “low risk” injectors and report involvement in a violent/aggressive incident than “low risk” injectors. Participants mainly reported seeking information about performance- and image-enhancing drugs from internet sites (62 %) and friends (55 %). It was concluded that over-reliance on personal networks and internet forums limits this groups' access to objective harm reduction advice and primary care services [08148].

Injection policies

High-performance sporting organizations should have strict policies in place around injection therapy. Injection therapy in high-performance sport should be limited to the treatment of illness or injury by the medical practitioner. There is no role for injection therapy as part of a supplementation programme. If routine injection therapy is taking place as part of a supplementation programme, one of the two things occurs:

1. The person doing the injecting is misleading the athlete and the sporting organisation by injecting substances such as vitamins or other substances, for which there is no scientific basis in the preparation of elite athletes.
2. The person doing the injecting is committing a doping violation.

No member of the support staff should be permitted to administer injections apart from the medical practitioner. No one in a sporting organisation should be permitted to possess injection equipment (syringes and hypodermic needles) other than the team doctor, the only exception being those athletes who have certified medical conditions such as diabetes, anaphylactoid reactions, etc [13027].

Gynecomastia

Drugs are estimated to cause about 10-25 percent of all cases of gynecomastia. Over the course of several decades, multiple medications have been implicated in the development of gynecomastia mostly in the form of case reports and case series. However, these reports suffer from a multitude of deficiencies, including poor quality of evidence. Studies were selected for this review by performing an extensive electronic and hand-search using

618
BIOSIS, EMBASE and Medline, from 1940 to present, for all reported drug associations of gynecomastia and their possible pathophysiology. Quality of evidence was assessed on a three-point scale: good, fair and poor, and each of the drugs reported to cause gynecomastia was assigned a level of strength. The pathophysiology of gynecomastia is also discussed in detail for each of the drugs found to have a good or fair evidence of association with gynecomastia. Most of the reported drug-gynecomastia associations were based on poor quality evidence. The drugs definitively associated with the onset of gynecomastia are spironolactone, cimetidine, ketoconazole, hGH, estrogens, hCG, anti-androgens, GnRH analogs and 5-α reductase inhibitors. Medications probably associated with gynecomastia include risperidone, verapamil, nifedipine, omeprazole, alkylating agents, HIV medications (Efavirenz), anabolic steroids, alcohol and opioids [12143].

Testosterone is broken down to form estradiol in the peripheral tissues of the body. In supratherapeutic doses of testosterone and its metabolites, the peripheral aromatization will lead to a major increase in estradiol. Serum levels of estradiol are often seven times the levels prior to onset of AAS use and approach medium levels of hormones found in normal women. In male athletes these levels of estradiol will often lead to gynecomastia and a heightened voice. Gynecomastia may cause breast pain and undesirable cosmetic affects for athletes. Some athletes even resort to taking estrogen-blocking medications such as tamoxifen to prevent these effects, although no scientific data support this practice. In most cases, gynecomastia will remit upon discontinuation of the steroids. However, in more prolonged use of AAS, gynecomastia can be permanent and thus require surgical resection for cosmesis [06031].

Testosterone is converted to estrogens by aromatase and to DHT by way of 5-alpha reductase. The estrogens lead to feminizing effects in men, such as gynecomastia and an increase in voice pitch. Although the breast tissue that develops becomes softer and less prominent after cessation of AAS use, this effect may be irreversible and require surgical correction [07058].

Gynecomastia in males is a common adverse effect of chronic AAS abuse. These effects are not readily reversible, particularly in adolescents. Adverse effects in women following chronic AAS use include masculinization (male pattern baldness and hirsutism), acne, oily skin, and breast atrophy. The virilizing effects of AAS use by women are similar to the clinical features of the virilizing syndrome associated with congenital adrenal hyperplasia and adrenal carcinoma [13003].

**Bone**

Testosterone is the major gonadal sex steroid produced by the testes in men. Androgens induce male sexual differentiation before birth and sexual maturation during puberty; in adult men, they maintain the function of the male genital system, including spermatogenesis. Testosterone is also produced in smaller amounts by the ovaries in women. The adrenal glands produce the weaker androgens dehydroepiandrosterone, dehydroepiandrosterone sulfate, and androstenedione. Because testosterone can be metabolized to estradiol by the aromatase enzyme, there has been controversy as to which gonadal sex steroid has the greater skeletal effect. In this respect, there is increasing evidence that at least part of the effects of androgens in men can be explained by their aromatization into estrogens. The current evidence suggests that estradiol plays a greater role in maintenance of skeletal health than testosterone, but that androgens also have direct beneficial effects on bone [12142].
The definition of bone quality is evolving particularly from the perspective of anabolic agents that can enhance not only bone mineral density but also bone microarchitecture, composition, morphology, amount of microdamage, and remodeling dynamics. From a MEDLINE search (1996-2010), articles were identified by the search terms “bone quality” (1851 articles), “anabolic agent” (5044 articles), “PTH or parathyroid hormone” (32,229 articles), “strontium” or “strontium ranelate” (283 articles), “prostaglandin” (77,539 articles), and “statin” or “statins” (14,233 articles). The search strategy included combining each with the phrase “bone quality.” Another more limited search aimed at finding more novel potential agents. Parathyroid hormone is the only US Food and Drug Administration-approved bone anabolic agent in the United States and has been the most extensively studied in in vitro animal and human trials. Strontium ranelate is approved in Europe but has not undergone Food and Drug Administration trials in the United States. All the studies on prostaglandin agonists have used in vivo animal models and there are no human trials examining prostaglandin agonist effects. The advantages of statins include the long-established advantages and safety profile, but they are limited by their bioavailability in bone. Other potential pathways include proline-rich tyrosine kinase 2 (PYK2) and sclerostin (SOST) inhibition, among others. The ongoing research to enhance the anabolic potential of current agents, identify new agents, and develop better delivery systems will greatly enhance the management of bone quality-related injuries and diseases in the future [11325].

**Effect on bone density**

One study evaluated the relationship between anabolic androgenic steroid (AAS) use and body constitution. Dual-energy x-ray absorptiometry was used to measure bone mineral density (BMD, g/cm²) of the total body, arms, and legs. Total gynoid and android fat mass (grams) and total lean mass (grams) were measured in 10 strength trained athletes (41 ± 8 years) who had used AASs for 5-15 years (Doped) and 7 strength trained athletes (29 ± 6 years) who had never used AASs (Clean). Seventeen sedentary men (30 ± 2 years) served as controls. Doped athletes had significantly more lean body mass and a greater index of fat-free/fat mass compared with Clean athletes and controls. Doped athletes also had significantly less gynoid fat mass compared with that of Clean athletes. There were no differences in BMD between the athletes but both groups had significantly higher BMDs at all sites compared with that of controls. Thus, long-term AAS use seems to alter body constitution, favoring higher muscle mass and reduced gynoid fat mass without affecting BMD [12148].

**Influence on muscle and tendon injury and injury healing**

In the past 20 years, there has been an increase in the incidence of upper extremity tendinous injuries, especially in sports including strong physical activity, such as in weight lifting, as well as with the concurrent use of anabolic steroids. Today, there are more than 200 cases describing rupture of the pectoralis major muscle in athletes. Twenty athletes with pectoralis major muscle (PMM) rupture were studied; 10 had surgical treatment, and the other 10 were treated nonoperatively. The average follow-up was 36 months (range, 48-72 months). Injuries were diagnosed by history, physical examination, and subsidiary tests. Functional evaluation and isokinetic evaluation were performed on all 20 patients. It was concluded that total pectoralis major muscle rupture in athletes showed a better functional result after surgical treatment than after nonsurgical treatment [10058].
The indiscriminate use of anabolic-androgenic steroids has been shown to induce pathologic changes in the Achilles tendon in several situations. To study tendon remodeling in rats treated with anabolic-androgenic steroids combined with an exercise program Wistar rats were grouped as follows: sedentary (group I), injected with anabolic-androgenic steroids only (group II), trained only (group III), and trained and injected with anabolic-androgenic steroids (group IV). The trained groups performed jumps in water: 4 series of 10 jumps each, with an overload of 50 to 70 percent of the animal's body weight and a 30-second rest interval between series, for 6 weeks. Anabolic-androgenic steroids (5 mg/kg) were injected subcutaneously. Activity of matrix metalloproteinases, a marker for tendon remodeling, was analyzed in tissue extracts by zymography on gelatin-sodium dodecyl sulfate-polycrylamide gel electrophoresis. Morphological analyses of tendons showed that in group II, the most external layer that covers the tendon was thicker with aggregation of the collagen fibers, suggesting an increase in collagen synthesis. In group IV, an inflammatory infiltrate and fibrosis in tendons as well as a pronounced increase of the serum corticosterone level were observed. This training protocol upregulated matrix metalloproteinase activity, whereas anabolic-androgenic steroid treatment strongly inhibited this activity. The appearance of lytic bands with molecular masses of approximately 62 and 58 kDa suggests the activation of matrix metalloproteinase-2. It was concluded that anabolic-androgenic steroid treatment can impair tissue remodeling in the tendons of animals undergoing physical exercise by down-regulating matrix metalloproteinase activity, thus increasing the potential for tendon injury. Since the AAS abuse is so widespread, a better comprehension of the pathological effects induced by these drugs may be helpful for the development of new forms of therapy of AAS-induced lesions [06089].

Although testosterone administration elicits well documented anabolic effects on skeletal muscle mass, the enhancement of muscle regeneration after injury has not been widely examined. The purpose of one study was to determine if anabolic steroid administration improves skeletal muscle regeneration from bupivacaine-induced injury. Male C57BL/6 mice were castrated 2 weeks prior to muscle injury induced by an intramuscular bupivacaine injection into the tibialis anterior muscle. Control mice received an intramuscular PBS injection. Anabolic steroid (nandrolone decanoate, 6 mg/kg) or sesame seed oil was administered at the time of initial injury and continued every 7 days for the study's duration. Mice were randomly assigned to one of 4 treatment groups for 5, 14 or 42 days of recovery as follows: 1) Control (uninjured); 2) nandrolone decanoate only (uninjured + nandrolone); 3) Bupivacaine only (Injured); or 4) Bupivacaine + nandrolone. Tibialis anterior morphology, protein and gene expression was analyzed at 14 and 42 days after injury, protein expression was analyzed at 5 days after injury. After 14 days of recovery the Injury and Injury + nandrolone treatments induced small diameter myofiber incidence, and also decreased mean myofiber area. The increase in small myofiber incidence was 65 percent greater in Injury + nandrolone muscle when compared to Injury alone. At 14 days, Injury + nandrolone induced a 5-fold increased in muscle IGF-1 mRNA expression, which was greater than injury alone. Muscle Akt activity and GSK3beta activity were also induced by Injury + nandrolone at 14 days of recovery, but not by Injury alone. Nandrolone had a main effect for increasing muscle MyoD and Cyclin D1 mRNA expression at 14 days. After 42 days of recovery, Injury + nandrolone increased large diameter myofiber incidence compared to Injury only. It was concluded that nandrolone decanoate administration can enhance castrated mouse muscle regeneration during the recovery from bupivacaine-induced injury [09063].

Experimental evidence exists that the use of AASs combined with intense exercise can cause structural and biomechanical alterations of tendons resulting in rupture. Structurally, the collagen fibril alignment is highly disorganized. From a biomechanical perspective, when muscle strength is increased with AAS use, the tendon becomes stiffer, absorbs less energy, and is more likely to fail during physical activity. Premature growth cessation due to physeal...
closures in younger users has not been studied systematically. Such case reports of the resultant permanent short stature have been described for several decades [07008].

Muscle mass seems to be affected greatly by AAS dosing. Higher doses have been shown to garner increases in muscle mass. Muscle mass gains are larger when AAS use is combined with strength training compared with AAS use alone. AASs increase the number of myonuclei. Strenuous exercise seems to increase the number of androgen receptor sites on the muscle. Body weight increases can be in the range of 2 to 5 kg after 10 weeks of AAS use. With more androgen receptors present in the upper regions of the body, the neck, shoulders, thorax, and upper arms gain the most new bulk. The thigh muscles require higher doses to show measurable increases in mass and are not as likely to show increases in the number of androgen receptors. Upon discontinuation, muscles shrink and strength declines over a period of 6 to 12 weeks. Androgens stimulate osteoblast proliferation and differentiation and inhibit the osteoclast. At the start of puberty, androgens stimulate bone formation. At the end of puberty, they induce epiphyseal closure. In adulthood, the sex hormones slow the rate of bone remodeling, protect against bone loss, encourage bone formation, and increase bone density. The adolescent AAS user risks an increased rate of muscle strains or ruptures. Unlike muscles, tendons do not increase in strength so with more intense training, they have a greater risk for rupture. In a developing adolescent, the growth plate cartilage is considered the “weakest link,” and generally is more prone to injury compared with ligaments. A rapid increase in the intensity, frequency, or volume of training is noted consistently in athletes who present with overuse injuries. Injury to the growth plate from weight training has long been a subject of controversy; power lifting may increase the risk for injury, even in adolescents not taking AAS [07058].

Long-standing rotator cuff tendon tearing is associated with retraction, loss of work capacity, irreversible fatty infiltration, and atrophy of the rotator cuff muscles. Although continuous musculotendinous relengthening can experimentally restore muscular architecture, restoration of atrophy and fatty infiltration is hitherto impossible. Continuous relengthening with pharmacological stimulation of muscle growth using an anabolic steroid or insulin-like growth factor (IGF) can reverse atrophy and fatty infiltration as well as improve the work capacity of chronically retracted rotator cuff muscles in sheep. Sixteen weeks after tenotomy of the infraspinatus (ISP) tendon, atrophy and fatty infiltration had developed in the retracted ISP muscle. The musculotendinous unit was continuously relengthened in 14 sheep during 6 weeks: Four sheep were treated without pharmacological stimulation, 4 with intramuscular administration of an anabolic steroid, and 6 with IGF before final repair and rehabilitation (12 weeks). Changes were documented by intraoperative measurements of muscle work capacity, histology, and computed tomography/magnetic resonance imaging. Musculotendinous relengthening by continuous traction resulted in gains of length ranging from 0.7 cm in the IGF group to 1.3 cm in the control group. Fatty infiltration progressed in all groups, and the muscle’s cross-sectional area ranged from 71 to 74 percent of the contralateral side at sacrifice and did not show any differences between groups in weight, volume, histological composition, or work capacity of the muscle. The contralateral muscles in the anabolic steroid group, however, showed significantly higher (mean ± standard deviation) muscle work capacity of 10 ± 0.9 N·m than the contralateral muscles of the control group (6.8 ± 2.4 N·m). This was accompanied by an increased mean muscle fiber area as well as by an unusual gain in the animals’ weight after injection of the anabolic steroid. It was concluded that subcutaneous continuous relengthening of a chronically retracted musculotendinous unit is feasible and advances the retracted musculotendinous junction toward its original position. This does not change the muscle work capacity. Whereas anabolic steroids have been shown to be effective in preventing classic degenerative muscle changes after tendon tears, neither an anabolic steroid nor IGF contributes to regeneration of the muscle once degenerative changes are established. Thus, the findings demonstrate that
muscle cells lose Reactiveness to an anabolic steroid and IGF once retraction has led to fatty infiltration and atrophy of the muscle. Retraction of the muscle after tendon tears must be avoided by early repair, particularly in an athlete, as no regeneration can be achieved by mechanical or pharmacological means at this time [12147].

Muscle healing in power-lifters

Power-lifters have hypertrophic muscle fibers with fissures seen in cross-sections, called as fiber splitting. Whether this phenomenon is due to real splitting or defective regeneration has not been settled. To elucidate this matter, we have examined biopsies from the trapezius and vastus lateralis of power lifters (P group) and power lifters self-administrating anabolic steroids (PAS group). For this purpose, immunohistochemical staining of serial cross-sections was used. The PAS group had significantly more fibers with fissures than the P group in the vastus lateralis but not in the trapezius muscle (1.7 % in both groups). Serial sections revealed that the fibers with fissures changed their profile profoundly over short distances. Some such fibers had a mature staining profile, whereas other fibers indicated recent degeneration and/or regeneration. Activation of satellite cells and formation of aberrant segments were also evident. It was concluded that the so-called split fibers are due to defect regeneration. Some fibers with fissures are the results of old events of segmental muscle fiber damage, whereas the others reflect an ongoing process. The normal regenerative process is most likely disturbed in power-lifters by their continuous training with repeated high mechanical stress on the muscles [06090].

Tendon adaptation

Combined androgenic-anabolic steroids (AAS) and overloading affects tendon collagen metabolism and ultrastructure and is often associated with a higher risk of injury. The aim of this prospective study was to investigate whether such effects would be reflected in the patellar tendon properties of individuals with a history of long-term resistance training and AAS abuse (RTS group), compared with trained (RT) and untrained (CTRL) nonsteroids users. Tendon cross-sectional area (CSA), stiffness, Young's modulus, and toe limit strain were measured in vivo, from synchronized ultrasonography and dynamometry data. The patellar tendon of RT and RTS subjects was much stiffer and larger than in the CTRL group. However, stiffness and modulus were higher in the RTS group (26 % and 30 %, respectively) than in the RT group. Conversely, tendon CSA was 15 percent larger in the RT group than in RTS, although differences disappeared when this variable was normalized to quadriceps maximal isometric torque. Yet maximal tendon stress was higher in RTS than in RT (15 %), without any statistical difference in maximal strain and toe limit strain between groups. The present lack of difference in toe limit strain does not substantiate the hypothesis of changes in collagen crimp pattern associated with AAS abuse. However, these findings indicate that tendon adaptations from years of heavy resistance training are different in AAS users, suggesting differences in collagen remodeling. Some of these adaptations (e.g., higher stress) could be linked to a higher risk of tendon injury [13121].

Compartment syndrome in upper limb

Acute compartment syndrome, a surgical emergency, is defined as increased pressure in an osseofascial space. The resulting reduction of capillary perfusion to that compartment requires prompt fasciotomy. Treatment delay has a poor prognosis, and is associated with muscle and nerve ischemia, resultant infarction, and late-onset contractures. It was reported a case of traumatic bilateral upper limb acute compartment syndrome associated with anabolic steroids, requiring bilateral emergency fasciotomies. A 25-year-old male
bodybuilder taking anabolic steroids, with no past medical history, presented to the Emergency Department 25 min after a road traffic accident. Secondary survey confirmed injuries to both upper limbs with no distal neurovascular deficit. Plain radiographs demonstrated bilateral metaphyseal fractures of the distal humeri. Within 2 h of the accident, the patient developed clinical features that were consistent with bilateral upper arm compartment syndrome. Bilateral fasciotomies of both anterior and posterior compartments were performed, confirming clinical suspicion [13122].

**Rhabdomyolysis**

A 34-year-old bodybuilder presented at the emergency room with fever, vomiting and muscle cramps that had started during a bodybuilding session. Several days before he started training he had used tablets and intramuscular injections containing the anabolic steroids: dehydro-chloro-methyltestosterone, boldenone and trenbolone. In addition, he had taken clenbuterol tablets, liothyronine tablets and subcutaneous injections of phosphatidylcholine. Laboratory investigations revealed massive rhabdomyolysis. The patient was treated with intravenous fluid replacement and sodium bicarbonate to alkalinize the urine. He recovered quickly and his renal function remained unaffected. Doping among amateur athletes in the Netherlands occurs frequently. Apart from long term side-effects, doping can also cause acute health problems. Therefore it is important to ask about doping use during history taking in amateur athletes [06091].

The use of supraphysiologic doses of testosterone increases muscle mass and maximal voluntary strength in a dose-dependent manner, but there is no definitive evidence that testosterone improves performance in endurance events. Rare case reports associate a general necrotizing myopathy with the chronic use of AAS. A 23-year old man developed diffuse myalgias and severe rhabdomyolysis with anuria. Although he developed a viral upper respiratory tract infection 1 week prior to the development of rhabdomyolysis, muscle biopsy and serum testing revealed no evidence of inflammation or immune disease. He had a history of chronic AAS abuse. Other case reports associate localized rhabdomyolysis of the deltoid muscles with chronic AAS injections in the same location. There are few data in the medical literature regarding the effect of chronic AAS abuse on the function of connective tissue. Several case reports associate the chronic abuse of AAS with disruption of connective tissues including spontaneous rupture of ligaments and muscles [13003].

Rhabdomyolysis (breakdown of skeletal muscle tissue) may be caused by mechanical, physical, chemical, or biological factors. It was presented the unique case of a bodybuilder who developed localized rhabdomyolysis of the deltoid muscle after injection of steroids into the shoulder region. Polymyositis and dermatomyositis, mild injury, infectious myositis without phlegmon or abscess formation, radiation therapy, subacute denervation, compartment syndrome, early myositis ossificans, rhabdomyolysis, and sickle cell crisis were differential diagnoses. The patient was treated with intravenous fluid replacement and sodium bicarbonate to alkalinize the urine. Four days after admission, his pain had decreased, he had regained range of motion, and his renal function remained unaffected. This was the first description of localized rhabdomyolysis in the area of an AAS injection [09062].

AAS users also are at risk for rhabdomyolysis or acute skeletal muscle destruction. Rhabdomyolysis has been reported after vigorous weight lifting and may be more likely in patients escalating and supplementing weight training with AASs. Physicians should consider the creatine phosphokinase and gamma-glutamyl transpeptidase levels as essential
elements in distinguishing muscle damage from liver damage when evaluating enzyme elevations in patients who use anabolic steroids [07058].

Renal effects

Renal side effects of AAS are uncommon and have been documented in a few isolated case reports. These reports noted a minimal effect of AAS use on renal function with a mild elevation in serum creatinine. The combination of AAS and creatine supplementation, commonly abused by weightlifters, may increase renal damage. One case of membranoproliferative glomerulonephritis has been cited in an athlete with prolonged AAS abuse. An unusual cancer in adults, Wilms' tumor, has been seen in a few athletes who were self-administering AAS over several years. In one case report, a 23-year-old bodybuilder on cycles of 8 weeks of oral stanozolol and oxymetholone and parenteral nandrolone, testosterone, and boldenone, along with a high-protein diet, developed profound symptoms after only 6 months of use. Of note, the patient was also limiting sodium and water intake and taking the diuretic torasemide. The patient presented with acute confusion, asthenia, and anorexia of 1 month's duration [06031].

Other side effects

Anabolic-androgenic steroids (AAS) are synthetic drugs derived from testosterone. Illegally, these drugs are regularly self-administered by bodybuilders and powerlifters to enhance their sporting performance. Adverse side effects of AAS include sexual dysfunction, alterations of the cardiovascular system, psyche and behavior, and liver toxicity. However, severe side effects appear only following prolonged use of AAS at high dose and their occurrence is limited. Occasionally, AAS abuse may be linked to certain social and psychological traits of the user, like low self-esteem, low self-confidence, suffered hostility, childhood conduct disorder, and tendency to high-risk behavior. The overwhelming stereotype about AAS is that these compounds cause aggressive behavior in males. However, the underlying personality traits of a specific subgroup of the AAS abusers, who show aggression and hostility, may be relevant, as well. Use of AAS in combination with alcohol largely increases the risk of violence and aggression. The dependence liability of AAS is very low, and withdrawal effects are relatively mild. Based on the scores for acute and chronic adverse health effects, the prevalence of use, social harm and criminality, AAS were ranked among 19 illicit drugs as a group of drugs with a relatively low harm [10059].

Side effects of anabolic steroids with relevance in forensic medicine are mainly due to life-threatening health risks with potential fatal outcome and cases of uncertain limitations of criminal liability after steroid administration. Both problems are typically associated with long-term abuse and excessive overdose of anabolic steroids. Side effects may be due to direct genomic or nongenomic activities (myotrophic, hepatotoxic), can result from down-regulation of endogenous biosynthesis (antiandrogenic) or be indirect consequence of steroid biotransformation (estrogenic). Logically, there are no systematic clinical studies available and the number of causally determined fatalities is fairly limited. A compilation reviews typical abundant observations in cases where nonnatural deaths (mostly liver failure and sudden cardiac death) were concurrent with steroid abuse. Moreover, frequent associations between structural characteristics and typical side effects may be explained [10060].

Regarding the health risks associated with the abuse of performance-enhancing drugs in sport data from randomized clinical trials may not be sufficient to identify the complete range
of adverse effects possible with the abuse; more specific studies are necessary to assess their actual and full toxic potential [08139].

**Thyroidal effects**

The most prominent effect on human thyroid function of anabolic steroids is reduction of thyroxine binding globulin (TBG), with consequent reductions of total serum T3 and T4, depending however on the susceptibility of the drug to aromatization and subsequent transformation into estrogen. In rats, anabolic steroids also act in the peripheral metabolism of thyroid hormones and seem to exert an important proliferative effect on thyroid cells [08145].

The use of anabolic steroids to increase physical performance and for aesthetic ends has reached alarming indices in the last three decades. Besides the desired actions, several collateral effects have been described in the literature, such as the development of some types of cancer, gynecomastia, peliosis hepatis, renal insufficiency, virilization, amongst others. The most prominent effect on human thyroid function is the reduction of thyroxine binding globulin (TBG), with consequent reductions of total serum T3 and T4, depending however on the susceptibility of the drug to aromatization and subsequent transformation into estrogen. In rats, anabolic steroids also act in the peripheral metabolism of thyroid hormones and seem to exert an important proliferative effect on thyroid cells. Thus, the aim of the present paper was to review data on the effect of supraphysiological doses of anabolic steroids on thyroid function, showing the danger that indiscriminate use of these drugs can cause to health [07073].

Thyroid cells have androgen receptors, and AASs may directly influence thyroid function. Some studies have shown effects on thyroid function, including a decrease in total triiodothyronine, thyroxine (T₄), and thyroid-binding globulin. Some studies have shown an increase in thyrotropin and free T₄, whereas others have shown no change in these concentrations. It is unclear if this relative impairment in thyroid function leads to a clinical effect. These changes may be due to direct block of thyroid hormone release or synthesis or some other mechanism [07058].

**Hematological**

AASs increase renal synthesis of erythropoietin. They also promote erythropoietic stem cell differentiation. Subsequently, hemoglobin and hematocrit may become elevated, which could result in erythrocytosis or sludging. Two adult cases of intramuscular testosterone-induced polycythemia were reportedly reversed by switching to transdermal testosterone; however, a 65-year-old man developed hypertension and polycythemia during daily testosterone application to his scrotum for 5 years (estimated dose 10 mg/d). Polycythemia and hypertension resolved when testosterone was discontinued. Mild, but significant, increases in mean red blood cell, hematocrit, hemoglobin, and white blood cell concentrations in 33 men were reported following intramuscular testosterone enanthate, 200 mg every 3 or four weeks for 24 weeks. The men remained asymptomatic. Increased platelet count and aggregation also may occur. AASs may potentiate platelet aggregation and be thrombogenic in humans; however, another study found only non-significant trends, including thrombocytosis and increased aggregation [07058].

**Hypercalcemia**
A 26-year-old male bodybuilder was admitted to the surgical department of a Danish community hospital for hematemesis. During the clinical interview, he revealed that he had recently finished a course of anabolic steroids and erythropoietin. The patient also had a previous history of infections and chronic ulcers due to paraffin-oil injections in both upper arms one year before. Over the course of the next few hours, the patient developed signs of multi-organ dysfunction, including pancreatitis, hemorrhagic gastritis, nephropathy with temporary anuria, and respiratory insufficiency, and was transferred to the ICU. After manometric monitoring on the patient's upper arms proved difficult, invasive blood pressure monitoring was used and revealed that the patient was in a state of hypertensive crisis. This case of multi-organ dysfunction was possibly caused by multi-substance-induced hypercalcemia [11067].

Effect on gingival tissues

Anabolic androgenic steroid (AAS) is the familiar name for synthetic derivatives of the male sex hormone, testosterone. A large number of young adults abuse AAS to enhance performance and physical appearance. The aim of one study was to evaluate the effects of AAS abuse on the gingival tissues in a group of bodybuilders and weight lifters. The test group was composed of 24 athletes aged between 17 and 29 years who had been using AAS for >1 year. All subjects were clinically examined for plaque levels (plaque index), gingival inflammation (gingival index), and gingival enlargement. The results were compared to a control group of 20 bodybuilders who had never used AAS drugs and who matched for age, educational level, and oral habits according to the data obtained from the test group. Although there were no statistical differences between the plaque index and gingival index scores of the study group and the control group, the AAS abusers had statistically higher scores of gingival thickness, extent of gingival encroachment, and total gingival enlargement scores compared to non-users. It was concluded that the results of this study have revealed that the prolonged use of AAS is closely associated with significant levels of gingival enlargement. Because recreational abuse and abuse in non-competitive sports seem to increase despite legislation, dentists and periodontists should be familiar with the adverse effects of these synthetic derivatives of testosterone on the gingival tissues [06087].

Peridontit

One study aimed to evaluate periodontal microbiological differences between systemically healthy nonsmoker males taking anabolic androgenic steroids (AASs) and non-AAS users and to find associations between disease severity and AAS use. Ninety-two men practicing bodybuilding were included in the study. They were divided into AAS users and a matched control nonuser group and subgrouped based on their most severe periodontal condition. Pooled subgingival samples from each individual were cultured to evaluate specific periodontopathogen infection. AAS users had significantly higher prevalence of severe periodontitis. AAS users had greater gingival inflammation and clinical attachment loss of ≥3 mm than nonusers (odds ratio 2.4;). AAS users were 4.9 times more likely to be infected with Prevotella intermedia than AAS nonusers (OR 4.9). The OR of presenting subgingival Aggregatibacter actinomycetemcomitans was 8.2 times higher in AAS users (OR 8.2). AAS users were 5.6 times more likely to present subgingival Candida spp. than nonusers (OR 5.6). AAS users were 14.8 times more likely to present subgingival Candida parapsilosis than nonusers (OR 14.8). The likelihood of AAS users presenting subgingival Candida tropicalis was 4.3 times higher than nonusers. A. actinomycetemcomitans was mostly isolated in individuals with severe periodontitis and was associated with subgingival Porphyromonas gingivalis, P. intermedia, and Candida spp. It was concluded that AAS use may increase the risk for severe periodontitis and may cause a subgingival selection of certain Candida species. Specific periodontopathogens, such as Candida dubliniensis and
Candida albicans, seem to be negatively affected by AAS use. The higher risk for disease progression in AAS users may be explained by the significantly higher proportions of A. actinomycetemcomitans, P. gingivalis, P. intermedia, and Candida species as compared to controls. Data on the influence of AAS on subgingival periodontopathogens and disease progression are scarce. Higher proportions of specific periodontopathogens are plausible in AAS users. AAS users had a higher prevalence of severe periodontitis, gingival inflammation, and clinical attachment loss. Men taking AAS are at greater risk of periodontitis and specific periodontopathogen infection [13155].

*Effect on inflammation*

Aging can alter the skeletal muscle growth response induced by overload. The initiation of overload induces muscle extracellular matrix expansion, increased cellularity, and inflammatory gene expression, which are all related to processes important for myofiber growth. These remodeling processes are also biological targets of testosterone. It is not certain how aging affects the inflammatory response to functional overload and whether anabolic steroid administration can alter this response. The effect of anabolic steroid administration on inflammatory processes during functional overload is not known. The purpose of this study was to determine if age altered the skeletal muscle inflammatory response at the onset of functional overload and whether anabolic steroid administration would modulate this response in young or older animals. Five-month and 25 month F344 x BRN rats were given nandrolone decanoate (ND) (6 mg/kg bw/wk) or sham injections for 3 weeks, and then the soleus muscle was overloaded (OV) for 3 days by synergist ablation. ND alone induced a 230% increase in ED1(+) cells in 5 month muscle. Three days of OV had no effect on ED1(+) cell number at either age. OV combined with ND induced a 90% increase in ED2(+) cells in 5 month muscle, while there was no effect of either treatment alone at this age. In 25 month muscle, OV induced a 40 percent increase in ED2(+) cells. Regardless of age, OV induced muscle TNF-alpha mRNA expression (300%) and IL-6 mRNA expression (900 %). ND attenuated OV-induced IL-6 mRNA but not TNF-alpha expression in both age groups. The overload induction of IL-1beta mRNA was 3-fold greater in 25 month muscle (1400 %), compared to 5 month muscle (400 %). ND administration ablated the overload IL-1beta mRNA induction in 25 month muscle. Anabolic steroid administration can suppress inflammatory cytokine gene expression at the onset of overload and this effect is age dependent [06088].

*In basket ball players*

It was analyze the outcome on testosterone (T) and cortisol (C) responses in 12 professional basketball players during a season of competition. Serum adrenocorticotropic hormone (ACTH), C, total testosterone (TT), and free testosterone (FT) levels were analyzed in October, December, March, and April. A day after the games, blood samples were taken. Serum ACTH levels were maintained at the initial levels during the season. However, basal C significantly changed during the season, with lower levels in December and in April. Basal serum TT levels increased during the season until a maximum in March. No differences were presented in the TT values in December, March, and April. Basal FT presented high levels in October and December, followed by a low level in March, remaining low in April. The T/C increased during the season, attaining a maximum level in December, followed by a significant decrease in March. Free T/C ratio decreased during the season (lower level in March). In conclusion, the players maintained a good anabolic-catabolic balance [10062].

*Side effects in elderly*
Testosterone supplementation has been shown to increase muscle mass and strength in healthy older men. The safety and efficacy of testosterone treatment in older men who have limitations in mobility have not been studied. Community-dwelling men, 65 years of age or older, with limitations in mobility and a total serum testosterone level of 100 to 350 ng per deciliter (3.5 to 12.1 nmol per liter) or a free serum testosterone level of less than 50 pg per milliliter (173 pmol per liter) were randomly assigned to receive placebo gel or testosterone gel, to be applied daily for 6 months. Adverse events were categorized with the use of the Medical Dictionary for Regulatory Activities classification. The data and safety monitoring board recommended that the trial be discontinued early because there was a significantly higher rate of adverse cardiovascular events in the testosterone group than in the placebo group. A total of 209 men (mean age, 74 years) were enrolled at the time the trial was terminated. At baseline, there was a high prevalence of hypertension, diabetes, hyperlipidemia, and obesity among the participants. During the course of the study, the testosterone group had higher rates of cardiac, respiratory, and dermatologic events than did the placebo group. A total of 23 subjects in the testosterone group, as compared with 5 in the placebo group, had cardiovascular-related adverse events. The relative risk of a cardiovascular-related adverse event remained constant throughout the 6-month treatment period. As compared with the placebo group, the testosterone group had significantly greater improvements in leg-press and chest-press strength and in stair climbing while carrying a load. It was concluded that in a population of older men with limitations in mobility and a high prevalence of chronic disease, the application of a testosterone gel was associated with an increased risk of cardiovascular adverse events [10329].

Other infections

Pyomyositis

Pyomyositis, a deep bacterial infection of skeletal muscle, is due to the combination of bacteremia and local muscle injury. It most commonly occurs in children and young adults, with a male predominance; the lower extremities and pelvic girdle are typically involved, with the quadriceps muscle being the most common site of infection. Recognized risk factors are muscle trauma (including strenuous exercise), immunosuppression, diabetes mellitus, malignancy, cirrhosis, rheumatologic disorders, and injections such as illicit drug abuse. The infectious agent is S. aureus in 75-90 percent of cases, with group A Streptococcus a distant second. Most patients will have a leukocytosis with neutrophilia and an elevated erythrocyte sedimentation rate, but muscle enzymes such as creatinine kinase are typically normal. Ultrasonography can be used to locate fluid collections within the musculature and assist with needle aspiration, but CT imaging may better show muscle abnormalities such as edema and abscess formation. Magnetic resonance imaging is thought to be even more sensitive and is the preferred modality in early stages of the disease. A case was presented regarding a previously healthy 45-year-old amateur bodybuilder who reported progressive right thigh pain and swelling for 3 days. He admitted injecting anabolic steroids into the lateral aspect of his right thigh 1 week prior. Although both thighs were quite large, the right was clearly swollen; erythema, warmth, and tenderness were present but there was no crepitus or fluctuance. Laboratory studies found an elevated white blood cell count of 19,000/mm³ with 86 percent neutrophils. A computed tomographic (CT) scan of the right thigh revealed a loculated collection involving the vastus lateralis muscle from just below the lesser trochanter to above the knee (17 cm length by 9 cm transverse by 6.5 cm anterior-posterior), with fluid and inflammatory changes of the rectus femoris and tensor fascia lata and overlying subcutaneous edema [13154].

Tuberculosis

It was presented a case report depicting masking of symptoms of intestinal tuberculosis by anabolic androgenic steroids (AAS) causing delay in diagnosis which lead to a major
surgery. Negative tuberculosis skin test (TST) probably due to immunomodulating effects of AAS also contributed to the delay. Patient had early dependence on AAS and rapid growth of scrotal sebaceous cysts, findings of which have not yet been reported. Patient initially did not reveal his AAS abuse to physician. This is in line with previous studies which report that majority of AAS abusers distrust medical professionals and do not disclose it to their treating physicians. During hospitalisation he reported it probably due to seriousness of his condition, impending major surgery and concurrent use of other medicines. The patient had large sebaceous cysts on scrotum with history of rapid growth in last six months. Although this is not a known side effect of AAS use, increase in growth of sebaceous glands and resulting acne have been documented. It is possible that this side effect might have been missed in previous studies as patients are usually reluctant to reveal it unless specifically asked. Further, most studies involved young age group who are more prone to acne, a clearly visible symptom having significant importance in that age. Usually period for development of AAS dependence is 9-12 months but this patient developed dependence in just four months probably because AAS withdrawal symptoms were associated with appearance of symptoms of tuberculosis [12149].

Genotoxicity (cancer risks)

The abuse of anabolic steroids for doping raises concerns. Many of these compounds have never been examined for their toxicological properties. Aside from hormonal (androgenic) activity, anabolic steroids may also exert genotoxic effects. In one study, it was determined the potencies of the "designer steroid" madol and the anabolic prohormone 19-norandrostenedione to induce micronuclei in V79 cells in vitro. CREST analysis was used to differentiate between aneugenic and clastogenic mechanisms of micronucleus induction. Cytotoxicity of the steroids and their influence on the cell cycle were assessed in parallel. In addition, the ability of the drugs to increase production of reactive oxygen species and to induce apoptosis were studied. Both agents caused a concentration-dependent increase in the rates of micronuclei in V79 cells, exceeding a doubling of the background micronucleus rates of untreated controls, which was evident at 27 μM and 29 μM for madol and 19-norandrostenedione, respectively. The steroid-induced micronuclei were predominantly kinetocho (CREST)-negative, pointing to a clastogenic mode of action. As cytotoxicity of both compounds is weak, cytotoxicity was unlikely to contribute to their genotoxicity. The observed genotoxicity of both compounds was due neither to apoptosis induction nor to production of reactive oxygen species. However, the ability of both steroids to induce micronuclei appears related to their lipophilicity. Therefore, a "non-specific" chromosomal genotoxicity of madol and 19-norandrostenedione, based on hydrophobic interactions, appears likely. This could well result in biologically relevant increases in chromosomal damage as soon as critical concentrations of the agents are reached in vivo. Regarding the current misuse of the steroids for doping, the uncontrolled administration of very high doses must be considered. Therefore it cannot be ruled out that these drugs present genotoxic hazards under current misuse conditions by athletes in sports or in body building [08149].

To evaluate genotoxicity of anabolic androgenic steroids (AAS) in male bodybuilders by a micronucleus assay in buccal mucosa cells 11 male bodybuilders volunteered to participate in a study and two groups were formed: group 1 (n=6), without AAS consumption and group 2 (n=5), with AAS consumption. A sample of buccal epithelium was taken from each participant once a week for 6 weeks. Samples were fixed, stained and analysed by a light microscope, and 2000 cells were counted from each slide. Results are expressed as micronucleated cells (MNC) per 1000 cells and were analysed by the Mann-Whitney U test and Wilcoxon's test. A marked increased in MNC was seen in bodybuilders with AAS consumption compared with those without AAS consumption (mean 4.1 MNC/1000 cells vs 0.4 MNC/1000 cells, respectively). Intragroup comparisons showed no differences in the
MNC frequencies during the sampling time in group 1, whereas the MNC frequency in group 2 varied significantly, reaching the highest MNC frequencies in the third and fourth week of sampling; frequency in the first sampled week was 1.1 MNC/1000 cells. Significant differences in all sampled weeks were found between the two groups. It was concluded that AAS consumption increased the frequency of MNC from buccal mucosa in bodybuilders [07078].

**Fatal events**

Anabolic-androgenic steroids (AAS) are widely abused, but the potential for dependence and addiction remains unclear. Recent studies from our laboratory have shown that male and female hamsters will voluntarily self-administer testosterone and other AAS. Furthermore, it was observed fatal androgen overdose during self-administration. This suggests that AAS are potentially addictive, independent of their effects on muscle mass or athletic performance [06093].

**Multiple organ failure**

It was reported a 42-year-old male amateur body builder and user of anabolic androgenic steroids, who developed ARDS, acute kidney injury, and refractory supraventricular tachycardia. He required extracorporeal membrane oxygenation, continuous veno-venous hemodialysis, and catheter ablation. It was believed that long-term anabolic androgenic steroid abuse predisposed the patient to multiple organ dysfunction syndrome, from its immunomodulatory effects in an otherwise healthy patient. Anabolic androgenic steroid use should be part of the history taking process, since it may complicate diagnosis, disease progression, and prognosis [13156].

**Side effects of topical anabolic steroids**

Androstanolone is an androgen with effects and efficacy comparable to dihydrotestosterone. Topical sexual steroid hormones may easily penetrate human skin and cause systemic effects. Topical androgens like testosterone have been used for decades to treat vulvar lichen sclerosus et atrophicus with a doubtful efficacy. Due to systemic absorption of testosterone virilism expressed as hirsutism may develop. Topical application of testosterone causes also systemic effects. Indeed, sexual steroids including androgens may show a significant percutaneous absorption leading to changes in the serum levels. Precursors like pregnenolone or androstendione fail to alter serum levels because of rapid metabolism. In controlled trials carefully monitored low-dose testosterone therapy in women efficiently increased female sexual interest and desire in the postmenopausal period. An up to two years administration in clinical trials did not cause serious side effects. It is, however, recommended that testosterone levels should be carefully monitored. It was reported five cases with different patterns of adverse reaction due to non-critical and/ or unmonitored use of anabolic steroids: deepening of the voice due to topical use of AAS in an anti-cellulite cream; circumscribed hypertrichosis and late onset acneiform eruptions due to testosterone replacement therapy after ovariectomy in women. Homolateral gynecomastia and infertility, acne and striae distensae was noted in males using injectable AAS. Cellulite is frequently observed in middle aged women and is characterized by furrowed and edematous skin on thighs, hips and buttocks. It seems to be caused by weakened muscular septa and a diffuse pattern of extrusion of underlying adipose tissue into thedermis. An increase of hypodermal adipose tissue and weakened fibrous septa extending perpendicularly from the bones to the
skin surface, have been observed. A higher body mass index is associated with an increased grade of cellulite. Life-style drugs may bear a significant risk of adverse effects, some irreversible or fatal [07013].

Long-term effects on social-medicine demography

Swedish data

The acute effects of AAS-use on mental health are well described in the literature, for example, increased aggression and irritability, anxiety and depressive symptoms, cognitive impairment, suicidal behaviours, hypomanic symptoms, enthusiasm and increased self-confidence. There are studies showing minor or no alterations in aggressive behaviours in past AAS-abusers who had been abstinent for a year or longer. On the other hand, one study found that past (abstinent period not defined) AAS-abusers had significantly more psychiatric diagnoses, as diagnosed by DSM-IV, than current abusers. A Finnish study, concerning a 12-year follow-up study of 62 male-elite Finnish powerlifters, where there was a high suspicion of AAS-use, reported a 4.6 times higher death rate compared with the general population. The main causes of death were myocardial infarctions and suicides. The high occurrence of suicide in populations with a strong suspicion of previous AAS-use is in line with preliminary results from our own study group, showing that death risk from suicide was increased by 2-4 times. One study aimed to investigate whether previous AAS-use affects mental health, present sociodemographic data, sport activity and substance abuse in a retrospective 30-year follow-up study of former elite athletes. During 2004, a questionnaire including structured questions concerning sociodemographic variables, previous and past sport activity, lifetime prevalence of seeking professional help for mental-health problems and previous and past substance use was sent to 996 Swedish male-elite power sport athletes on the top 10 national ranking lists during any of the years 1960-1979 in wrestling, Olympic lifting, powerlifting and the throwing events in track and field answered a questionnaire. At least 20 percent of the former athletes admitted previous AAS-use. A reminder to fill in the questionnaire was sent to those athletes who had not answered, and finally 683 (69 %) subjects had answered the questionnaire including the specific questions in the questionnaire concerning whether they had ever used AAS, and if so, when in relation to their sport career. Regarding their past sport activity, the former AAS-users were significantly older when they started training in the sport discipline within which they reached their highest ranking and they spent more hours per week training during their sport active years compared with non-AAS-users. There was no difference in mean age between the groups when they discontinued elite power sports. Compared with non-AAS-users, former AAS-users had significantly more often sought professional help for depression (13 % vs 5 %), anxiety (13% vs 6 %), melancholy (13 % vs 4 %), concentration deficit (4 % vs 1 %) and worry for mental health (8 % vs 3 %). The two groups did not differ regarding frequency of present alcohol consumption. Concerning tobacco use, the former AAS-users were less often present tobacco users compared with the non-AAS-users. Regarding previous tobacco use, the two groups did not differ. The former AAS-users showed higher lifetime prevalence of illicit drug use compared with the non-AAS-users but AAS-users had more often been offered AAS compared with the non-AAS-users (87 % vs 20). All the former AAS-users (n=143) reported having used AAS during their active sports career. The percentage of AAS-users in specific sport disciplines were: powerlifting (57 %), Olympic lifting (47 %), track and field (28 %) and wrestling (6 %). Forty-two percent (n=60) had administrated AAS by tablets, 3 percent (n=5), by injections and 55 percent (n=78) by both tablets and injections. The main reasons for using AAS were: to achieve better sport results (81 %), to train harder (56 %), a suspicion that their competitors used AAS (45 %) and faster recovery (43 %). There were no significant differences between former high and low AAS-users concerning
sociodemographic variables and present and past sport activity. Furthermore, there were no
differences in past and present substance use (tobacco, alcohol and illicit drugs). However,
the former high AAS-users significantly more often combined AAS with the use of illicit drugs
and with the use of alcohol compared with the former low AAS-users. The AAS-users also
differed in former sport activity pattern compared to non AAS-users. It was concluded that a
relationship exists between use of AAS and mental-health problems. Thus, the results from
this study of former male-elite athletes in sport disciplines, where increased muscle strength
has a marked influence on performance, showed that at least 20% admitted AAS-use during
their active sport career. The study indicates that the former AAS-users had a higher
frequency of lifetime prevalence of seeking professional expertise for several mental
problems. Furthermore, former AAS-users more often had used illicit drugs. Former AAS-
users were significantly older when they started training in their sport discipline in which they
had been most successful and they also spent more hours per week training. Furthermore, if
the former AAS-using athletes used AAS for a longer time than 2 years, they had more often
sought professional expertise for anxiety, irritation and anger and they had also more often a
combined use of alcohol and illicit drugs together with AAS. These high-consumers of AAS
reported having experienced more side effects of AAS, compared with those athletes having
used AAS no longer than 2 years. A former use of AAS does not seem to have a negative
long-term effect on either present substance abuse and present sport activity or on whether
they presently lived in a relationship or not. The present results can be compared with
previous results regarding lifetime prevalence of AAS-use among elite athletes active in the
60s and 70s. For example, in 1972, it was estimated that one-third of the Swedish-elite track
and field athletes used AAS. The present study is, however, based on a larger number of
athletes and in four power sports. In the same year’s Olympic Games, 68 percent of the
participants in the track and field events reported prior steroid abuse. The former elite
athletes admitting AAS-use were significantly younger compared with the non-users. This
age difference might explain why the former AAS-users are, to a higher degree, in their
present employment. However, this difference disappeared when the old-age pensioners,
that is, above 65 years of age, were excluded from the analysis [13157].

Increased mortality in former users of anabolic steroids

Physical training has been shown to reduce mortality in normal subjects, and athletes have a
healthier lifestyle after their active career as compared with normal subjects. Since the
1950s, the use of anabolic androgenic steroids (AAS) has been frequent, especially in power
sports. The aim of the present study was to investigate mortality, including causes of death,
in former Swedish male elite athletes, active 1960-1979, in wrestling, powerlifting, Olympic
lifting, and the throwing events in track and field when the suspicion of former AAS use was
high. Results indicate that, during the age period of 20-50 years, there was an excess
mortality of around 45 percent. However, when analyzing the total study period, the mortality
was not increased. Mortality from suicide was increased 2-4 times among the former athletes
during the period of 30-50 years of age compared with the general population of men.
Mortality rate from malignancy was lower among the athletes. As the use of AAS was
marked between 1960 and 1979 and was not doping-listed until 1975, it seems probable that
the effect of AAS use might play a part in the observed increased mortality and suicide rate.
The otherwise healthy lifestyle among the athletes might explain the low malignancy rates
[13158].

Tour de France (1947-2012)

In the context of recent concerns regarding performance enhancing techniques and potential
negative health effects of high-level physical activity, data on the long-term outcomes and
causes of death in elite endurance cyclists are of particular interest. Characteristics and vital status of all French participants in the Tour de France were collected for the 1947-2012 period. Causes of death were obtained from 1968. Overall and disease-specific mortalities were compared with the French male population using overall and specific standardized mortality ratios (SMRs) with their 95 percent confidence intervals (CIs). Among the 786 French cyclists who participated at least once between 1947 and 2012, 208 (26%) died by 1 September 2012. Neoplasms and cardiovascular diseases accounted for 61 percent of deaths. It was observed a 41 percent lower mortality in French cyclists (SMR: 0.59, 95% confidence interval 0.51 to 0.68), which did not change over time. It was observed for main mortality causes: for neoplasms (SMR: 0.56; 95% confidence interval 0.42 to 0.72) and for cardiovascular death (SMR: 0.67; 95% confidence interval 0.50 to 0.88), except mortality related to external causes (SMR: 1.06, 95% confidence interval 0.71 to 1.53). It was observed a substantially and significantly lower mortality in participants in the Tour de France, compared with the general male population. However, the results do not allow us to assess in detail the balance between positive effects of high-level sports activity and selection of healthy elite athletes, versus any potential deleterious effects of excessive physical exercise or alleged doping [13159].
TESTOSTERONE

Testosterone is more than a "male sex hormone". It is an important contributor to the robust metabolic functioning of multiple bodily systems. The abuse of anabolic steroids by athletes over the years has been one of the major detractors from the investigation and treatment of clinical states that could be caused by or related to male hypogonadism. The unwarranted fear that testosterone therapy would induce prostate cancer has also deterred physicians from pursuing more aggressively the possibility of hypogonadism in symptomatic male patients. In addition to these two mythologies, many physicians believe that testosterone is bad for the male heart. The classical anabolic agents, 17-alkylated steroids, are indeed, potentially harmful to the liver, to insulin action, and to lipid metabolism. These substances, however, are not testosterone, which has none of these adverse effects. The current evidence, in fact, strongly suggests that testosterone may be cardioprotective. There is virtually no evidence to implicate testosterone as a cause of prostate cancer. It may exacerbate an existing prostate cancer, although the evidence is flimsy, but it does not likely cause the cancer in the first place. Testosterone has stimulatory effects on bones, muscles, erythropoietin, libido, mood and cognition centres in the brain, penile erection. It is reduced in metabolic syndrome and diabetes and therapy with testosterone in these conditions may provide amelioration by lowering LDL cholesterol, blood sugar, glycated hemoglobin and insulin resistance. The best measure is bio-available testosterone which is the fraction of testosterone not bound to sex hormone binding globulin. Several forms of testosterone administration are available making compliance much less of an issue with testosterone replacement therapy [08063].

Testosterone is the principal male sex hormone. As with all natural steroids, it is biosynthesized from cholesterol. Phase I metabolism employs some very specific enzymes and pathways. Phase II metabolism and excretion follow more general patterns. Synthetic and endogenous steroids differ in this measure. Numerous xenobiotic compounds have been derived from testosterone. The modifications typically aim at a reduction of the androgenic properties while maintaining the anabolic potential. Most of these compounds have been withdrawn from the legal market. However, they are found to be illicitly added to otherwise inefficient nutritional supplements. These products represent a major problem to doping control. Recently, clinical trials with selective androgen receptor modulators have been started [10078].

Testosterone causes the hypertrophy of muscle fibers and an increase in the number of myonuclei and satellite cells. This metabolic action follows from the binding of testosterone to the androgen receptor. Its ability to transduce the binding of testosterone in the cytoplasm of the cell to its anabolic action (altering gene function in the nucleus) is inversely proportional to the number of CAG repeats in the first exon of the androgen receptor, a member of the nuclear transcription factor family of receptors and located in virtually all tissues. There are many reports of muscle hypertrophy and increases in strength in athletes who take anabolic steroids. Virtually none are controlled trials of just testosterone or one of other anabolically-active steroids, for many ingest and inject multiple potentially active drugs [10001].

The primary screening method for the detection of doping by athletes using synthetic versions of endogenous steroids such as testosterone relies on measurement of the ratio of testosterone (T) to epitestosterone (E) in urine. In 2005 the World Anti-Doping Agency (WADA) lowered the T/E value at which samples undergo further investigation from six to four. This has resulted in a large increase in the number of athletes with naturally elevated T/E ratios undergoing investigation without a corresponding increase in the number of proven
doping offences involving testosterone. The objective was to develop a new simple screening protocol that can, with high probability, not only distinguish athletes whose natural T/E values exceed four from those whose T/E values have been elevated by testosterone doping but also detect those athletes with naturally low T/E values that do not exceed four despite being administered testosterone. Testosterone (250 mg Sustanon) was administered weekly to a group of 47 young adult males for five weeks in a double-blind placebo controlled study and urine samples collected. The samples were analysed for steroid concentrations using GC/MS and for luteinizing hormone (LH) by immunoassay. The elevation of T/E that occurred in all subjects was accompanied by a significant reduction in urinary LH concentrations to levels that are rare in normal subjects. The appropriate measurement of urinary LH, with the measurement of T/E values, can markedly improve the efficiency of detection of doping with testosterone by male athletes, particularly those who have low natural T/E ratios [09085].

**Theoretical, overviewing, aspects**

It is suggested that the sex steroid hormones testosterone and estrogen (SSH) provide receptor cells with reliable information on protein synthesis and on the level of oxidative metabolism in the cells of the gonads. The SSH are derived from the oxidation of cholesterol. This oxidation is a side reaction of the oxidative processes in the mitochondria that generate most of the energy to the organism. The amount of SSH that is synthesized is correlated to the partial pressure of oxygen at the synthesizing cells. The amount of free SSH that a cell can hold is checked by the damage that free steroids may cause. This damage is prevented by proteins that bind with SSH. As a result, SSH levels are correlated also with the ability of the SSH synthesizing cell to produce proteins that bind with them. A cell can only synthesize SSH in relation to the oxidative processes within it and to its ability to produce the binding proteins necessary to prevent the damage caused by SSH. As a result, the information conveyed by SSH is reliable. It was examined the specific damage caused by testosterone and estrogen, and suggest why each of them is best suited for its function. Although both SSH can provide similar information on the metabolism in the cells that synthesize them, there are secondary reasons why testosterone and estrogen were selected to serve particular functions. Testosterone improves the efficiency of the proton pump at the mitochondria in producing ATP, but increases oxidative damage. Estrogen on the other hand decreases oxygen damage but also decreases the efficiency of the proton pump. These differences between the two SSH may explain why females use estrogen to inform the body about the activity of the cells in their gonads while males do it by testosterone. The increased oxidative damage may also explain why in males the testosterone that reaches the brain is turned into estrogen. It was also suggested why fish use 11-keto testosterone and why insects do not use these two steroids [11326].

Testosterone is among the oldest drugs in medicine. It has a long efficacy and safety record for its prime role of androgen replacement therapy in men with androgen deficiency. Testosterone and synthetic analogue androgens have also been used in pharmacological androgen therapy (PAT) to produce androgenic effects on marrow, muscle or bone. Although PAT is increasingly being superseded by newer, more expensive drugs, androgens remain cost-effective in many older applications. Androgen misuse is the systematic over-prescribing for unproven medical indications. Misuse is increasingly evident for male ageing ("andropause") and some other clinical conditions. Further trials for new indications for androgens require reliable safety data, but rising costs may make it increasingly attractive to circumvent the need for evidence by promoting off-label mass marketing. Androgen abuse is the illicit self-administration of often massive doses of androgens for non-medical purposes - notably power sports and body building. In parallel with effective detection reducing
androgen abuse in elite sports, more focus is needed on non-sporting cosmetic, recreational and occupational androgen abuse. Despite ongoing androgen misuse and abuse, testosterone remains under-prescribed for younger men with classical androgen deficiency that frequently remains undiagnosed [06050].

Testosterone therapy is prescribed for millions of men each year, and the number is increasing rapidly. Prescription sales of testosterone increased by 500 percent in the United States between 1993 and 2000. Most testosterone prescriptions are written to treat nonspecific symptoms, such as fatigue or sexual dysfunction, when accompanied by testosterone levels below the laboratory reference range. Currently, testosterone levels that are at least 2 SD below the mean value for healthy young adults are classified as low. Although convenient, this classification fails to consider the physiological consequences of specific testosterone levels. More than 80 percent of circulating estradiol in men is derived from the aromatization of testosterone. Thus, as serum testosterone levels decline, there is a concomitant decline in serum estradiol levels. Nevertheless, the consequences of male hypogonadism are routinely attributed solely to androgen deficiency; the potential role of the concomitant decline in estrogens is typically ignored. It has become clear, however, that estrogen deficiency may be important in the pathogenesis of some consequences of male hypogonadism, such as bone loss. The potential role of estrogen deficiency in the pathogenesis of other consequences of hypogonadism, such as alterations in body composition or sexual function, is largely unknown. Information on the role of estrogens in male hypogonadism may help identify men at risk for specific manifestations of the condition and may provide a rationale for novel approaches to its management. We sought to determine the relative degree of testosterone deficiency, estradiol deficiency, or both at which undesirable changes in body composition, strength, and sexual function begin to occur and whether those changes are due to androgen deficiency, estrogen deficiency, or both. It was recruited two cohorts of men who were 20 to 50 years of age and healthy. All the men had normal serum testosterone levels. All participants received goserelin acetate (Zoladex®), at a dose of 3.6 mg subcutaneously at weeks 0, 4, 8, and 12, to suppress endogenous gonadal steroids. Participants were then randomly assigned to receive 0 g (placebo), 1.25 g, 2.5 g, 5 g, or 10 g of a topical 1 percent testosterone gel (AndroGel®) daily for 16 weeks. Participants in cohort 2 also received anastrozole (Arimidex®) at a dose of 1 mg daily to block the aromatization of testosterone to estrogen. Participants were unaware of the study-group assignments. Participants were seen every 4 weeks. At each visit, fasting blood samples were obtained to measure gonadal steroid levels, and questionnaires were administered to assess physical function, health status, vitality, and sexual function. At baseline and week 16, body fat and lean mass were assessed by means of dual-energy x-ray absorptiometry (DXA); subcutaneous- and intraabdominal-fat areas and thigh-muscle area were measured by means of computed tomography (CT); and lower-extremity strength was determined by means of a leg press. Data on bone homeostasis (bone-turnover markers and bone mineral density), risk factors for cardiovascular disease (blood pressure, lipids, and insulin sensitivity), and levels of leptin and prostate-specific antigen were also collected but are not included in the present report. It was enrolled 198 men in cohort 1 and 202 men in cohort 2. There were no significant differences in baseline testosterone levels among dose groups or between cohorts. In the study, it was found that the dose of testosterone required to prevent adverse changes in a variety of measures varies considerably. When aromatization was intact, fat accumulation began with mild gonadal steroid deficiency (a testosterone level of approximately 300 to 350 ng per deciliter), whereas lean mass, thigh-muscle area, and muscle strength were preserved until gonadal steroid deficiency was more marked (a testosterone level ≤200 ng per deciliter). Sexual desire and erectile function, the two major domains of sexual function, showed distinct patterns of change as serum testosterone levels were reduced. The variation in tissue sensitivity to androgens could be due to polymorphisms affecting polyglutamine repeat length in the androgen-receptor gene.
tissue-specific differences in androgen-receptor expression or local hormone metabolism, or, as shown in the study, variation in the roles of androgens and estrogens in the regulation of target-tissue responses. Observational studies have shown that lean mass and strength are reduced and fat mass is increased in men with low testosterone levels. Men with hypogonadism report less sexual activity, fewer sexual thoughts, and fewer spontaneous erections than men with normal testosterone levels. Moreover, testosterone replacement increases lean mass, decreases fat mass, and can improve sexual function in men with hypogonadism. These observations have led to the widespread belief that undesirable changes in body composition and sexual dysfunction in men with hypogonadism are due to androgen deficiency. However, because estradiol is a metabolite of testosterone, it is difficult to distinguish the effects of androgens from those of estrogens in observational studies, or even in randomized, controlled trials if aromatizable androgens are used without the administration of an aromatase inhibitor. By administering a variety of testosterone doses with and without concomitant aromatase inhibition, it was found that changes in lean mass, thigh-muscle area, and leg-press strength were attributable to changes in testosterone levels, whereas changes in fat measures were primarily related to changes in estradiol levels. Both androgens and estrogens contributed to the maintenance of normal libido and erectile function. Although these results may be surprising, they are consistent with studies showing that body fat is increased in humans and male mice with null mutations of the aromatase gene or the estrogen-receptor α gene and that sexual function is markedly impaired in mice and humans with these genetic defects. These observations may have important clinical implications. First, they provide a physiological basis for interpreting testosterone levels in young and middle-aged men and identifying the adverse consequences that are most likely to occur at various gonadal steroid levels. Second, because increases in visceral fat reduce insulin sensitivity and are associated with diabetes and the metabolic syndrome, the marked increase in intraabdominal fat with aromatase inhibition could portend an increase in cardiovascular disease with long-term estrogen deficiency. Finally, because lean mass, thigh-muscle area, and erectile function were reduced at a testosterone dose (1.25 g per day) that elicited a mean serum level of approximately 200 ng per deciliter, testosterone supplementation seems justified in men with testosterone levels in this range. However, some men have alterations in these functional outcomes at lower or higher testosterone levels, and other consequences of hypogonadism, such as increases in body fat and loss of sexual desire, routinely develop at higher mean testosterone levels. Thus, each person's specific clinical scenario should be considered when interpreting the clinical significance of the circulating testosterone level. These findings may also have implications for older men. Serum testosterone levels decline modestly as men age, such that 20 percent of men older than 60 years of age and 50 percent of men older than 80 years of age have testosterone levels at least 2 SD below the mean level in young men. The finding that estrogens have a fundamental role in the regulation of body fat and sexual function, coupled with evidence from prior studies of the crucial role of estrogen in bone metabolism, indicates that estrogen deficiency is largely responsible for some of the key consequences of male hypogonadism and suggests that measuring estradiol might be helpful in assessing the risk of sexual dysfunction, bone loss, or fat accumulation in men with hypogonadism. For example, in men with serum testosterone levels of 200 to 400 ng per deciliter, sexual-desire scores decreased by 13 percent if estradiol levels were 10 pg per milliliter or more and by 31% if estradiol levels were below 10 pg per millilitre. The findings also suggest that treatment with aromatizable androgens would be preferable to treatment with nonaromatizable androgens in most men with hypogonadism [13188].

Normal values
Normal total plasma testosterone levels in males are in the range of 300 to 1,000 ng/dL. Most is bound by sex hormone-binding protein and is inactive; free testosterone, the active form, makes up only 2 to 3 percent of circulating testosterone. Testosterone is metabolized into dihydrotestosterone, which is 10 times more potent than testosterone, and estradiol, which has feminizing effects [07031].

Reference ranges are essential for partitioning testosterone levels into low or normal and making the diagnosis of androgen deficiency. It was established reference ranges for total testosterone (TT) and free testosterone (FT) in a community-based sample of men. TT was measured using liquid chromatography tandem mass spectrometry in nonobese healthy men, 19-40 years old, in the Framingham Heart Study Generation 3; FT was calculated. Values below the 2.5th percentile of reference sample were deemed low. It was determined the association of low TT and FT with physical dysfunction, sexual symptoms (European Male Aging Study, EMAS, only), and diabetes mellitus in three cohorts: Framingham Heart Study generations 2 and 3, EMAS, and the Osteoporotic Fractures in Men Study. In a reference sample of 456 men, mean (SD), median (quartile), and 2.5th percentile values were 724 (221), 699 (297), and 348 ng/dL for TT and 142 (45), 134 (60), and 70 pg/mL for FT, respectively. In all three samples, men with low TT and FT were more likely to have slow walking speed, difficulty climbing stairs, or frailty and diabetes than those with normal levels. In EMAS, men with low TT and FT were more likely to report sexual symptoms than men with normal levels. Men with low TT and FT were more likely to have at least one of the following: sexual symptoms (EMAS only), physical dysfunction, or diabetes. It was concluded that the reference ranges generated in a community-based sample of men provide a rational basis for categorizing testosterone levels as low or normal. Men with low TT or FT by these criteria had higher prevalence of physical dysfunction, sexual dysfunction, and diabetes. These reference limits should be validated prospectively in relation to incident outcomes and in randomized trials [11327].

Problems in evaluating serum testosterone values

Testosterone (T) and other androgens are incorporated into an increasingly wide array of human sexuality research, but there are a number of issues that can affect or confound research outcomes. One review addressed various methodological issues relevant to research design in human studies with T; unaddressed, these issues may introduce unwanted noise, error, or conceptual barriers to interpreting results. Topics covered are (1) social and demographic factors (gender and sex; sexual orientations and sexual diversity; social/familial connections and processes; social location variables), (2) biological rhythms (diurnal variation; seasonality; menstrual cycles; aging and menopause), (3) sample collection, handling, and storage (saliva vs. blood; sialogogues, saliva, and tubes; sampling frequency, timing, and context; shipping samples), (4) health, medical issues, and the body (hormonal contraceptives; medications and nicotine; health conditions and stress; body composition, weight, and exercise), and (5) incorporating multiple hormones. Detailing a comprehensive set of important issues and relevant empirical evidence, the review provided a starting point for best practices in human sexuality research with T and other androgens that may be especially useful for those new to hormone research [13194].

Half life

Designer AAS are altered so as to increase the bioavailability and prolong the desired effects. For example, testosterone’s half-life is measured in minutes, whereas fluoxy-mesterone, a synthetic AAS, has a half-life of 9.2 hours [06031].
Testosterone/epitestosterone concentration ratio (T/E)

Determining the origin of testosterone and other steroids in human urine is a major issue in doping control. According to the latest published laboratory statistics of 2009 from WADA, 65 percent of reported adverse and atypical analytical findings by the accredited anti-doping laboratories belonged to the substance group of anabolic agents. Out of these, testosterone is by far the most common agent reported, constituting as much as 70 percent of the findings. An atypical analytical finding of testosterone, however, must not be confused with an anti-doping rule violation. The detection of the administration of testosterone is primarily based on a population reference interval of a urinary ratio of testosterone to epitestosterone, excreted as glucuronide, referred to as the T/E ratio. Since it is well known that natural outliers of a normal steroid profile exist, a further investigation is mandatory after a finding of an elevated T/E ratio. For this reason, samples with a T/E ratio that equals or exceeds 4 are amongst other parameters recommended to be submitted to isotope ratio mass spectrometric (IRMS) analysis. The IRMS analysis is based on differences in isotope ratio between endogenous and synthetic testosterone and provides the basis for confirmatory analysis. The $^{13}$C/$^{12}$C ratio in natural compounds, such as steroids, is determined by the pathway by which they were produced. Consequently, synthetic testosterone is generally less enriched in $^{13}$C and shows a different $^{13}$C/$^{12}$C ratio than human endogenous testosterone. Out of the samples with T/E ratios above 4 submitted to IRMS analysis in one laboratory, about 8 percent were reported as adverse analytical findings for the application of synthetic testosterone or testosterone prohormones [11574].

The use of biomarkers of doping is not new. For example, the testosterone/epitestosterone concentration ratio (T/E) was introduced by several sports organizations in the 1970s to deter the administration of anabolic steroids. Because epitestosterone is only a minor product of testosterone metabolism and does not increase after exogenous testosterone administration, the net effect of the latter is an increase in T/E. In 1983, a T/E in excess of 6.0 was considered indicative of steroid doping by the International Olympic Committee. The introduction of this rule was mitigated, however, by the discovery a few years later that some individuals may have naturally increased T/E, a phenomenon that has recently been attributed to the discovery of genetic polymorphisms that are associated with the metabolism of anabolic steroids. Currently, in addition to the T/E, a urinary steroid profile that includes multiple testosterone metabolites and precursors is used to detect steroid doping, in addition to doping with other anabolic agents, such as designer steroids, gonadotropins, estrogen antagonists, aromatase inhibitors, androgen precursors, and selective androgen receptor modulators [11426].

T/E in police-seized drugs
One possible explanation for some of the negative findings at IRMA in T/E-positiva cases might be the use of testosterone with endogenous-like delta values. The isotopic ratio depends on the manufacturing process and on the carbon feed stocks of the starting materials. Hence, it could be possible to produce testosterone products with carbon isotope ratios in, or close, to the endogenous range, by using $^{13}$C enriched starting materials. In one study, the content of a number of black market testosterone products collected in Austria were analyzed. Additionally, $^{13}$C/$^{12}$C ratios were measured for testosterone in the products after cleavage of the testosterone ester. The aim was to determine whether some of these products had similar $^{13}$C/$^{12}$C ratios to those normally found for endogenous testosterone, which could prevent a positive isotopic ratio mass spectrometric (IRMS) finding in doping control. Moreover, it was investigated to what extent the preparations contained the masking agent epitestosterone, in order to lower the testosterone/epitestosterone (T/E) ratio in urinary steroid profiles. Out of 30 analyzed products, the declared ingredients differed from the actual content in 10 cases. Epitestosterone, however, could not be found in any of the
products. The products displayed $^{13}CVPDB$ values between 24 and 29 percent. Formore than half of these products, the values were within a range reported for endogenous urinary steroids [11574].

**Androsterone to epitestosterone ratio**
The conspicuous interindividual differences in metabolism and urinary excretion of testosterone and its metabolites make it challenging to reveal testosterone doping. The variation in testosterone glucuronide excretion is strongly associated with a deletion polymorphism in the uridine diphosphate-glucuronosyltranferase (UGT) 2B17 gene. The objective of one study was to identify additional biomarkers to detect testosterone abuse and to elucidate alternative pathways for testosterone elimination in individuals devoid of the UGT2B17 enzyme. For this purpose a new ultraperformance liquid chromatographic tandem mass spectrometric method for simultaneous determination of 10 different sulfo- and glucuronide-conjugated steroids was developed. Fifty-four healthy male volunteers with two, one, or no allele (ins/ins, ins/del, or del/del) of the UGT2B17 gene participated in the study. Intervention included a single im dose of 500 mg testosterone enanthate. Urinary sulfo- and glucuronide-conjugated steroids were measured. Testosterone sulfate levels decreased in all individuals after the dose. The individual differences in the excretion of all sulfated metabolites were large. Thus, these metabolites will not serve as appropriate biomarkers for testosterone abuse. However, androsterone glucuronide excretion increased in all of our study subjects after the testosterone dose. Etiocholanolone sulfate was excreted at significantly higher levels in UGT2B17 del/del individuals. It was proposed that the androsterone glucuronide to epitestosterone glucuronide ratio may serve as a complementary biomarker to reveal testosterone abuse [11575].

**Free testosterone/cortisol ratio**

**In golf**
The purpose of one investigation was to study the effects of 36 continuous holes of competitive golf on salivary testosterone, cortisol, and testosterone-to-cortisol ratio and their relation to performance in eight elite male collegiate golfers (age 20 years). Thirty-six holes of a 54-hole NCAA golf tournament were played on the first day of the competition. A saliva sample was taken 45 minutes prior to the round and immediately following each hole for a total of 37 samples per subject. Time matched baseline samples were collected on a different day to account for circadian variation. Six-hole areas under the curve (AUC) values were calculated for endocrine measures. Significant increases were noted for cortisol during competition, however, testosterone did not change during competition compared to baseline. Testosterone-to-cortisol (T/C) ratio was significantly lower throughout the competition compared to baseline measures. Thirty-six-hole AUC testosterone-to-cortisol ratio response was correlated to 36-hole score. There was a high correlation between pre-round testosterone, T/C ratio response, and 36-hole score. CSAI-2 somatic anxiety was correlated to pre-round cortisol and testosterone response. These results indicate a significant hormonal response during 10 hours of competitive golf. Good golf performance (low golf scores) in this competition was related to low T/C ratio. Additionally, results from this investigation validated CSAI-2 somatic anxiety with physiological measures of anxiety [06059].

**In soccer**
The following up of some hormonal parameters during the professional soccer training process could be one of the indicators of the training effects. On the other hand, overreaching and overtraining as an opposite adaptation of supercompensation could be detected by following up some hormonal changes. The aim of one study was to evaluate the changes in some hormonal parameters in professional soccer players during a half-season
competition. It was included 30 professional soccer players from a soccer club of our National Soccer League in this study. All sport medical examinations were conducted three times: before the preparation phase, before the competition phase (after previous phase) and after finishing the competition phase. There were significant differences in all evaluated hormones between three phases of soccer training process, including significant decrease in T/C of more than 30 percent at the end of the competition phase (phase III). The decrease in muscle mass after the preparation phase and the increase in fat mass at the end of competition phase were insignificant. The hormonal changes indicated that some indices could indicate overreaching and overtraining at the end of professional soccer competition season. Although insignificant, the decrease in muscle mass after the preparation phase and the increase in fat mass at the end of competition phase were undesirable effects for us [06060].

The free testosterone:cortisol ratio (FTCR) is widely used for studying and preventing overtraining syndrome in various sports. The use of FTCR for following overtraining syndrome was proposed originally with two approaches: FTCR lower than $0.35 \times 10^{-3}$, calculated on free testosterone (FT) in nanomoles per liter (nmol/L) and on cortisol (C) in micromoles per liter (mmole/L) or a decrease of the ratio of 30 percent or more in comparison with the previous value. In our experience, the use of an absolute value as a threshold is not useful, whereas the evaluation of the concentrations of hormones and their ratio in comparison with previous ones is more useful. These classical approaches are not, however, sufficient to describe the various possible physiological modifications linked to training excess and/or incomplete recovery. It was collected samples from 32 professional soccer players of an Italian First Division team, during the period 2001-2003. It was analyzed the values of 21 athletes during the season 2001-2002 and of 11 athletes during the season 2002-2003 (6 out of 11 were examined also during the previous one) always present when the 4 (first season) or 5 (second season) blood drawings have been performed. We applied an original, pragmatic and easy-to-use classification of FTCR values, in association with classical interpretations based on decreases of the values in comparison with previous athlete’s result. It was used the traditional approaches in two consecutive seasons in a professional soccer team: the evaluation of the decrease >30% of the parameter in comparison with the previous value or with the basal (preseason) value are shown. The statistical differences between the FTCR values of the six athletes followed in both seasons were not significant. Thus, the classification method that was proposed is advantageous in comparison with traditional interpretative schemes, because identify different risk categories, stratifying the interval between the values 0.35-0.8 [06061].

**Time-course of testosterone action**

The classic model of steroid action is that steroid hormones have a relatively slow time-course of action by acting as transcription factors after binding to intracellular receptors Athletes know that the anabolic gains realized while on a pre-competition steroid “cycle” will persist for weeks after AAS use is discontinued. However, research in animals has demonstrated behavioral effects of testosterone that occur within minutes. In the 1980s, East German scientists developed an androgen nasal spray to enhance aggression and competitiveness without systemic effects. Similarly, intranasal 4,16-androstadien-3-one induces an amphetamine-like “high” in human volunteers [12100].

**Stability in the urine**

The stability of testosterone glucuronide (TG), epitestosterone glucuronide (EG) and the T/E ratio in urine has been studied. Samples were analyzed by gas chromatography coupled to
mass spectrometry (GC/MS). Urine samples were submitted to a solid-liquid cleanup followed by extraction of unconjugated testosterone (T) and epitestosterone (E) with tert-butyl methyl ether (free fraction). The remaining aqueous phase was hydrolyzed with beta-glucuronidase and extracted at alkaline pH with n-pentane. Analytes were analyzed by GC/MS as their enol-trimethylsilyl (TMS) derivatives. The urine for stability testing was obtained from an excretion study after the administration of T to healthy volunteers. The homogeneity of the sample was verified before starting the stability study. The stability of TG and EG was evaluated at different storage conditions. For long-term stability testing, analyte concentration in urine stored at 4 degrees C and -20 degrees C was determined at different time intervals for 22 months. For short-term stability testing, analyte concentration was evaluated in urine stored at 37 degrees C for 3 and 7 days. The effect of repeated freezing (at -20 degrees C) and thawing (at room temperature) was studied for up to three cycles. Data obtained in this work demonstrated the stability of TG, EG and the T/E ratio in sterilized urine samples stored at 4 and -20 degrees C for 22 months and after going through repeated freeze/thaw cycles. Decreases in concentration were observed after 7 days of storage at 37 degrees C due to the partial cleavage of the glucuronide conjugates; however, the T/E ratio was not affected. These results show the feasibility of preparing reference materials containing TG and EG to be used for quality control purposes [06051].

**Testosterone prohormones**

The term prohormone strictly refers to a post-translational peptide that is cleaved into a variety of bioactive hormones. In the supplement context, prohormones refer to androgenic precursors which, when ingested, become enzymatically activated to testosterone derivatives. An understanding of the biochemical pathways emphasises the similarity between testosterone and its precursors. Users see prohormones as a natural means to improve muscle strength, body composition and general well-being with fewer side effects than testosterone or synthetic androgenic steroids and a more practical (capsule) form of intake. The marketing strategy of commercial websites is to promote prohormones as “legal alternatives” to testosterone with similar anabolic effects. Of course, many consumers are unaware that these prohormones are included on the WADA list of prohibited substances as well as being illegal for sale or importation in many countries. Prohormones have another concerning role in sports nutrition as contaminants in other sports supplements which account for a large proportion of inadvertent doping offences. From cholesterol, pregnenolone is produced which converts to testosterone via dihydroepiandrosterone (DHEA). The path via DHEA produces androstenedione (DIONE) and androstenediol (DIOL) which convert to testosterone. Importantly, however, these precursors can also be converted to the estrogens, which may cause effects such as gynaecomastia and liver dysfunction. To counteract this effect, some users of prohormones alternate between 1 month on and 2 months off, allowing restoration of intrinsic function within each cycle. Users also often stack differing prohormones of differing oestrogenicity within each cycle, and take N-acetyl cysteine to prevent liver dysfunction. In addition, selective estrogen receptor modulators or aromatase inhibitors are taken to mitigate oestrogenic effects, and androgenic herbal compounds taken to reduce the low period between cycles. Despite these sophisticated multidrug regimens and marketing claims, research fails to demonstrate any anabolic or ergogenic effects of taking DHEA, DIONE or DIOL, which confirms the risk of adverse side effects for DIONE and DIOL. For example Broeder et al gave 200 mg/day DIONE or DIOL to middle-aged men over a 12-week resistance training programme, and showed a significant 16 percent increase in testosterone levels after 1 month of use which had returned to prestudy levels by 12 weeks. DIOL did not significantly increase blood testosterone levels. The major fate of the ingested DIONE and DIOL appears to be aromatisation, since blood estrogen levels were increased by about 63 percent. There was no enhancement in muscle strength during resistance training above placebo but, conversely, an 11 percent increase in
the LDL-Cholesterol/HDL-Cholesterol lipid ratio, corresponding to a significant increase in the cardiovascular disease risk. Twelve weeks of supplementation reduced blood luteinising hormone levels, which may serve to decrease inherent testicular and adrenal testosterone production. The last major literature review, by Brown et al, confirmed the findings that DIONE, DIOL and DHEA do not augment muscle size and strength gains observed from resistance training alone, and that use of DIONE and DIOL may predispose users to serious health risks [12459].

An area for concern is the abuse of a precursor to AAS known as prohormones, such as androstenediol. Many prohormone synthetic chemicals have been banned, but numerous clones have been made with a minor change in the formulation. These prohormones increase hormone levels within the body leading to many of the same benefits and side effects as the AAS [12119].

The first steroids introduced on the prohormone market were dehydroepiandrosterone (DHEA) and androst-4-ene-3,17-dione in 1996, shortly thereafter followed by androst-4-ene-3beta,17beta-diol, androst-5-ene-3beta,17beta-diol and androst-5-ene-3,17-dione. These steroids can be regarded as prohormones of testosterone as they are claimed to metabolise to testosterone after oral administration. Several studies on the metabolism of DHEA androst-4-ene-3,17-dione, androst-4-ene-3beta,17beta-diol, androst-5-ene-3beta,17beta-diol and androst-5-ene-3,17-dione have indicated the possibilities of using alterations in the steroid profile to detect misuse of these steroids. Based upon these studies, monitoring the concentration and ratios of several steroids can be used for the detection of the administration of a testosterone prohormone. A similar approach was already successfully implemented for testosterone and dihydrotestosterone misuse. Amongst the most powerful indicators, besides the increase in the individual urinary concentrations of the administered steroids, were the ratio of androsterone/etiocholanolone and testosterone/epitestosterone.

These parameters were already well established in doping control analysis to detect the administration of testosterone. Besides these parameters, other steroids have been proposed for the detection of misuse of androst-4-ene-3,17-dione, including 6alpha-OH-androstenedione, 6beta-OH-androsterone, 6beta-OH-etiocholanolone, 6beta-OH epiandrosterone and 4-OH-androstenedione. For the detection of DHEA administration, 7beta-OH-DHEA, 7-keto-androsterone, 16alpha-OH-androsterone and 16alpha-OH-etiocholanolone can be used as indicative parameters. 5alpha-Androstane-3beta,17beta-diol is a prohormone of dihydrotestosterone (DHT) and is marketed as such. Administration of this steroid resulted in elevated urinary levels of endogenous 5alpha-steroids, similarly as after DHT administration as well as in high urinary concentrations of epiaandrosterone sulphate. It was therefore suggested that for the detection of the abuse of 5alpha androstane-3beta,17beta- diol, the same criteria as for DHT-misuse could be applied, in addition to the increase in urinary 5alpha-androstane-3beta,17beta- diol glucuronide concentration [06004].

5alpha-Androstane-3,17-dione is an endogenous steroid and an intermediate in the conversion of androstenedione to androsterone and etiocholanolone and is naturally present in urine in low concentrations. In microbiologically contaminated urine however, the concentration of this steroid might rapidly increase. 5alpha-Androstane-3,17-dione is listed as a controlled substance in the 2004 ASCA but was in spite of that detected as a contaminant in a nutritional supplement [06004].

Seasonal variations

Humans' endogenous testosterone concentrations vary over a number of temporal scales, with little known about variation longer than monthly cycles. Past studies of seasonal or
circannual variation have principally used male participants and have produced inconsistent results. Thus, little is known about how testosterone concentrations fluctuate throughout the year, whether such variation differs between men and women, and whether there are influences of hormonal contraceptive use. The present study collected saliva samples from a large sample (n=718) of men and women, each collected at one time point within a relatively uniform distribution over a full calendar year. Both men and normally-cycling women displayed seasonal variation in salivary testosterone concentrations, such that testosterone concentrations are maximal in the fall and minimal in the summer. Notably, normally-cycling women had testosterone concentrations that were over 100 percent greater at their maximum in fall compared to their minimum in summer. Women using hormonal contraceptives not only had consistently lower endogenous testosterone concentrations, but also showed a flatter seasonal testosterone profile [11577].

**Ethnic differences in steroid-related diseases**

Differences in circulating steroid hormone levels have been hypothesized to explain ethnic differences in steroid-related diseases. The aim of one study was to determine the serum levels of a wide panel of steroid hormones, both androgens and estrogens, in healthy middle-aged African-Caribbean and European men. Serum steroid hormone levels were determined in men participating in a systematic public health study funded by the French National Health Insurance system. Blood was collected in the morning from 304 healthy African-Caribbean and European men aged between 40 and 69 years. Serum steroids were measured by mass spectrometry-gas chromatography, except for DHEAS and sex hormone-binding globulin, which were determined by RIA. Data were analyzed in 10-year age intervals by analysis of covariance, with adjustment for age, body mass index, waist-to-hip ratio, tobacco and alcohol consumption, and season of sampling. Compared with Europeans, African-Caribbean men presented significantly higher serum levels of measured bioavailable testosterone, 4-androstenedione (4-dione), and estrone (E1) regardless of the age group, of 5-androstenediol (5-diol) in those aged 40-49 and 50-59 years, and of testosterone (TT) and dihydrotestosterone in those aged 40-49 years. In contrast, European men aged 40-69 years showed significantly higher serum levels of DHEA and DHEAS. It was concluded that significant differences in serum steroid hormone levels were observed in middle-aged African-Caribbean and European men. Whether such differences could contribute to ethnic differences in disease risk in adult men remains to be investigated. Some steroids, such as bioavailable TT, 4-dione, 5-diol, and E1, deserve particular attention [11578].

It is hypothesised that seemingly disparate and unrelated phenomena clustering in persons of African descent living in the Americas such as outstanding sprinting ability and high prostate cancer incidence and mortality are in fact related and emerge from enhanced testosterone responsiveness in descendants of African slaves surviving the transatlantic trade in Africans. It is postulated that the ability to have survived the middle passage was positively correlated with greater responsiveness of the androgen receptor to its primary ligands dihydrotestosterone and testosterone, and that slaves possessing more responsive androgen receptors experienced a survival advantage engendered by the enhanced anabolic effects which accrued such as increased red cell mass and therefore greater oxygen carrying capacity and tissue oxygen delivery enabling these slaves to tolerate stifling conditions in the hull of the slave ship, increased lean muscle mass and therefore greater surface area to volume ratio resulting in easier ability to dissipate heat and remain cool, and increased skin thickness and sebum production resisting the macerating effect of lying in admixed bodily fluids below deck. These androgen effects as well as others would have produced a survival advantage under the severe selection pressure created by the inhumane and physiologically challenging circumstances under which the slaves were transported from the interior of the African continent and West Africa to the 'New World'. This would result in a population shift...
favouring increased androgen receptor responsiveness in descendants of African slaves populating the Americas and a corresponding geographic and racial distribution of androgen related phenomena such as sprinting prowess and prostate cancer. African-Americans having the highest prostate cancer incidence rate and the Caribbean having the highest prostate cancer mortality rates in the world are consistent with this hypothesis as is the observation that the 10 fastest men and 9 fastest women of all time are exclusively the descendants of West African slaves who survived the middle passage. It is predicted that as yet undiscovered as well as known biological correlates of enhanced androgen receptor responsiveness such as relatively short CAG-repeats in the poly Q tail of exon 1 of the androgen receptor gene will be more prevalent among African-Americans and Afro-Caribbean peoples than among West Africans. It is also predicted that African-Americans and Afro-Caribbean peoples will have relatively shorter CAG-repeats in the androgen receptor gene compared to West Africans [11579].

**Urinary levels of testosterone and epitestosterone in a Korean male population**

Cannabis, or marijuana, the most commonly used illicit drug in the world, has been shown to be responsible for suppressing the production and secretion of androgens, particularly testosterone. However, despite such findings in animals, the chronic effects of marijuana use on human endocrine systems have proved to be inconsistent. Here, it was investigated the reference ranges of urinary levels of testosterone (T) and epitestosterone (E) as well as their metabolic ratio of T/E in a Korean male population (n=337), which would enable an evaluation of abnormal changes in steroid metabolism induced by habitually administered cannabis. The T/E ratio was significantly decreased in the marijuana group (n=18), while the urinary testosterone concentrations were also tended to decrease. This study is the first to provide data for the reference values of two urinary androgens and T/E values among control Korean males, and, furthermore, suggests that the T/E ratio, though not testosterone levels, might be used to understand the suppression of human male gonadal function affected by smoking marijuana [13219].

**Status of lean elite athletes**

It was investigated the endocrine profile, body composition, and state of mood in male Olympic athletes participating in sports that do or do not emphasize leanness. Forty-four Swedish male Olympic athletes participating in 26 different sport disciplines were studied. Body composition was determined by dual-energy x-ray absorptiometry, and blood levels of steroid hormones and biomarkers of nutritional status were analyzed. In addition, states of mood were assessed employing the profile of mood states (POMS) test. The athletes were divided into 2 groups on the basis of whether their sporting discipline emphasized leanness or not. In all subjects, body composition, hormone levels, and POMS scores were within normal ranges. However, the leanness athletes (n=18) displayed significantly lower proportion of body fat, higher spinal bone mineral density, lower serum levels of free testosterone and leptin, and higher serum levels of insulin-like growth factor binding protein 1 than nonleanness athletes (n=26). Leanness athletes also had higher POMS scores for depression and anger, and a higher global POMS score, the latter being positively correlated to the frequency of illness before the Olympic Games. It was concluded that although there were no indications of energy deficiency or endocrine disturbance in the leanness athletes, their higher POMS scores and frequency of illness may indicate the potential harmfulness of their pursuit of outstanding athletic performance [13195].

**Levels in male Olympics**
To investigate the endocrine profile, body composition, and state of mood in male Olympic athletes participating in sports that do or do not emphasize leanness 44 Swedish male Olympic athletes participating in 26 different sport discipline were investigated. Body composition was determined by dual-energy x-ray absorptiometry, and blood levels of steroid hormones and biomarkers of nutritional status were analyzed. In addition, states of mood were assessed employing the Profile of Mood States (POMS) test. The athletes were divided into 2 groups on the basis of whether their sporting discipline emphasized leanness or not. In all subjects, body composition, hormone levels, and POMS scores were within normal ranges. However, the leanness athletes (n=18) displayed significantly lower proportion of body fat higher spinal bone mineral density, lower serum levels of free testosterone and leptin and higher serum levels of insulin-like growth factor binding protein 1 than nonleanness athletes (n=26). Leanness athletes also had higher POMS scores for depression and anger, and a higher global POMS score, the latter being positively correlated to the frequency of illness before the Olympic Games. CONCLUSION:: Although there were no indications of energy deficiency or endocrine disturbance in the leanness athletes, their higher POMS scores and frequency of illness may indicate the potential harmfulness of their pursuit of outstanding athletic performance [12105].

**Older men**

Little information exists about longitudinal changes in body composition and physical function in relation to sex hormone levels in older men. The aim of one study was to determine associations of testosterone, estradiol, and SHBG with changes in body composition and physical function. It was conducted a prospective cohort study within the Osteoporotic Fractures in Men (MrOS) study at six US clinical centers. A total of 5994 ambulatory men aged 65 years or older enrolled in the MrOS. It was examined 1183 men with complete measures of sex steroid hormones, body composition, and some measure of physical function. Intervention: There were no interventions. Sex steroids were measured by mass spectrometry in serum collected at baseline. Measurements of body composition using dual-energy x-ray absorptiometry and physical performance (grip strength, leg power, timed chair stands, narrow walk, and 6-m walk) were performed at baseline and repeated 4.5 years later. Overall, men lost 1.3 kg (± 4.4) weight between study visits. Lean mass, especially appendicular, declined less at higher baseline testosterone levels. These associations were most evident in the 40 percent of men who lost more than 2.0 kg during follow-up. In weight losers, higher testosterone was associated with less decline in timed chair stands. Estradiol was not related to body composition or physical function changes. Higher SHBG was associated with less loss of appendicular lean mass and grip strength. Higher endogenous testosterone is associated with reduced loss of lean mass and lower extremity function in older men losing weight. Endogenous testosterone may contribute to healthy aging [11580].

Cycling androgens has been reported by athletes to improve physical performance by enhancing muscle mass and strength, a paradigm that has not been studied, and may have clinical value in older men being treated with testosterone. It was investigated the efficacy of a monthly cycled testosterone regimen that uses half the testosterone dose as the current standard of care continuous therapy on body composition and muscle strength in older men. Twenty-four community-dwelling older men 70 ± 2 years of age with total testosterone levels below 500 ng/dL were randomized at the Institute for Translational Sciences-Clinical Research Center into a 5-month double-blind placebo-controlled trial. Subjects were dosed weekly for 5 months, receiving continuous testosterone (TE, n=8; 100 mg testosterone enanthate, im injection), monthly cycled testosterone (MO, n=8; alternating months of testosterone and placebo), or placebo (PL, n=8). Main outcomes included body composition by dual-energy x-ray absorptiometry and upper and lower body muscle strength. Secondary
outcomes included body weight, serum hormones, and mixed-muscle protein fractional synthesis rate (FSR). Total lean body mass was increased and percent fat was reduced after 5 months in TE and MO. Upper body muscle strength increased in TE, and lower body muscle strength increased in TE and MO. FSR increased in TE and MO but not in PL. It was concluded that cycled testosterone improved body composition and increased muscle strength compared with placebo and increased FSR similarly to continuous testosterone [11581].

Testosterone in Older Men with Mobility Limitations Trial determined the effects of testosterone on muscle performance and physical function in older men with mobility limitation. Trial's Data and Safety Monitoring Board recommended enrollment cessation due to increased frequency of adverse events in testosterone arm. It was concluded that cycled testosterone improved body composition and increased muscle strength compared with placebo and increased FSR similarly to continuous testosterone [11581].

The measurement of serum testosterone in women is challenging due to lack of trueness, precision, and sensitivity of various available testosterone assays. Accurate assessment of testosterone in women is crucial especially in conditions associated with alleged over- or under-production of testosterone, such as in polycystic ovary syndrome (PCOS) or primary ovarian insufficiency (POI). The aim of this study was to measure and compare androgen concentrations in women with PCOS, POI, and female controls and to evaluate the performance of extraction RIA and liquid chromatography-tandem mass spectrometry (LC-MS/MS) in these women. Carefully phenotyped women with POI (n=208) or PCOS (n=200) and 45 healthy, regularly cyclic female controls were included. Method comparison analyses were performed for total testosterone, androstenedione (AD), and DHEA, as measured by
LC-MS/MS and extraction RIA. All androgen levels were significantly elevated in women with PCOS compared with POI patients and controls. Women with POI presented with similar androgen concentrations as controls, except for AD. Compared with measurements by extraction RIA, testosterone, DHEA, and AD concentrations measured by LC-MS/MS were systematically lower. However, using extraction RIA and LC-MS/MS, testosterone, DHEA, and AD measurements were shown to have good agreement as assessed by Bland-Altman analysis and intraclass correlation coefficient: 0.95, 0.83, and 0.96, respectively. It was concluded that LC-MS/MS, compared with a labor-intensive extraction RIA, shows good precision, sensitivity, and high accuracy for measuring female testosterone, DHEA, and AD concentrations under various clinical conditions. LC-MS/MS, therefore, represents a convenient and reliable assay for both clinical and research purposes, where androgen measurement in women is required [11439].

To evaluate the role of physiologic levels of androgens and their precursors in the regulation of body composition, energy and substrate metabolism and aerobic capacity in healthy, cycling, premenopausal women it was evaluated 30 young (27 ± 1 year) premenopausal, non-obese (23 ± 0.5 kg/m²), normally-cycling women, without clinical or chemical evidence of hyperandrogenism or hyperinsulinemia, for parameters of total and regional body composition, glucose tolerance, aerobic capacity and resting energy expenditure and substrate oxidation. Serum was assayed for androgens and androgen precursors by techniques optimized to assess the low androgen levels in this population. Higher serum testosterone levels correlated with greater fat mass but not abdominal adiposity or other metabolic/physiologic variables. Additionally, dehydroepiandrosterone (DHEA) was negatively related to visceral fat content. Other serum androgens did not correlate with total or regional adiposity, skeletal muscle mass, aerobic capacity, glucose tolerance, or resting energy and substrate metabolism. It was concluded that this group of non-obese, premenopausal women with no clinical or chemical evidence of hyperandrogenemia, serum testosterone levels were positively related with fat mass, but not with abdominal adiposity; whereas, DHEA was negatively related to visceral adiposity. The data suggest that within the normal physiologic range, testosterone is a predictor of overall adiposity, but that this effect does not appear to be associated with concomitant alterations in resting energy or substrate metabolism that could predispose to weight gain [11440].

Androgen therapy is being increasingly used in the management of postmenopausal women. The most common indication is to improve sexual function. The aim of one review was to evaluate current knowledge pertaining to testosterone and sexual function in postmenopausal women. The change of testosterone levels during the menopause transition remains controversial. A correlation of endogenous testosterone levels and sexual function is still inconclusive. A Cochrane Review and recent randomized control trials have, however, consistently demonstrated that short-term testosterone therapy in combination with traditional hormone therapy regimens improves sexual function in postmenopausal women, particularly surgically menopausal women with hypoactive sexual desire disorder. An adverse effect on the lipid profile has been identified which appears to be mostly associated with oral methyltestosterone. Data for other effects of testosterone and long-terms risks are lacking. Testosterone may act in a variety of ways in different tissues. This is, however, an area that requires further investigation. Thus, testosterone therapy is a promising option for treating women with hypoactive sexual desire disorder after surgical menopause. Two remaining questions need to be answer: who is most likely to benefit from testosterone therapy and what are the long-term health risks? [06052].

**Testosterone after use of chlorinated swimming pools**
The goal of one study was to evaluate the associations between testicular hormones at adolescence and the exposure to chlorination by-products when attending chlorinated swimming pools. We obtained serum samples from 361 school male adolescents (aged 14-18 years) who had visited swimming pools disinfected with chlorine or by copper-silver ionization. We analysed serum concentrations of inhibin B (two different assays), total and free testosterone, sex hormone-binding globulin, luteinizing hormone (LH), follicle stimulating hormone (FSH) and dehydroepiandrosterone sulphate (DHEAS). There were strong inverse associations between serum levels of inhibin B (both assays) or of total testosterone, adjusted or unadjusted for gonadotropins and the time adolescents had spent in indoor chlorinated pools, especially during their childhood. Adolescents having attended indoor chlorinated pools for more than 250 h before the age of 10 years or for more than 125 h before the age of 7 years were about three times more likely to have an abnormally low serum inhibin B and/or total testosterone (<10th percentile) than their peers who never visited this type of pool during their childhood (odds ratio 2.83 and 3.67, respectively). Such associations were not seen with free testosterone, LH, FSH and DHEAS or with the attendance of outdoor chlorinated pools or of the copper-silver pool. Swimming in indoor chlorinated pools during childhood is strongly associated with lower levels of serum inhibin B and total testosterone. The absorption of reprotoxic chlorination by-products across the highly permeable scrotum might explain these associations [11441].

**Testosterone prohormones**

Testosterone prohormones such as androstenedione, androstenediol, and dehydroepiandrosterone (DHEA) have been heavily marketed as testosterone-enhancing and muscle-building nutritional supplements for the past decade. Concerns over the safety of prohormone supplement use prompted the United States Food and Drug Administration to call for a ban on androstenedione sales, and Congress passed the Anabolic Steroid Control Act of 2004, which classifies androstenedione and 17 other steroids as controlled substances. As of January 2005, these substances cannot be sold without prescription. Here, we summarize the current scientific knowledge regarding the efficacy and safety of prohormone supplementation in humans. We focus primarily on androstenedione, but we also discuss DHEA, androstenediol, 19-nor androstenedione, and 19-nor androstenediol supplements. Contrary to marketing claims, research to date indicates that the use of prohormone nutritional supplements (DHEA, androstenedione, androstenediol, and other steroid hormone supplements) does not produce either anabolic or ergogenic effects in men. Moreover, the use of prohormone nutritional supplements may raise the risk for negative health consequences [06122].

**Salivary testosterone (and testosterone to cortisol ratio)**

Salivary testosterone (T) and cortisol (C) concentrations were monitored across a sports competition. Data were compared using two enzyme-immunoassay (EIA) methods and two sample preparations to determine their influence on hormone concentrations. A group of male athletes (n=19) provided a saliva sample the morning before and one day after (24h post) an international rugby union match. Following an extraction procedure, the samples were analysed for T and C concentrations using a commercial kit (CM_e) and an in-house method (IH_e). Raw samples (no extraction procedure) were also tested using the commercial kit (CM_r). There were no significant changes in T and C levels from pre to post competition with each EIA method and sample preparation, but significant differences in T (IH_e>CM_e>CM_r) and C (CM_r)>IH_e and CM_e) concentrations were seen when both samples were pooled. Bland-Altman analyses confirmed the presence of fixed and proportional bias. Strong and significant correlations were demonstrated between the IH_e and CM_e measures of
salivary T and C. The T and C values from the raw and extracted samples were also strongly correlated. The measurement of salivary T and C concentrations across an international sports event was influenced by different EIA methods and sample preparations, but all measures were strongly correlated with some bias. Both T and C were unresponsive to the sports event, but within the group results large individual variation was seen [12106].

To prepare efficiently for competition, wrestlers usually train physically for a period of approximately 12-20 weeks. Numerous physical qualities must be developed during this period of preparation: aerobic fitness, maximal strength, muscular endurance, power, and speed. However, numerous studies have concluded that it is difficult to concurrently develop strength and aerobic fitness for several reasons, in particular antagonistic endocrine variations. The study involved 15 elite junior wrestlers who trained at a sports training school for 15 weeks. To investigate the effects of long-term training and to assess the relationships between hormonal concentrations (salivary testosterone [T] and cortisol [C]) and performance changes during simultaneous strength and aerobic fitness training, 6 saliva samples and 3 physical tests and 2 measures of body composition were made during the training period. Wrestlers had a significant increase (+1.5 kg) in body weight without changes in percentage body fat. Apart from the 20-m maximal shuttle speed, all performances increased significantly during the 15 weeks of training: maximum mechanical power output (Pmax: +13 %), mean power during 30 seconds (Pmean: +11 %), bench press (+6 %), squat (+23 %), power clean (+6 %), time to 3,000- and 30-m sprints (-3.6, -1.3 % respectively). During the period that the C increased, there was no significant variation for the T. The T/C ratio followed a variation pattern contrary to that of the C. It was found strong correlations between salivary T, C, and T/C and the variation in explosive strength. The results suggest that data about subjects’ salivary C, T, and T/C may be employed to optimize the training process for sports people who need to develop strength and aerobic fitness simultaneously [12107].

It was developed a simple and sensitive method for the simultaneous determination of testosterone (TES), cortisol (CRT), and dehydroepiandrosterone (DHEA) in saliva by automated online in-tube solid-phase microextraction (SPME) coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Discovery HS F5 column. The optimum in-tube SPME conditions were 25 draw/eject cycles of 40 μL of sample at a flow rate of 200 μL/min using a Supel-Q PLOT capillary column as an extraction device. The extracted compounds were easily desorbed from the capillary by passage of the mobile phase, and no carryover was observed. The in-tube SPME LC-MS/MS method showed good linearity with correlation coefficients r ≥ 0.9998 for TES, CRT, and DHEA using their respective stable isotope-labeled internal standards. The intra-day and inter-day precisions (relative standard deviations) were below 4.9 and 8.5 percent (n=5), respectively. This method was successfully utilized to analyze TES, CRT, and DHEA in saliva samples without any other pretreatment or interference peaks, and the quantification limits (S/N = 10) of TES, CRT and DHEA were about 0.01, 0.03 and 0.29 ng/mL saliva, respectively. The recoveries of these compounds spiked into saliva samples were each above 94 percent. This method was applied to analyze changes in salivary TES, CRT, and DHEA levels resulting from stress and fatigue load [12108].

In women
To compare the baseline free testosterone (T) and cortisol (C) concentrations of elite and non-elite female athletes 18 females from different sports (track and field, netball, cycling, swimming, bob skeleton) were monitored over a 12-week period. Baseline measures of salivary free T and C concentrations were taken weekly prior to any training. The elites (n=9) and non-elites (n=9) were classified as international and national level competitors, respectively, with both groups matched by sport. The pooled free T concentrations of the
elites (87 pg/ml) were significantly higher than the non-elites (41 pg/ml) and consistently so across all weekly time points. Pooled free C concentrations were also greater in the elite group (2.90 ng/ml) than the non-elites (2.32 ng/ml). The pooled baseline T and C measures were higher in elite female athletes than non-elites. Higher free T and C concentrations could indicate a greater capacity for physical performance at higher work rates, which is commensurate with the demands of elite sport. Speculatively, the T differences observed could influence female behavior and thereby help to regulate sporting potential [12109].

**Laboratory techniques**

A sensitive and rapid liquid chromatographic (LC) method for the simultaneous determination of testosterone (T) and epitestosterone (E) in human urine samples has been developed and elaborated. The ratio of the both steroids (T/E) in human urine is a widely used as doping control indicator. A sample pretreatment by solid-phase extraction (SPE) after hydrolysis using 36 percent hydrochloric acid for determination of total level of T has been applied. Unconjugated (free) form of the both androgens was determined without hydrolysis steps, what makes novelty of the method, because simplifies the proposed procedure. In turn, the measurements of urinary free T and E provided the diagnostic information for excess adrenal production of steroids. The proposed LC assay was evaluated by analyzing a series of urine samples containing T, E and methyltestosterone (MT) as internal standard at the range of concentration 2-300 ng/mL of both analyzed hormones. The proposed method was fully validated for specificity, linearity, limits of detection and quantitation, precision and trueness according to the current requirements concerning analytical methods. Interestingly, the developed LC method allows to obtain a sensitive enhancement with respect to UV detection with the quantitation limit for T and E equaled 2 ng/mL. The method was selective and reliable for identity and enables to detect changes of endogenous levels of T and E in urine independently of fluctuations characteristic for both analyzed endogenous hormone level in plasma [12110].

**Biological action**

The anabolic hormone testosterone induces muscle hypertrophy, but the intracellular mechanisms involved are poorly known. It was addressed the question whether signal transduction pathways other than the androgen receptor (AR) are necessary to elicit hypertrophy in skeletal muscle myotubes. Cultured rat skeletal muscle myotubes were preincubated with inhibitors for ERK1/2 (PD98059), PI3K/Akt (LY294002 and Akt inhibitor VIII) or mTOR/S6K1 (rapamycin), and then stimulated with 100 nM testosterone. The expression of α-actin and the phosphorylation levels of ERK1/2, Akt and S6K1 (a downstream target for mTOR) were measured by Western blot. mRNA levels were evaluated by real time RT-PCR. Myotube size and sarcomerization were determined by confocal microscopy. Inhibition of AR was assessed by bicalutamide. Testosterone-induced myotube hypertrophy was assessed as increased myotube cross-sectional area (CSA) and increased alpha-actin mRNA and alpha-actin protein levels, with no changes in mRNA expression of atrogenes (MAFbx and MuRF-1). Morphological development of myotube sarcomeres was evident in testosterone-stimulated myotubes. Known hypertrophy signaling pathways were studied at short times: ERK1/2 and Akt showed an increase in phosphorylation status after testosterone stimulus at 5 and 15 min, respectively. S6K1 was phosphorylated at 60 min. This response was abolished by PI3K/Akt and mTOR inhibition but not by ERK1/2 inhibition. Similarly, the CSA increase at 12 h was abolished by inhibitors of the PI3K/Akt pathway as well as by AR inhibition. These results suggest a crosstalk between pathways involving fast intracellular signaling and the AR to explain testosterone-induced skeletal muscle hypertrophy [13189].
Anabolic steroids are performance enhancers, this being particularly apparent in women, although there is a high risk of virilization despite the favourable myotrophic-androgenic dissociation that many xenobiotic steroids confer. Modulation of androgen receptor expression appears to be key to partial dissociation, with consideration of both intracellular steroid metabolism and the topology of the bound androgen receptor interacting with co-activators. An anticatabolic effect, by interfering with glucocorticoid receptor expression, remains an attractive hypothesis. Behavioural changes by non-genomic and genomic pathways probably help motivate training. It is important not to exaggerate the medical risks associated with their administration for sporting or bodybuilding purposes but to emphasize to users that an attitude of personal invulnerability to their adverse effects is certainly misguided [08064].

Androgens are modulators of skeletal muscle adaptation and regeneration processes. The control of satellite cell activity is a key mechanism during this process. In this study, it was analyzed the ability of dihydrotestosterone (DHT) and anabolic steroids to induce and modulate the differentiation of myoblastoma cells toward myotubes. Myoblastoma cells were dose-dependently treated with DHT and anabolic steroids. The time-dependent effects on differentiation were measured and correlated with the expression of genes involved in the regulation of satellite cell activity. The distribution of myoblastoma cells within the cell cycle was measured by flow cytometry and differentiation by creatine kinase (CK) activity. Gene expression was analyzed using quantitative real-time PCR and confocal microscopy. The treatment with DHT and anabolic steroids resulted in a stimulation of myoblastoma cell proliferation and CK activity. The antiandrogen flutamide was able to antagonize this effect. The expression of the androgen receptor, SOX8, SOX9, Delta, Notch, myostatin, and paired box gene7 (Pax7) was modulated by androgens. The treatment with DHT and anabolic steroids resulted in a strong stimulation of myostatin expression not only in undifferentiated cells but also in myotubes. The stimulation could be antagonized by flutamide. The expression of Pax7 was detectable in myoblastoma cells early after treatment with DHT. The results demonstrate that the key mechanisms of satellite cell differentiation are modulated by androgens. Androgens stimulate the proliferation of myoblastoma cells, accelerate the process of differentiation, and increase the expression of myostatin in undifferentiated and differentiated cells [08065].

Important mechanisms behind the myotrophic effects of testosterone were uncovered both in athletes using steroids for several years and in short-term controlled studies. Both long-term and short-term steroid usage accentuates the degree of fibre hypertrophy in human skeletal muscle by enhancing protein synthesis. A mechanism by which testosterone facilitates the hypertrophy of muscle fibres is the activation of satellite cells and the promotion of myonuclear accretion when existing myonuclei become unable to sustain further enhancement of protein synthesis. Interestingly, long-term steroid usage also enhances the frequency of fibres with centrally located myonuclei, which implies the occurrence of a high regenerative activity. Under the action of testosterone, some daughter cells generated by satellite cell proliferation may escape differentiation and return to quiescence, which help to replenish the satellite cell reserve pool. However, whether long-term steroid usage induces adverse effects of satellite cells remains unknown. Testosterone might also favour the commitment of pluripotent precursor cells into myotubes and inhibit adipogenic differentiation. The effects of testosterone on skeletal muscle are thought to be mediated via androgen receptors expressed in myonuclei and satellite cells. Some evidence also suggests the existence of an androgen-receptor-independent pathway. Clearly, testosterone abuse is associated with an intense recruitment of multiple myogenic pathways. This provides an unfair advantage over non-drug users. The long-term consequences on the regenerative capacity of skeletal muscle are unknown [08066].
There is strong evidence that androgen administration in men increases skeletal muscle mass, maximal voluntary strength and muscle power. However, there is no good experimental evidence to support the presumption that androgen administration improves physical function or athletic performance. Androgens do not increase specific force or whole body endurance measures. The anabolic effects of testosterone on the skeletal muscle are mediated through androgen receptor signaling. Testosterone promotes myogenic differentiation of multipotent mesenchymal stem cells and inhibits their differentiation into the adipogenic lineage. Testosterone binding to androgen receptor induces a conformational change in androgen receptor protein, causing it to associate with beta-catenin and TCF-4 and activate downstream Wnt target genes thus promoting myogenic differentiation. The adverse effects of androgens among athletes and recreational bodybuilders are under reported and include acne, deleterious changes in the cardiovascular risk factors, including a marked decrease in plasma high-density lipoproteins (HDL) cholesterol level, suppression of spermatogenesis resulting in infertility, increase in liver enzymes, hepatic neoplasms, mood and behavioral disturbances, and long term suppression of the endogenous hypothalamic-pituitary-gonadal axis. Androgens are often used in combination with other drugs which may have serious adverse events of their own [08067].

One study examined the anabolic-hormone response to carbohydrate (CHO) supplementation at rest and after resistance exercise. Nine recreationally trained men randomly underwent 4 testing conditions: rest with placebo, rest with CHO, resistance exercise with placebo, and resistance exercise with CHO. The resistance-exercise protocol was four sets of Smith machine squats with a 10-repetition-maximum load, with 90-s rests between sets. Participants then consumed either a placebo or CHO (24 % CHO, 1.5 g/kg) drink. Blood was taken before exercise, immediately after testing, and then 15, 30, and 60 min after drink ingestion. Blood was analyzed for cortisol, glucose, insulin, and total testosterone (TTST). Cortisol did not change significantly in any condition. Glucose concentrations increased significantly. Insulin concentrations increased significantly under resistant conditions. There were no significant changes in total testosterone concentrations during rest with or without carbohydrate in contrast to the exercise groups. It was concluded that ingesting carbohydrates after resistance exercise resulted in decreased total testosterone concentrations during recovery, although the mechanism is unclear [00868].

Many hormones (e.g. catecholamines, growth hormone, adrenal steroids, androgens, etc.) influence health status, exercise/sport performances and the physiological adaptation to exercise-related stress in athletes. In addition to classic reproductive and sexual effects (e.g. sexual behaviour, penis growth, erection, secondary sexual characteristics and spermatogenesis), and also depending on the role of CAG repeat polymorphism on androgen receptors biological activity, endogenous testosterone exert a wide spectrum of actions in males. Particularly, testosterone can differently influence body composition (e.g. muscles growth, fat mass, bone density), central nervous system maturation and functions (e.g. behavior characteristics, aggression and cognitive processes), endocrine and metabolic pathways (glucose metabolism, insulin and leptin), muscles physiology and motor behaviour, erythropoiesis, and adaptation to stress. A biologically normal testosterone secretion appears therefore fundamental in males to guarantee both a physiological exercise adaptation and safe sport participation. The reproductive system is highly sensitive to the effects of exercise-related stress and the reproductive hormones may both increase and decrease after different acute or chronic exercises. Exercise and sport participation may positively or negatively influence andrological health status depending on the type, intensity and duration of performed physical activity and on individual health status. In addition, prohibited substances administration (e.g. androgenic-anabolic steroids, and so forth) in competitive and non-competitive athletes represents the main cause of iatrogenic andrological diseases [12094].
Hypothalamic-pituitary-gonadal (HPG) axis

The hypothalamic-pituitary-gonadal (HPG) axis is regulated by a negative feedback mechanism. Testosterone inhibits the frequency and amplitude of gonadotropin-releasing hormone (GnRH) release from the hypothalamus and also the secretion of luteinizing hormone (LH) from the pituitary. The Sertoli cells of the testes, in addition to stimulating spermatogenesis, also secrete the glycoprotein hormone inhibin, which provides negative feedback to the pituitary, inhibiting the secretion of follicle stimulating hormone (FSH).

Testosterone is converted to dihydrotestosterone (DHT) by 5-alpha-reductase enzymes or to estradiol by P450 aromatase in target cells. Testosterone and DHT both bind to the androgen receptor where they exert their biological effects. Approximately 20 percent of the DHT in the circulation is produced directly by testicular secretion, with the remaining 80 percent being derived from conversion of testosterone in peripheral tissues. Target cells that contain 5-alpha-reductase are concentrated in the prostate, reproductive system, and skin. Aromatase containing cells predominate in the liver, adipose tissue, and regions of the brain.

Total serum testosterone is composed of 3 components added together. Roughly half of testosterone is bound to the carrier molecule sex hormone-binding globulin (SHBG), almost all of the remainder is bound to albumin, and 1-2 percent is unbound or free. Testosterone binds so tightly to SHBG that it is functionally unavailable to cells. In contrast, albumin-bound testosterone dissociates readily, meaning that this component and the free component are available to cells. The term “bioavailable testosterone” refers to a combination of the albumin-bound and free portions [12095].

Brain

Sex steroids readily pass the blood-brain barrier, and receptors for them are abundant in brain areas important for the regulation of emotions, cognition and behaviour. The sex steroid receptors are ligand-activated transcription factors that bind to specific hormone response elements in their target genes. There are two subtypes of estrogen receptors: alpha and beta [08069]. In addition, several isoforms of each subtype have been reported [08070]. The two estrogen receptor subtypes have comparable affinities to estradiol, but many other ligands show preferential binding to one or the other of them. The two subtypes also differ with respect to tissue distribution and coregulator interactions [08071, 08072]. The human estrogen receptor α gene (ESR1) is located on chromosome 6q25.1 [08073] and composed of 8 exons. A large number of polymorphisms in this gene have been identified, none of which has as yet been shown beyond doubt to be functional. The human estrogen receptor β gene (ESR2) is located on chromosome 14q22–24. The gene is composed of 8 exons [08074] and has several polymorphisms.

So far, there seems to be only a single subtype of the androgen receptor and the progesterone receptor. Alternative splicing of the amino terminal of androgen receptor and progesterone receptor genes, however, results in different isoforms displaying differences in both expression and function [08075, 08076].

The human progesterone receptor gene (PGR) is located on chromosome 11q22-23 [077] and composed of 8 exons. The receptor exists in 2 molecular forms, PR-A and PR-B; these differ only at the amino terminus, with PR-B containing an additional stretch of amino acids. This domain plays an important role in identifying target genes that can be activated by the PR-B protein but not by the PR-A protein. The expression ratio of the 2 PR isoforms in the brain varies during fetal development and as a result of the estrous cycle and also differs between males and females. Administration of estrogen and progesterone has been shown
to influence the expression ratio, and some of these variations may therefore be induced by these hormones. Notably, PR-A has recently been shown to play a key role in both hormone-dependent and hormone-independent facilitation of female sexual behavior [08078].

The androgen receptor gene (AR) is located on chromosome Xq11-12 and composed of 8 exons [08079, 08080]. Preliminary evidence from several studies also suggests that AR repeat polymorphisms may be of importance for interindividual differences in personality traits.

Coregulators of sex steroid receptors play an important role for tissue-specific actions of sex steroids. Several coregulators of importance for brain function have been identified [08081]; for example, both the steroid receptor coactivator gene and CREB-binding protein have been shown to be involved in estrogen receptor–mediated effects on sexual behavior [08082]. Moreover, the coactivator estrogen receptor–associated protein 140, which interacts with both estrogen receptor α and estrogen receptor β, displays its highest expression in the brain [083]. Another protein expressed in the brain that, among other tasks, serves as coactivator for sex steroid receptors, is E6-associated protein (UBE3A) [08084].

The aromatase enzyme converts androgens into estrogens. The human aromatase gene (CYP19) is located on 15q21.1 and contains several genetic variants [08085, 08086].

Notably, enzymes required for the synthesis of sex steroids, as well as for functionally active sex steroid metabolites, are expressed locally within the brain; some of the sex steroids present in the central nervous system are thus probably produced locally [08087]. For example, within the brain, progesterone is metabolized to allopregnanolone [08088, 08089] that may influence behaviour by interacting with GABA_A receptors. The genes for the 5 alpha-reductase type 1 and type 2 enzymes, which are critical for this conversion, contain functional polymorphisms [08090] that could be relevant for the study of psychiatric disorders for which allopregnanolone has been attributed importance, such as premenstrual dysphoric disorder.

Animal experiments have revealed that sex steroids have both an important early and permanent influence on brain development and an ongoing influence on brain neurotransmission in the adult organism. The influence exerted by sex steroids on animal behaviour, including sexual activity and aggression, is exerted by both these mechanisms [08091-08094]. That sex steroids also influence behavior in humans is shown by the reduction in libido that often follows a decrease in serum sex steroids and by conditions such as premenstrual dysphoric disorder (where the symptoms coincide with sex steroid fluctuations in serum and can be abolished by means of ovariectomy or treatment with ovulation inhibitors), postpartum depression, dysphoria induced by oral contraceptives and changes in behavior induced by anabolic steroids [08094-08097]. The hypothesis that sex hormones play a role in the regulation of mood and behavior also gains support from the fact that a large number of psychiatric conditions, including depression, panic disorder, generalized anxiety disorder, social phobia and eating disorders, are more prevalent in women than in men. In contrast, alcoholism, attention-deficit hyperactivity disorder and autism are more common in men. As well, with respect to normal personality traits, there are subtle but clear differences between women and men at the group level (e.g. with respect to anxiety-related traits). Similarly, certain aspects of cognitive abilities appear to differ slightly between the sexes. Autism is a disorder of particular interest in regard to the possible role of sex steroids; there is evidence suggesting that subjects with autism are characterized by a brain that, in certain aspects, may be regarded as unusually masculinized [08093, 08094].

Anabolic androgenic steroids at supratherapeutic doses seem to improve physical
appearance and the drug user becomes more bold and courageous. Investigations of the possible neurochemical effects of AAS have focused partially on the monoaminergic systems, which are involved in aggressive behaviours and the development of drug dependence. In one study, it was administered nandrolone decanoate (3 or 15 mg/kg/day for 14 days) and measured mRNA expression of dopaminergic and serotonergic receptors, transporters and enzymes in the male rat brain using quantitative real-time polymerase chain reaction. Expression of the dopamine D1-receptor transcript was elevated in the amygdala and decreased in the hippocampus while the transcript level of the dopamine D4-receptor was increased in the nucleus accumbens. No changes in transcriptional levels were detected among the serotonin-related genes examined in this study. The altered mRNA expression of the dopamine receptors may contribute to some of the behavioural changes often reported in abusers of anabolic steroids of increased impulsivity, aggression and drug-seeking [08098].

Heart

The aim of one study was to investigate the effects of anabolic androgenic steroids on the cardiac structure and the plasma lipoprotein profile isolated and in combination with exercise. Transgenic mice with a human lapaemic phenotype (expressing cholesteryl ester transfer protein on the LDL receptor knockout background) were used in this study. Sedentary and exercised mice (treadmill running, five times per week for 6 weeks) were treated with mesterolone (2 microg/g body weight) or vehicle (control-C) in the last 3 weeks. Four groups were compared: (i) exercise + mesterolone (Ex-M), (ii) exercise + vehicle (Ex-C), (iii) sedentary + mesterolone (Sed-M) and (iv) sedentary + vehicle (Sed-C). Arterial blood pressure and body mass increased in all groups along time, but Sed-M reached the highest values and Ex-C the lowest. Treatment with mesterolone increased total cholesterol, triglyceride, low-density lipoprotein cholesterol (LDL-c) and very LDL-c (VLDL-c) plasma levels. However, exercise blunted some of these deleterious effects by increasing high-density lipoprotein cholesterol and decreasing LDL-c, VLDL-c and triglycerides. Exercise training induced beneficial effects, such as physiological cardiomyocyte hypertrophy, increase in myocardial circulation and decrease in cardiac interstitium. However, mesterolone impaired such physiological gains and in addition increased troponin T plasma levels both in sedentary and exercised mice. Thus, while mesterolone induced pro-atherogenic lipoprotein profile and pathogenic cardiac hypertrophy, exercise counteracted these effects and modified favourably both the lipoprotein profile and the cardiac remodelling induced by mesterolone [08099].

Tendon

Matrix metallopeptidases (MMPs) are responsible for degradation of the extracellular matrix components and tissue remodeling. To achieve a better understanding of anabolic steroids effects in rat tendon, MMP-2 activity in the proximal and distal regions of the calcanear tendon and proximal, intermediate and distal region of superficial and deep flexor tendons after mechanical load exercise associated with anabolic androgenic steroids was investigated. In animals both proximal and distal regions of the calcanear tendon showed the lowest MMP-2 concentration and the highest proportion in MMP-2 active form. The intermediate region of the superficial flexor tendons differed significantly from the proximal and distal regions with higher proportion of active MMP-2 in the sedentary group. The proportion of active MMP-2 decreased in the proximal region of the calcanear tendon. The differences in the response to exercise and androgenic anabolic steroid treatment are a result of distinct metabolism and recruitment of these tendon regions in the exercise program employed in this study [08100].
The growing and indiscriminate use of high doses of anabolic androgenic steroid (AAS) among youth and athletes has raised serious concerns about its hepatotoxic effects. Now the influence of AAS in the nuclear phenotype of hepatocytes was investigated in sedentary and trained mice heterozygous for the human CETP (cholesteryl ester transfer protein) transgene and for LDL-receptor null allele (CETP+/−/LDLr+/−) by image analysis. Sedentary blank control mice showed the lowest chromatin condensation and highest Feulgen-DNA content, polyploid nuclei frequency, nuclear area and perimeter, suggesting gene activation. Contrarily, exercised mice showed the highest chromatin condensation, and a significant decrease of Feulgen-DNA content and decreased frequency of polyploid nuclei, which suggest gene silencing. Image analysis of the nuclear phenotype offered a coherent descriptive picture of the changing patterns of chromatin organization, which were shown to be congruent with the levels of Feulgen-DNA content, geometric nuclear parameters and hepatocyte activity. In the study, the image analysis permitted the monitoring of the nuclear response to mesterolone and physical exercise action in liver cells, the molecular mechanism of which is in prospect [08101].

Use of testosterone enanthate has been shown to significantly increase strength within 6-12 weeks of administration, however, it is unclear if the ergogenic benefits are evident in less than 6 weeks. The two objectives of this study were to establish if injection of 3.5 mg/kg testosterone enanthate once per week could increase muscular strength and cycle sprint performance in 3-6 weeks; and if the WADA-imposed urinary T/E ratio of 4:1 could identify all subjects being administered 3.5 mg/kg testosterone enanthate. Sixteen healthy young men were match-paired and were assigned randomly in a double-blind manner to either a testosterone enanthate or a placebo group. All subjects performed a structured heavy resistance training program while receiving either testosterone enanthate (3.5 mg/day) or saline injections once weekly for 6 weeks. One repetition maximum (1RM) strength measures and 10-second cycle sprint performance were monitored at the pre (week 0), mid (week 3), and post (week 6) time points. Body mass and the urinary T/E ratio were measured at the pre (week 0) and post (week 6) time points. When compared with baseline (pre), 1RM bench press strength and total work during the cycle sprint increased significantly at week 3 and week 6 in the testosterone enanthate group, but not in the placebo group. Body mass at week 6 was significantly greater than at baseline in the testosterone enanthate group, but not in the placebo group. Despite the clear ergogenic effects of testosterone enanthate in as little as 3 weeks, 4 of the 9 subjects in the testosterone enanthate group (approximately 44 %) did not test positive to testosterone under current WADA urinary T/E ratio criteria [07087].

In myocytes
In myocytes, testosterone binds to the intracellular androgen receptor (AR), initiating an activation cascade with conformational changes and nuclear translocation of the AR-steroid complex. Binding of the complex to androgen responsive elements (ARE) in the DNA results in specific activation or repression of the transcription in target genes [12096].

Breast
Gynecomastia is a common finding in adolescent men. While gynecomastia has long been attributed to an imbalance between estrogen and androgen concentrations, recent literature has begun to illuminate other potential mechanisms for breast development in adolescent men. Increased leptin levels, as well as human chorionic gonadotropin and luteinizing hormone receptors on male breast tissue, may play a role. Newer treatment strategies, such as the antiestrogen raloxifene, have shown promising results; however, further studies are
needed to determine long-term efficacy. As a result of the limited pharmaceutical treatment options, many more adolescents are seeking surgical intervention. Careful attention should be paid to both the breast and testicular examination. A detailed history should include an inquiry regarding the use of illicit substances, anabolic-androgenic steroids, herbal products, and medications. The impact of gynecomastia on the adolescent's mental health should be assessed. Reassurance remains the standard of care for physiologic gynecomastia [08102].

**Liver**

Anabolic steroid abuse is associated with a number of medical complications. Reported hepatic complications include cholestasis, elevation of aminotransferases, jaundice, benign hepatic adenomas, and rare cases of hepatocellular carcinoma. Histologic findings include peliosis hepatis, a lesion characterized by hepatic sinusoidal dilatation that is often cystic. Rupture of these cysts can cause fatal internal hemorrhage. The risk of androgen-associated liver tumors appears to correlate with the cumulative androgen dose and the potency of the steroid used [08103-08110].

It was described a case of a 27-year-old male bodybuilder with multiple hepatic adenomas induced by anabolic steroids. He initially presented with tumor hemorrhage and was treated with left lateral hepatic segmentectomy. Regression of the remaining tumors was observed with cessation of steroid use. However, 3 years and a half after his initial hepatic segmentectomy, he presented with recurrent tumor enlargement and intraperitoneal hemorrhage in the setting of steroid abuse relapse. This was the first reported case of hepatic adenoma re-growth with recidivistic steroid abuse, complicated by life-threatening hemorrhage [08111].

**Testosterone deficiency**

Testosterone deficiency (TD) afflicts approximately 30 percent of men aged 40-79 years, with an increase in prevalence strongly associated with aging and common medical conditions including obesity, diabetes, and hypertension. Clinical symptoms of TD include fatigue, decreased libido, ED, and negative mood states. TD also is associated with changes in body composition, including decreased lean body mass, increased fat mass, and decreased bone mineral density. A significant increased risk of TD is noted in association with common medical conditions such as obesity, type 2 diabetes mellitus (T2DM), and hypertension. In addition, a strong relationship was observed between TD and the metabolic syndrom. Further, recent studies in women with complete androgen insensitivity syndrome showed increased body fat, abnormal values of cholesterol, and homeostasis model assessment of insulin resistance (HOMA-IR), suggesting that disruption of androgen signaling in women also is associated with metabolic disorders. Repletion of testosterone (T) in T-deficient men with these co-morbidities may indeed reverse or delay their progression. Studies of testosterone replacement therapy (TRT) on sexual function and performance vary in quality, although findings are generally consistent. Most studies show that TRT increased sexual awareness and arousal, erectile function, and the frequency of spontaneous erections, but is less consistent in enhancing sexual behavior and performance. A number of TRT preparations are currently available. Intramuscular injections of short-acting testosterone derivatives achieve good serum concentrations within 2-3 days, with levels returning to baseline in most men by 2 weeks, resulting in an injection schedule of 1-2 weeks. Topical gels or patches provide a more stable serum-testosterone concentration over time than injections. Patches currently available are associated with a high rate of skin reaction, and their use has been largely supplanted by testosterone gels. The main disadvantages of testosterone gels are cost and a black box warning concerning transfer potential to women and children. A long-acting injection formulation (testosterone undecanoate) is dosed every
10-12 weeks, and is available internationally. Testosterone pellets provide 3-6 months of normal serum testosterone, and are placed subcutaneously in the gluteal region via an in-office surgical implantation procedure under local anesthesia; this formulation also has some disadvantages such as extrusion of pellets post surgical procedure. In addition, the genetic background relating to the patient responsiveness to androgens, hence, androgen receptor polymorphisms, are likely to play an inter-individual role. Adverse events that have been definitively associated with treatment are reversible with cessation of treatment. These include acne, gynecomastia, erythrocytosis, and edema. A number of additional risks have appeared in the literature, but their relationship to TRT is less well established. These include sleep apnea, worsening of urinary voiding symptoms, and prostate cancer. Standard forms of TRT do not appear to adversely affect lipid profiles and does not appear to cause liver toxicity, with the exception of oral alkylated testosterone preparations (e.g. methyltestosterone), which should not be used for testosterone replacement therapy for this reason. A key area of controversy relates to the biochemical determination of TD. There is no defined serum threshold for testosterone. Yet all published guidelines recommend one arbitrary threshold or another, generally ranging from 200 to 350 ng/dL (6.94-12.15 nmol/L)]. Variation in sex hormone-binding globulin levels also confounds the interpretation of bioavailable testosterone levels. There is general agreement that free or bioavailable testosterone provides a better estimation of testosterone status, but there is uncertainty about the reliability of those assays. In addition, genetic variation may influence response to circulating testosterone [11328].

**Opioid-induced androgen deficiency (OPIAD)**

Morphine sulfate, through its binding to opioid receptors, is known to act in several body regions from the gut to the brain. Previous studies have demonstrated that morphine induces a dramatic long-lasting decrease in testosterone, which persists during opioid therapy even if the treatment lasts for months or years, in both males and females. The effect can occur after a few hours, with testosterone concentrations reaching castration levels (< 1 ng/mL). Aloisi and colleagues have also shown that once opioid treatment is interrupted, testosterone levels recover in a few hours/days. Furthermore, spinal (intrathecal or epidural) administration of morphine resulted in a similar reduction in testosterone in both males and females. Opioid therapy is one of the most effective forms of analgesia currently in use. In the past few decades, the use of opioids as a long-term treatment for chronic pain has increased dramatically. Accompanying this upsurge in the use of long-term opioid therapy has been an increase in the occurrence of opioid associated endocrinopathy, most commonly manifested as an androgen deficiency and therefore referred to as opioid associated androgen deficiency (OPIAD). This syndrome is characterized by the presence of inappropriately low levels of gonadotropins (follicle stimulating hormone and luteinizing hormone) leading to inadequate production of sex hormones, particularly testosterone. Symptoms that may manifest in patients with OPIAD include reduced libido, erectile dysfunction, fatigue, hot flashes, and depression. Physical findings may include reduced facial and body hair, anemia, decreased muscle mass, weight gain, and osteopenia or osteoporosis. Additionally, both men and women with OPIAD may suffer from infertility. While the literature regarding OPIAD remains limited, it is apparent that OPIAD is becoming increasingly prevalent among chronic opioid consumers but often goes unrecognized. OPIAD can have a significant negative impact on the the quality of life of opioid users, and clinicians should anticipate the potential for its occurrence whenever long-term opioid prescribing is undertaken. Once diagnosed, treatment for OPIAD may be offered utilizing a number of androgen replacement therapy options including a variety of testosterone preparations and, for female patients with OPIAD, dehydroepiandrosterone (DHEA) supplementation. Follow-up evaluation of patients receiving androgen replacement therapy should include a review of any unresolved symptoms of hypogonadism, laboratory evaluation, and surveillance for
potential adverse effects of androgen replacement therapy including prostate disease in males [12095].

**Testosterone and motivation to compete**

It is possible that high-testosterone individuals have increased motivation to compete in sports. High-testosterone individuals may select into sports as a function of testosterone's positive influence on dominance striving, also known as power motivation. Basal testosterone is positively correlated with power motivation in men, whereas basal estradiol is positively correlated with power motivation in women. High concentrations of testosterone are also positively associated with selection into power-laden careers, e.g., trial law and acting. Knowing that power-motivated individuals are motivated to pursue dominance and find dominance experiences rewarding, the positive association between testosterone and power motivation suggests that high testosterone individuals may be the individuals most motivated to pursue athletic competition. Testosterone is also associated with reduced empathy, reduced perception of negative emotions enhanced attention to social threat, and enhanced amygdala responses to social threat. Additionally, testosterone has been linked to increased risk-taking in economic domains and social domains. Lastly, testosterone is associated with enhanced visuospatial ability, which may provide greater abilities in the perceiving critical targets and navigating the physical sports environment, i.e., field, rink, or court. Thus, high endogenous concentrations of testosterone may confer both psychological and physiological advantage in sports [12100].

It was tested the effects of different post-match recovery interventions on the subsequent hormonal responses to a physical stress-test and game performance in professional rugby union players. On four occasions, participants (n=12) completed a video session (1 h each) with accompanying coach feedback the day after a rugby union match. The interventions showed either video footage of player mistakes with negative coach feedback (NCF1) or player successes with positive feedback (PCF1). Both approaches were repeated (NCF2 and PCF2). In the following week, participants were assessed for their free testosterone (T) and cortisol (C) responses to a physical stress-test, pre-game T and game-ranked performance. The PFC1 and PCF2 approaches were both associated with significantly greater free T (36 % to 42 %) responses to the stress-test when compared to NCF1 and NCF2 (16 % to -3 %), respectively. The PCF interventions were also associated with higher (28 % to 51 %) pre-game T concentrations and superior game-ranked performances than the NCF approaches. In conclusion, the post-game presentation of specific video footage combined with different coach feedbacks appeared to influence the free hormonal state of rugby players and game performance several days later. Therefore, within the sporting context, future behaviour and performance might be modified through the use of simple psychological strategies. These data are applicable to generalised human stress responses and their modifiability by prior exposure to a stressor [12104].

**Effect of magnesium on testosterone levels**

One study was performed to assess how 4 weeks of magnesium supplementation and exercise affect the free and total plasma testosterone levels of sportsmen practicing tae kwon do and sedentary controls at rest and after exhaustion. The testosterone levels were determined at four different periods: resting before supplementation, exhaustion before supplementation, resting after supplementation, and exhaustion after supplementation in three study groups, which are as follows: group 1-sedentary controls supplemented with 10
mg magnesium per kilogram body weight; group 2-tae kwon do athletes practicing 90-120 min/day supplemented with 10 mg magnesium per kilogram body weight; and group 3-tae kwon do athletes practicing 90-120 min/day receiving no magnesium supplements. The free plasma testosterone levels increased at exhaustion before and after supplementation compared to resting levels. Exercise also increased testosterone levels relative to sedentary subjects. Similar increases were observed for total testosterone. The results show that supplementation with magnesium increases free and total testosterone values in sedentary and in athletes. The increases are higher in those who exercise than in sedentary individuals [10080].

**Psychological influence on testosterone levels**

Previous research indicates that testosterone concentrations are highly responsive to human competitive interactions and that winners have elevated testosterone concentrations relative to losers. Also, there is some evidence that simply observing others compete can have a similar effect on the endocrine system. Here, in two studies, it was examined the extent to which elite male hockey players would demonstrate an increase in testosterone concentrations after watching themselves engaged in a previous successful competitive interaction. Results indicated that watching a previous victory produced a significant increase in testosterone concentrations (42-44 % increase), whereas watching a previous defeat or a neutral video did not produce a significant change in testosterone (17 % and 6 %, respectively). Given that natural fluctuations in testosterone have been shown to influence future competitive and aggressive behaviours, the current studies may have important practical implications for individuals involved in competitive sports [09086].

**Influence of psychosocial environment**

The aim of one survey was to provide a literary review of current knowledge of the possible association between the psychosocial working environment and relevant physiological parameters measured in blood and urine. Literature databases (PubMed, Toxline, Biosis and Embase) were screened using the key words job, work-related and stress in combination with selected physiological parameters. In total, 51 work place studies investigated the associations between the psychosocial working environment and physiological changes, of which 20 were longitudinal studies and 12 population-based studies. The studied exposures in work place/population-based studies included: job demands (26/8 studies), job control (24/10 studies), social support and/or leadership behaviour (12/3 studies), effort-reward imbalance (three/one studies), occupational changes (four studies), shift work (eight studies), traumatic events (one study) and other (five studies). The physiological responses were catecholamines (adrenaline, noradrenaline) (14 studies), cortisol (28 studies), cholesterol (23 studies), glycated haemoglobinA(1c) (six studies), testosterone (nine studies), oestrogens (three studies), dehydroepiandrosterone (six studies), prolactin (14 studies), melatonin (one study), thyroxin (one study), immunoglobulin (Ig) A (five studies), IgG (four studies), IgM (one study) and fibrinogen (eight studies). In general, fibrinogen and catabolic indicators, defined as energy releasing, were increased, whereas the anabolic indicators defined as constructive building up energy resources were decreased when the psychosocial working environment was perceived as poor. In conclusion, in this review the association between an adverse psychosocial working environment and HbA(1c), testosterone and fibrinogen in serum was found to be a robust and potential candidate for a physiological effect of the psychosocial working environment. Further, urinary catecholamines appear to reflect the effects of shift work and monotonous work [09087].
**Victory or defeat**

In one study, it was reported evidence from sport competition that is consistent with the biosocial model of status and dominance. Results show that testosterone levels rise and drop following victory and defeat in badminton players of both sexes, although at lower circulating levels in women. After losing the match, peak cortisol levels are observed in both sexes and correlational analyses indicate that defeat leads to rises in cortisol as well as to drops in testosterone, the percent change in hormone levels being almost identical in both sexes. In conclusion, results show the same pattern of hormonal responses to victory and defeat in men and women [12097].

**Home or away game**

The authors examined the extent to which changes in testosterone concentrations before competition would be associated with performance among elite male hockey players. Saliva samples were collected on two noncompetition days (baseline) and before two playoff games (1 home game, 1 away game). Individual performance was assessed by the coaching staff after each game. Results indicated that changes in testosterone before competition predicted performance, but this effect was influenced by game location. Unexpectedly, the authors found a significant negative relationship between a rise in testosterone and performance for the away game and a nonsignificant positive relationship for the home game. These findings indicate that game location should be considered in studies examining the neuroendocrine correlates of athletic competition [12098].

**Spectators**

One field study investigated the release of testosterone and cortisol of a vicarious winning experience in Spanish fans watching the finals between Spain and the Netherlands in the 2010 FIFA World Cup Soccer. Spanish fans (n=50) watched the match with friends or family in a public place or at home and also participated in a control condition. Consistent with hypotheses, results revealed that testosterone and cortisol levels were higher when watching the match than on a control day. However, neither testosterone nor cortisol levels increased after the victory of the Spanish team. Moreover, the increase in testosterone secretion was not related to participants' sex, age or soccer fandom, but the increase in total cortisol secretion during the match was higher among men than among women and among fans that were younger. Also, increases in cortisol secretion were greater to the degree that people were a stronger fan of soccer. Level of fandom further appeared to account for the sex effect, but not for the age effect. Generally, the testosterone data from this study are in line with the challenge hypothesis, as testosterone levels of watchers increased to prepare their organism to defend or enhance their social status. The cortisol data from this study are in line with social self-preservation theory, as higher cortisol secretion among young and greater soccer fans suggests that especially they perceived that a negative outcome of the match would threaten their own social esteem [12099].

**Influence of stress on testosterone (and other anabolics) levels**

To determine whether cycling has an effect on serum PSA, gonadotropins, and uroflowmetric parameters a total of 34 healthy male athletes from the National Cycling Team and 24 healthy male student volunteers from University and medical staff were prospectively enrolled in a study. Blood samples for serum total prostate-specific antigen (tPSA), free PSA (fPSA, fPSA/tPSA, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and
testosterone determinations were obtained before and after cyclists completed 300 km bicycle ride and with each cyclist seated without changing posture and with minimal movement for 10 minutes before blood collection. The athletes and the control group were well matched by age. There was no significant difference between the 2 groups in terms of serum tPSA, fPSA, f/t PSA values, FSH, LH, and testosterone levels and uroflowmetric parameters. The postcycling serum testosterone level was significantly lower than precycling levels (mean, 604 ng/dL vs 425 ng/dL). There was no correlation between body mass index values, postcycling serum FSH, LH levels, age, and testosterone levels [09088].

**Hormone profile in men**

Steroid profiling provides valuable information to detect doping with endogenous steroids. Apart from the traditionally monitored steroids, minor metabolites can play an important role to increase the specificity and efficiency of current detection methods. The applicability of several minor steroid metabolites was tested on administration studies with low doses of oral testosterone (T), T gel, dihydrotestosterone (DHT) gel and oral dehydroepiandrosterone (DHEA). The collected data for all monitored parameters were evaluated with the respective population based reference ranges. Besides the traditional markers T/E, T and DHT, minor metabolites 4-OH-Adion and 6alpha-OH-Adion were found as most sensitive metabolites to detect oral T administration. The most sensitive metabolites for the detection of DHEA were identified as 16alpha-OH-DHEA and 7beta-OH-DHEA but longest detection up to three days (after oral administration of 50 mg) was obtained with non-specific 5β-steroids and its ratios. Steroids applied as a gel had longer effects on the metabolism but were generally not detectable with universal decision criteria. It can be concluded that population based reference ranges show limited overall performance in detecting misuse of small doses of natural androgens. Although some minor metabolites provide additional information for the oral testosterone and DHEA formulations, the topical administered steroids could not be detected for all volunteers using universal reference limits. Application of other population based threshold limits did not lead to longer detection times [10455].
Hormone profile in women

It is unclear whether hormone profiles obtained in two consecutive months are consistent within females. It was prospectively examined month to month consistency in daily, nadir, peak and mean hormone concentrations during the early follicular and luteal phases in recreationally active, young eumenorrheic females. Sixty healthy, non-smoking females who reported normal and consistent menstrual cycles lasting 26-32 days for the past 6 months were followed prospectively to obtain serum samples for the first 6 days of menses, and for 8 days following a positive ovulation test over two consecutive months. Month to month consistency of daily concentrations of estradiol (pg/mL), progesterone (ng/mL), testosterone (ng/dL), SHBG (nmol/L) and FAI were determined using linear mixed models. Month to month consistency in nadir, peak and mean concentrations were then assessed using intraclass correlation coefficients and standard error of the measurement to more precisely examine intra-individual consistency. Linear mixed models revealed stable hormone concentrations across cycles and cycles by day. Reliability estimates for nadir, peak, mean menses and mean postovulatory concentrations range from 0.56-0.86 for estradiol, 0.44-0.91 for progesterone, 0.60-0.86 for testosterone, 0.88-0.97 for SHBG, and 0.78-0.91 for FAI. It was concluded that hormone profiles were reproducible over two consecutive months. In order to reduce month to month intra-individual variations and improve measurement consistency, it was recommended that multiple samples be taken over consecutive days as opposed to a single sample [09102].

Influence of fasting (Ramadan)

The Ramadan fasting period is associated with changes in sleep habits and increased sleepiness, which may affect physical performance in athletes, and may induce metabolic, hormonal, and inflammatory disturbances. In 8 middle-distance athletes (25 ± 1 years), a maximal aerobic velocity (MAV) test was performed 5 days before Ramadan fasting period (day -5), and on days 7 and 21 of Ramadan fasting period. The same days, saliva samples were collected to determine cortisol and testosterone concentrations before and after the MAV test. During Ramadan fasting period, mean body mass and body fat did not statistically change. Compared with day -5, MAV values decreased significantly at days 7 and 21, while testosterone/cortisol ratio values did not change significantly. Nocturnal sleep time and energy intake were significantly lower at day 21 than before Ramadan fasting period. At the end of Ramadan fasting period (day 31), the fatigue score on the Profile of Mood States questionnaire was significantly increased. In conclusion, Ramadan fasting period is accompanied by significant metabolic, hormonal, and inflammatory changes. Sleep disturbances, energy deficiency, and fatigue during Ramadan fasting period may decrease physical performance in Muslim athletes who maintain training. Reduction of work load and (or) daytime napping may represent adequate strategies to counteract Ramadan fasting period effects for Muslim athletes [09089].

Circadian rhythm

The study investigated the effects of circadian rhythm of cortisol (C) and testosterone (T) on maximal force production ($F_{peak}$) and power output ($P_{peak}$). Twenty male university students (mean age 24 years) performed 4 time-of-day testing sessions consisting of countermovement jumps (CMJs), squat jumps (SJ), isometric midthigh pulls (IMTPs), and a 1-repetition maximum (1RM) squat. Saliva samples were collected at 0800, 1200, 1600, and 2000 hours to assess T and C levels on each testing day. Session rate-of-perceived exertion
(RPE) scores were collected after each session. The results showed that $F_{\text{peak}}$ and $P_{\text{peak}}$ presented a clear circadian rhythm in CMJ and IMTP but not in SJ. One repetition maximum squat did not display a clear circadian rhythm. Session RPE scores collected at 0800 and 2000 hours were significantly higher than those obtained at 1200 and 1600 hours. Salivary T and C displayed a clear circadian rhythm with highest values at 0800 hours and lowest at 2000 hours; however, no significant correlation was found between T and C with $F_{\text{peak}}$ and $P_{\text{peak}}$. A very strong correlation was found between $T_{\text{aural}}$ with $F_{\text{peak}}$ of CMJ and IMTP and $P_{\text{peak}}$ of CMJ. Thus, the study showed the existence of a circadian rhythm in $F_{\text{peak}}$ and $P_{\text{peak}}$ in CMJ and IMTP. The evidence suggests that strength and power training or testing should be scheduled later during the day. The use of $T_{\text{aural}}$ seemed to be a more effective indicator of physical performance than hormonal measures, and the use of session RPE should also be closely monitored because it may present a circadian rhythm [11095].

Genetic influence (polymorphism)

Doping with anabolic agents is regulated within a number of sports. Testosterone and its functional analogs are popular compounds for increasing muscle mass, physical performance, recovery, and reducing body fat. While routine tests for anabolic drugs exist (e.g. hair, urine, and blood analysis), the aim of the present study is to determine specific gene expression profiles (induced by testosterone and exercise) which may be used as effective biomarkers to determine the use of anabolic drugs. In one study, whole blood samples of 19 male volunteers were analyzed by semi-quantitative real-time polymerase chain reaction (RT-PCR) for gene expression profiles in the context of exercise and transdermal testosterone application (1.5 mg/kg body weight). The hormone application was monitored by urine and saliva analysis for testosterone. Both urinary and saliva levels indicate that transdermal testosterone application leads to an increase of testosterone, especially after exercise. RT-PCR results showed a clear variation in the expression of target genes as well as established housekeeping genes. Only one of the nine common housekeeping genes, cyclophilin b (PPIB), appears to be independent of both exercise and testosterone. Out of 14 candidate genes, five are unregulated; all others were more or less influenced by the mentioned variables. Only interleukin-6 appeared to be exclusively dependent on long-term testosterone application. This study indicates that many genes are not influenced by testosterone alone while exercise modulates gene expression in whole blood samples. As such, exercise must be considered when validating gene expression techniques for doping analysis [11442].

The ability to identify anabolic steroid use continues to improve. A sudden elevation of the testosterone–epitestosterone ratio typically indicates illegitimate use of an anabolic steroid. However, it is well known that some individuals may have naturally elevated ratios, and it has recently been recognized that 40 percent of those with a certain genotype found in two-thirds of people of Asian ancestry may not reach the threshold elevation of the ratio after administration of testosterone [08112].

The heritability of most behavioural traits, including personality, cognitive abilities and susceptibility to psychiatric illness, is considerable, but as yet, only few genes of definite importance in this context have been identified. Given the important role of sex steroids for brain function, it is unfortunate that relatively few studies so far have addressed the possible influence of sex steroid-related genes on interindividual differences with respect to personality, cognition and susceptibility to psychiatric disorders [08113].

Testosterone is excreted mainly as glucuronide conjugates after metabolism by uridine diphospho (UDP)-glucuronosyl transferases (UGT). It is well established that UGT2B7,
UGT2B15 and UGT2B17 are the principal catalysts of the glucuronidation of androgens and their metabolites in the human [08114]. Testosterone is mainly conjugated by UGT2B17 and, to a minor extent, by UGT2B15. The main androgen substrate of UGT2B15 is androstan-3α,17β-diol [115]. UGT2B17 shares 96 percent homology with UGT2B15 [08116], but its substrate specificity is broader [08115]. UGT2B7 has been shown to have the capacity to conjugate epitestosterone [08117] while testosterone is a poor substrate for this enzyme [08115].

Testosterone abuse is conventionally assessed by the urinary testosterone/epitestosterone (T/E) ratio, levels above 4.0 being considered suspicious. An alternative is determination of the $^{13}$C/$^{12}$C ratio of selected steroids (IRMS analysis) provides the possibility to distinguish between pharmaceutical and natural testosterone because exogenous compounds contain less $^{13}$C than their endogenous homologues [08118].

The large variation in testosterone glucuronide excretion and its strong association with a deletion polymorphism in the UGT2B17 gene challenge the accuracy of the T/E ratio test. Therefore, it was investigated in an open 3-armed comparative study whether genotype based cut-off values will improve the sensitivity and specificity of the test. Fifty-five healthy male volunteers with either two, one or no allele of the UGT2B17 gene were investigated after a single intramuscular dose of 500 mg testosterone enanthate. It was found that the degree and rate of increase in testosterone glucuronide excretion rate was highly dependent on the UGT2B17 genotype with a 20-fold average maximum difference. Forty percent of the subjects with deletions never reached the T/E ratio of 4.0 on any of the 15 days after the dose. This means that consideration of the genetic variation in disposition of androgens will improve the sensitivity and specificity of the testosterone doping test [203]. The polymorphism was considerably more common in a Korean Asian than in a Swedish Caucasian population, with 67 and 9 percent deletion/deletion homozygotes respectively [286, 287]. Continued experience of testing for anabolic steroids indicated that Asian individuals excrete lower amounts of testosterone glucuronide and hence have lower T/E ratios, thus increasing the risk of false-negative doping test results [08119]. This means that there are possible genetic differences between groups of individuals.

Testosterone is excreted in urine as water soluble glucuronidated and sulphatated conjugates. The ability to glucuronidate testosterone and other steroids depends on a number of different glucuronidases (UGT) of which UGT2B17 is essential. A clinical study of 116 healthy boys aged 8 to 19 years had UGT2B17 genotyping performed using quantitative PCR. Serum FSH, LH, T, estradiol (E$_2$) and SHBG were analysed by immunoassays, and urinary levels of androgen metabolites were quantitated by gas chromatography/mass spectrometry in all subjects. Ten out of 116 subjects (9 %) presented with a homozygote deletion of the UGT2B17 gene (del/del), while 52 and 54 boys were hetero- or homozygous carriers of the UGT2B17 gene (del/ins and ins/ins), respectively. None of the reproductive hormones were affected by UGT2B17 genotype. In all subjects, mean urinary T/E ratio was 1.56 ± 1.14 and unaffected by age or pubertal stage. Subjects with homozygous deletions of UGT2B17 had significantly lower urinary levels of T, and 5alpha- and 5beta-androstanediol. Mean urinary T/E was significantly reduced in del/del subjects (0.29 ± 0.30). It was concluded that in pubertal boys, a common homozygous deletion in the UGT2B17 gene strongly affected urinary excretion pattern of androgen metabolites, but did not influence circulating androgen levels [08120].

It has been known for more than a decade that urinary T/E ratios are significantly lower in certain ethnic groups. This observation has limited the effectiveness of a population-based T/E ratio as a screening test for testosterone use. Recent evidence has demonstrated that a deletion polymorphism in the UGT2B17 gene is responsible for reduced urinary testosterone
levels. UGT2B17 deletion polymorphism testing would be difficult to perform on every athlete at this time, and GC/C/IRMS is not a practical screening test for testosterone use [08016].

T/E ratio testing provided a solution for detecting synthetic testosterone use until Asian men were found to have a lower urinary T/E ratio (compared with Caucasians) more than a decade ago. Circulating concentrations of steroid hormones are controlled by the UDP-glucuronosyl transferase 2B (UGT2B) subfamily of uridine diphospho-glucuronosyl transferases, which facilitate urinary excretion by glucuronidation reactions that make steroid molecules more hydrophilic. UGT2B17 is the major enzyme in the UGT2B subfamily that conjugates glucuronide to testosterone, dihydrotestosterone, and androsterone in the liver and tissues. A common deletion polymorphism in the UGT2B17 gene was recently shown to differ among ethnic groups, being more common in whites than in African Americans. Further studies revealed that large differences in urinary testosterone concentrations are associated with a deletion polymorphism in the UGT2B17 gene. Men homozygous for the UGT2B17 deletion polymorphism have extremely low or undetectable urinary testosterone concentrations, and this genotype is 7 times more common in Korean men (67 %) than Swedish men (9 %). Epitestosterone concentrations are similar in the 2 ethnic groups, regardless of whether they have low or high urinary testosterone concentrations [08016].

The strong association of the UGT2B17 deletion polymorphism with testosterone excretion brings into question the ability of a population-based T/E ratio to detect testosterone use. Unfortunately, it would be difficult at this time for laboratories to incorporate genetic testing into their routine test menu and screen each athlete for UGT2B17 deletions. An alternative would be to use a low urinary T/E ratio (<0.2) as evidence for the del/del polymorphism. However, this would be problematic since it would incorrectly classify athletes that are doping with a combination of testosterone and epitestosterone (to lower their T/E ratio) as having the del/del polymorphism. In these cases, either genotyping or other tests to detect doping with epitestosterone would be required, such as the epitestosterone to 5-androstene-3β,17α-diol ratio or 13C/12C ratio of epitestosterone [08016].

Testosterone abuse is conventionally disclosed by urinary assay of the testosterone/epitestosterone (T/E) glucuronide ratio, which should not exceed 4. A noteworthy number of athletes, however, have higher natural ratios than 4, most likely because of decreased excretion of epitestosterone glucuronide. Urine from different study populations was analysed for androgen glucuronides by gas chromatography-mass spectrometry. All men were genotyped for the uridine diphospho-glucuronosyltransferase (UGT) 2B17 deletion polymorphism and single nucleotide polymorphisms in the cytochrome P-450c17alpha (CYP17), UGT2B15 and UGT2B7 genes. Expression of UGT2B15 mRNA in human liver samples was analysed using real-time PCR. A T>C (A1>A2) promoter polymorphism in the CYP17 gene was associated with the urinary glucuronide levels of epitestosterone and its putative precursor androstene-3beta, 17alpha-diol, resulting in 64 percent higher T/E ratios in A1/A1 homozygotes. Individuals devoid of UGT2B17 had significantly higher UGT2B15 mRNA levels in liver than individuals carrying two functional UGT2B17 alleles. The CYP17 promoter polymorphism may partly explain high natural (>4) T/E ratios. The data indicate that 5-androstene-3beta, 17alpha-diol is an important precursor of epitestosterone and that CYP17 is involved in its production. In addition, it was found that lack of the UGT2B17 enzyme may be compensated for by increase in UGT2B15 transcription [08121].

Testosterone and epitestosterone are endogenous steroids that differ in the configuration of the hydroxyl-bearing carbon at the C-17. Testosterone is the predominant male sex hormone while the role of epitestosterone is largely unclear. In humans, both androgens are mainly excreted as glucuronide conjugates and the urinary ratio of testosterone to epitestosterone
(T/E), used to expose illicit testosterone abuse by male athletes, indicates the relative concentrations of the respective glucuronides. Some male athletes have T/E above the accepted threshold value, 4.0, even without testosterone abuse. It was therefore analyzed athletes urine samples and found that the main reason for such "false positives" in doping tests was low epitestosterone glucuronide concentration, not high level of testosterone glucuronide. Sulfate conjugates of both testosterone and epitestosterone were also detected in the different urine samples. Glucuronidation assays with the 19 human UDP-glucuronosyltransferases (UGTs) of subfamilies UGT1A, UGT2A and UGT2B revealed that UGT2B17 is the most active enzyme in testosterone glucuronidation. UGT2B17 does not glucuronidate epitestosterone, but inhibition studies revealed that it binds epitestosterone with similar affinity as testosterone. Epitestosterone glucuronidation is mainly catalyzed by UGT2B7 and the Km of this reaction is significantly lower than the Km of UGT2B17 for testosterone. While UGT2B7 and UGT2B17 exhibited high, although converse, stereoselectivity in testosterone and epitestosterone glucuronidation, UGT2A1, an extrahepatic enzyme that is mainly expressed in the nasal epithelium, catalyzed the glucuronidation of both steroids at considerable rates and similar kinetics [08122].

To study the disposition of serum testosterone and seven of its metabolites before and after 2 days of an intramuscular dose (500 mg) of testosterone enanthate in relation to the phosphodiesterase (PDE7B) and the uridine 5'-diphospho-glucuronosyltransferase (UGT2B17) genotypes patients were genotyped for UGT2B17 deletion polymorphism and single nucleotide polymorphisms in the PDE7B gene. The involvement of PDE7B in hydrolysis of enanthate was assessed in human liver homogenates. Genetic variation in the PDE7B gene was found to be associated with the serum level of testosterone. Individuals homozygous for PDE7B rs7774640 G allele had a smaller increase (2.5-fold) in the serum testosterone levels compared with carriers of the A allele (3.9-fold). In addition, genetic variation in the PDE7B gene significantly influences the testosterone/epitestosterone ratio, a biomarker of testosterone doping. An in-vitro incubation studies confirmed that PDE7B serves as a catalyst of the hydrolysis of testosterone enanthate. The UGT2B17 deletion polymorphism did not show any significant association with serum testosterone levels or the other androgen metabolites investigated. It was concluded that it was found that PDE7B is involved in the hydrolysis of testosterone enanthate and that genetic variation in the PDE7B gene is a determinant of the systemic levels of testosterone after administration of testosterone enanthate. It is reasonable to believe that the genetic variation in testosterone bioavailability may be correlated to varying effects of this androgen, whether it is used for replacement therapy or abused in doping. Thus the results may be important to consider in doping test programmes and in therapeutics with androgens and other esterified drugs [11091].

The deletion polymorphism of the enzyme UGT2B17 is known to correlate with the level of the testosterone to epitestosterone (T/E) ratio in urine specimen. Due to the importance of the T/E ratio to detect testosterone abuse in doping analysis, a PCR-ELISA system was established to identify the UGT2B17 phenotype in urine samples. Epidemiological investigations in a set of 674 routine doping controls (in- and out-of-competition) resulted in 23 percent homozygote gene-deleted and 75 percent UGT2B17-positive athletes. The validated test system has shown to be robust and sensitive: in only 18 cases (3 %) isolation of cell material from urine failed. Following hydrolysis of glucuronidated conjugates, steroids were analyzed as bis-TMS derivatives by gas chromatography-mass spectrometry (GC-MS), for example, testosterone (T) and epitestosterone (E). Additionally, isotope ration mass spectrometry (IRMS) analysis and luteinizing hormone (LH) measurement were applied. Mean T/E ratios significantly correlated with the UGT2B17 phenotype (del: T/E 0.9; pos: 1.7), however the values did not differ as distinctive as reported in previous studies. Additionally, the T/E ratios in the gene-deleted group did not show a normal curve of distribution (median
of T/E 0.5). Obviously, beside the UGT2B17 deletion further influences have to be taken into account, for example, polymorphisms or induction of other metabolizing enzymes. The results indicate that the UGT2B17 polymorphism might be insufficient when utilized solely as a crucial parameter for individual interpretation of T/E in urine. Nevertheless, the detection of the UGT2B17-gene deletion in urine samples would provide additional information important for gathering evidence in analysis of steroids in doping control [11444].

The conspicuous interindividual differences in metabolism and urinary excretion of testosterone and its metabolites make it challenging to reveal testosterone doping. The variation in testosterone glucuronide excretion is strongly associated with a deletion polymorphism in the uridine diphosphate-glucuronosyltranferase (UGT) 2B17 gene. The objective of one study was to identify additional biomarkers to detect testosterone abuse and to elucidate alternative pathways for testosterone elimination in individuals devoid of the UGT2B17 enzyme. For this purpose a new ultraperformance liquid chromatographic tandem mass spectrometric method for simultaneous determination of 10 different sulfo- and glucuronide-conjugated steroids was developed. Fifty-four healthy male volunteers with two, one, or no allele (ins/ins, ins/del, or del/del) of the UGT2B17 gene participated in the study. Intervention included a single im dose of 500 mg testosterone enanthate. Urinary sulfo- and glucuronide-conjugated steroids were measured. Testosterone sulfate levels decreased in all individuals after the dose. The individual differences in the excretion of all sulfated metabolites were large. Thus, these metabolites will not serve as appropriate biomarkers for testosterone abuse. However, androsterone glucuronide excretion increased in all of our study subjects after the testosterone dose. Etiocholanolone sulfate was excreted at significantly higher levels in UGT2B17 del/del individuals. It was proposed that the androsterone glucuronide to epitestosterone glucuronide ratio may serve as a complementary biomarker to reveal testosterone abuse [11445].

**Influence of exercise on testosterone levels**

The purpose of one study was to explore the mechanisms for increased exercise performance in conditions of competition. Endurance trained subjects (n=14) performed incremental treadmill running to exhaustion in control laboratory conditions (non-competition) and in conditions of simulated competition to assess performance (running duration). Heart rate and respiration gases were monitored continuously through each exercise condition. Blood lactate, cortisol, growth hormone and testosterone concentrations were also determined at pre- (rest) and postexercise in each condition. Results indicated competition exercise performance was significantly increased as was peak VO$_2$ response versus non-competition. No significant differences were found in peak measurements of minute ventilation, respiratory exchange ratio, ventilation threshold, post-exercise lactate, heart rate, or the ventilation equivalent for O$_2$ between the exercise conditions. In both conditions growth hormone and testosterone concentrations increased significantly in response to exercise, whereas cortisol responses post-exercise were significantly elevated in the competition but not in the control condition. These findings support that in competitive situations the affective state (motivation) experienced by athletes can enhance performance in exercise events, and lead to an increased peak oxygen uptake. The magnitude of the improvement is of a substantial nature and of a level seen with some training programs. Competitive conditions also augment the cortisol response to exercise, suggesting that enhanced sympatho-adrenal system activation occur in such situations which may be one of the key "driving forces" to performance improvement [10081].

Although adaptations to water-based resistance exercise and conventional water-based
exercise have been investigated, little is known regarding acute anabolic and catabolic hormonal responses to these two types of exercise. The purpose of this study was to investigate the acute responses of salivary testosterone and cortisol to two water-based exercise protocols in which the different intensities were determined using Borg’s perceived exertion scale. Ten young (24 ± 3 yr) and 7 elderly men (65 ± 6 yr) who were familiar with exercise in water were subjects of the study. Salivary samples were collected at rest and 5 minutes after the 2 water-based exercise protocols. One session involved intermittent water resistance training at a Borg-scale intensity of 19 (W19), whereas the other involved continuous water aerobic training at an intensity of 13 (W13). The samples were used to determine salivary levels of free testosterone and cortisol. There was a significant increase on salivary testosterone in both groups after the W19 protocol, but no such alteration was observed after W13. The testosterone response to the W19 protocol was significantly higher in young than in elderly men. Although no modification on salivary cortisol was observed after either protocol, in young men, the cortisol response to W19 was higher than in elderly men. Water-based exercise with emphasis on strength development was found to stimulate a more acute increase on salivary testosterone than water-based aerobic exercise, probably as a result of the higher intensity used in that training protocol. Given the known relationship between acute hormonal responses and chronic neuromuscular adaptations, the testosterone response after W19 should be considered when prescribing water-based exercise, especially to older populations [09090].

It was investigated whether the myosin heavy chain (MyHC) proportion and androgen receptor (AR) concentration in skeletal muscle differ following 21 weeks of strength, endurance and combined training in untrained older men. Strength (S) and endurance (E) groups trained twice per week and combined (S+E) group trained four times per week (two strength and two endurance). Muscle biopsies were obtained before and after the training period from m. vastus lateralis (VL) and AR mRNA and protein concentration and MyHC proportion were determined. 1RM increased during the training period in S, S+E and E but the changes were greater in S and S+E than in E. Statistically significant increases were observed only in S and S+E in maximal isometric force as well as in VL thickness. VO$_{2\text{max}}$ increased significantly only in E. MyHCIIa proportion increased in S, while MyHCIIa proportion decreased and MyHCI increased significantly in the endurance group. No statistically significant changes were observed in serum testosterone and in AR mRNA or protein concentrations. The present results indicate that 21 weeks of strength, endurance or combined training changed MyHC proportion according to the training method but did not have an effect on androgen mRNA or protein expression in skeletal muscle at rest [09091].

One study compared the neuromuscular performance (speed, power, strength) of elite rugby union players, by position, and examined the relationship between player performance and salivary hormones, by squad and position. Thirty-four professional male rugby players were assessed for running speed (10-, 20- or 30-m sprints), concentric mean and peak power during a 70-kg squat jump and 50-kg bench press throw, and estimated 1 repetition maximum (1RM) strength for a box squat and bench press. Tests were performed on separate days with absolute and normalized (power and strength only) values computed. Saliva was collected before each test and assayed for testosterone and cortisol. The testosterone and/or cortisol concentrations of players correlated significantly to speed, power, and strength, especially for the backs, thereby confirming relationships between neuromuscular performance and hormone secretion patterns. Based on these findings, it was suggested that training to increase whole-body and muscle mass might facilitate general performance improvements. Training prescription might also benefit from acute and chronic hormone monitoring to identify those individuals likely to respond more to hormonal change [09092].
The effect of a single exercise as well as exercise training on the growth hormone (GH)-insulin-like growth factor (IGF-I) axis and inflammatory cytokines was studied mainly in adults participating in individualized endurance-type sports. The gender-specific effect of exercise on these systems in adolescents is unknown. Therefore, the purpose of one study was to evaluate the effect of a typical volleyball practice on anabolic (GH, IGF-I, and testosterone) and catabolic hormones (cortisol) and inflammatory mediators (interleukin-6) in elite, national team level, male (n=14) and female (n=13) adolescent volleyball players (13-18 years, Tanner stage 4-5). Exercise consisted of a typical 1-hour volleyball practice. Blood samples were collected before and immediately after the practice. Exercise led to significant increases in GH in men and women, testosterone (6.1 ± 0.9 to 7.3 ± 1.0 and 2.4 ± 0.6 to 3.3 ± 0.7 ng x mL, in men and women, respectively), and interleukin-6. There were no gender differences in the hormonal response to training. Changes in GH and testosterone after the volleyball practice suggest exercise-related anabolic adaptations [09093].

In trained and in not-trained

It is a common view that strength and sprint trained athletes are characterized by high plasma/serum testosterone (T) concentration, which is believed to be partly responsible for their performance level. This opinion, however, has poor scientific background. The aim of one study was to give evidence-based information on this issue. It was examined gonadal hormone status at rest after overnight fasting in high and top-class track and field sprinters (n=16) and in untrained men (n=15). It was shown that basal T, free testosterone (fT), bioavailable testosterone (bio-T), and sex hormone-binding globulin concentrations were not significantly different in sprinters versus untrained subjects. Further comparison of the results of the basal serum T concentration in 8 sprinters showed its significant changes during an annual training period. Significantly higher T concentration during a low-intensity training period (beginning of December) than during heavy sprint specific training period (end of March) was observed in these athletes. It was concluded that basal gonadal hormone concentration in high and top-class athletes (sprinters and jumpers) did not appear to be significantly different when compared with untrained subjects. Moreover, basal T concentration in sprinters can differ significantly during an annual training period. This fact should be taken into consideration when interpreting the results of gonadal hormone status in athletes at varied training stages [11082].

Effects of hard exercise

In the last years, mainly 2 high-intensity-training (HIT) protocols became common: First, a Wingate-based “all-out” protocol and second, a 4×4 min protocol. However, no direct comparison between these protocols exists, and also a comparison with high-volume-training (HVT) is missing. Therefore, the aim of the present study was to compare these 3 endurance training protocols on metabolic, hormonal, and psychological responses. Twelve subjects performed: 1) HVT [130 min at 55 % peak power output (PPO)]; 2) 4×4 min at 95 % PPO; 3) 4×30 s all-out. Human growth hormone (hGH), testosterone, and cortisol were determined before (pre) and 0', 30', 60', 180' after each intervention. Metabolic stimuli and perturbations were characterized by lactate, blood gas (pH, BE, HCO₃⁻, pO₂, PCO₂), and spirometric analysis. Furthermore, changes of the person’s perceived physical state were determined. The 4×30 s training caused the highest increases in cortisol and hGH, followed by 4 × 4 min and HVT. Testosterone levels were significantly increased by all 3 exercise protocols. Metabolic stress was highest during and after 4×30 s, followed by 4×4 min and HVT. The 4×30 s training was also the most demanding intervention from an athlete’s point of view. In conclusion, the results suggest that 4×30 s and 4×4 min promote anabolic processes more than HVT, due to higher increases of hGH, testosterone, and the T/C ratio. It can be
speculated that the acute hormonal increase and the metabolic perturbations might play a positive role in optimizing training adaptation and in eliciting health benefits as it has been shown by previous long term training studies using similar exercise protocols [13197].

**Effect of endurance training**

The aim of one study was to compare the levels of serum immunoglobulin (IgA, IgM, IgG), testosterone and cortisol in semi-endurance elite runners during general preparation and competition phase of training. Thirteen semi-endurance elite male runners with an average age of 19 years volunteered to take part in this study. The runners participated in the selected training for a period of 14 weeks and 12 sessions per week (in the morning and afternoon). Blood samples were collected during the three phases of training (before-preparation phase, after-preparation phase and before-competition phase). The levels of serum IgM in semi-endurance elite runners after preparation phase reduced significantly, while these levels during the competition phase increased even though significantly. The levels of serum IgG and IgA also reduced, however not significantly, during both phases. Moreover, after preparation phase, there was no significant change in serum IgA levels; though, these levels reduced, however not significantly, before competition phase. Cortisol levels significantly decrease after preparation phase; although, it increased before competition phase. Testosterone/cortisol ratio increases significantly after preparation phase, and it decreased before competition phase. Testosterone levels intangibility increased and decreased respectively after preparation and before competition phases. Findings indicated that long and intensive exercises weaken the immune system, while moderate and short drills strengthened this system [12101].

Acute exercise, depending on its characteristics, demands a physiological increase in testosterone. In fact, the majority of investigations showed that total and/or free testosterone acutely increased immediately after acute strenuous and/or prolonged sub-maximal endurance and resistance exercises. Unfortunately, the mechanisms responsible for testosterone increase after acute exercise are still unknown. Probably due to exercise standardization and/or to individual variability gonadotropins levels have been reported unchanged, increased or, rarely, decreased after both sub-maximal and maximal acute exercise. Consequently, other mechanisms, such as a possible adaptation of secretory capacity of the Leydig cells, adrenergic and/or lactate stimulation, modifications of clearance rate, plasma volume reductions and changes in testicular blood flow should be investigated. Studies on the effects of chronic exercise (e.g. training) frequently showed a reduction of free and total testosterone concentrations in endurance-trained men, and the few prospective studies showed contradictory results probably due to the features of the training period, the magnitude of training stimulus and the volume of training load employed. In addition, modifications of androgen receptors status have also been described [12094].

Generally, cardiovascular exercise and resistance training transiently increase testosterone concentrations in men although a few studies report null effects. Testosterone concentrations also vary both before and after competition in a systematic and consistent manner. Wingfield et al. in 1990) proposed the “challenge hypothesis”, which posits that during mating seasons and times of resource scarcity testosterone concentrations rise to facilitate competition, particularly amongst males. The challenge hypothesis is relevant to human competition in the world of sports. As predicted by the challenge hypothesis, pre-competition concentrations of testosterone rise in male and female athletes in anticipation of the impending competition. In men, testosterone commonly increases following victory and decreases following loss. However, this main effect of winning or losing on changes in men's testosterone is not always observed. Several studies have shown that other factors like context, individual
differences, e.g. power motivation, social anxiety, and motivation to win (Suay et al., 1999), as well as cognitive appraisal can play an important role in predicting post-competition testosterone changes. Dominance-motivated individuals, who positively value interpersonal dominance and dislike submission, are those most likely to experience outcome-dependent changes in testosterone. Competitors’ level of engagement is also relevant to testosterone changes, such that men’s testosterone increases are greatest when one’s opponents feel more confident. An elite athlete in an international competition is likely to be more engaged and to value victory and defeat much more significantly than a participant in laboratory manipulations with cognitive games. Accordingly, testosterone changes in situations of high value and importance are likely to be of greater magnitude. A winner will likely benefit from continued victory and increased access to resources, whereas a loser, who may be injured or still in the presence of the victory-primed winner, will likely benefit from disengagement. Several animal studies have elucidated the mediating effects of testosterone in the winner and loser effect, which have been subsequently studied in humans. A single sample collected at the peak of endogenous testosterone production has potential to produce a false positive result, when compared against a population-based average. There is a notable sexual dimorphism in testosterone responses to competition in humans. Testosterone responses to winning and losing appear to principally apply to men. Only a single study has reported an effect winning/losing on differential testosterone changes in women, whereas many more studies have failed to find an effect. This is likely a function of the different source glands for testosterone between the sexes, which include the testes and adrenals in men and ovaries and adrenals in women [12100].

One study examined the predictive relationships between the salivary free testosterone (T) concentrations of elite athletes and the expression of force and power. A group of elite male rugby players (n=64) were assessed for peak force (PF), peak rate of force development (PRFD), force at 100 milliseconds (F100 ms) and 250 milliseconds (F250 ms) during an isometric mid-thigh pull (IMTP), and/or peak power (PP) and height during a counter-movement jump (CMJ). Saliva samples were collected before testing and assayed for free T. Relationships between individual T concentrations and performance were assessed as a pooled group and 4 sub-groups of equal size. As pooled data sets, none of the IMTP and CMJ performance variables were significantly correlated with free T in either the PF or PP groups. The PF and PP abilities of the 4 sub-groups were significantly different, so that PF1>PF2>PF3>PF4 and PP1>PP2>PP3>PP4. When the 4 sub-groups were analysed, the T concentrations of the PF4 group were significantly correlated to PRFD and F100 ms during the IMTP, as was F100 ms in the PF1 group. In the PP1 group, free T also correlated to CMJ height. The key conclusion is that the expression of force and power in an elite athletic group may be dependent, to some extent, on individual variation in salivary free T concentrations and existing strength or power levels. The current results also confirm that the grouping of elite athletes of mixed strength or power ability may bias predictive results in a manner not reflective of sub-groups within this population [12102].

Responses to intensive interval versus steady-state endurance exercise
Free testosterone (FT) hormonal responses were compared between high-intensity interval exercise (IE) and steadystate endurance exercise (SSE) in endurance trained males (n=15). IE session was repeated periods of 90-sec treadmill running at 100-110 percent maximal oxygen uptake (VO\textsubscript{2max}) and 90-sec active recovery at 40 percent VO\textsubscript{2max} for 42-47 min. The SSE session consisted of a continuous 45-min run at 60-65 percent VO\textsubscript{2max}. Total work output was equal for each exercise session. A 45-min supine rest control session (CON) was also performed. All three sessions were on separate days. Pre-session (PRE), immediate post-session (POST), and 12-h post-session (12POST) blood samples were collected and used to determine FT, SHBG, LH, 3- alpha-androstenediol glucuronide (3-alphaDiol G) and cortisol. Analysis of variance compared IE and SSE biomarker responses to the reference
CON session. IE and SSE each caused an increase in FT, but IE more so than SSE. The 5alpha-reductase marker 3-alpha Diol G response at 12POST IE was elevated while FT was reduced; no such change occurred following SSE. These findings suggest IE might produce a more pronounced turnover of FT by androgen sensitive tissue than the SSE form of exercise [12103].

Effect of resistance training

One study assessed the effect of different resistance exercise scheme (RES) designs of similar total of load lifted on the responses of testosterone, cortisol, and creatine kinase (CK). Twenty-seven healthy males performed 1 of 4 bench press workouts described by the 1 repetition maximum (1RM) load: 4 sets of maximum repetitions at 50 percent-1RM (50%-1RM RES), 5 sets of maximum repetitions at 75 percent-1RM (75%-1RM RES), 10 sets of maximum repetitions at 90 percent-1RM (90%-1RM RES), or 8 sets of maximum repetitions at 110 percent-1RM (110%-1RM RES). Each RES was equated by the total volume of load lifted (repetitions x sets x load). Blood samples, collected pre-exercise (Pre) and post-exercise (Post) at 1 and 24 hours (24 h), were analyzed for total and free testosterone, total cortisol, and CK. In general, testosterone and cortisol showed little change within or between the different RES, possibly because of the relatively low volume lifted and/or the small muscle mass activated by the bench press exercise. Cortisol was elevated after the 75%-1RM RES at the Post sample, with this response also significantly exceeding the other RES. The 24 h CK response was also elevated after the 75%-1RM RES, thereby suggesting greater training strain for the same volume of load. These results confirm previous recommendations regarding the prescription of resistance exercise and the importance of total volume as a stimulus for activating the endocrine system and achieving long-term adaptation [09094].

One study examined the effects of heavy resistance training on physiological acute exercise-induced fatigue (5 x 10 RM leg press) changes after two loading protocols with the same relative intensity (%) (5 x 10 RM) and the same absolute load (kg) (5 x 10 RM) as in pretraining in men (n=12). Exercise-induced neuromuscular (maximal strength and muscle power output), acute cytokine and hormonal adaptations (i.e. total and free testosterone, cortisol, growth hormone (GH), insulin-like growth factor-1 (IGF-1), IGF binding protein-3 (IGFBP-3), interleukin-1 receptor antagonist (IL-1ra), IL-1beta, IL-6, and IL-10 and metabolic responses (i.e. blood lactate) were measured before and after exercise. The resistance training induced similar acute responses in serum cortisol concentration but increased responses in anabolic hormones of free testosterone and growth hormone. This enhanced hormonal and cytokine response to strength exercise at a given relative exercise intensity after strength training occurred with greater accumulated fatigue and metabolic demand (i.e. blood lactate accumulation). The magnitude of metabolic demand or the fatigue experienced during the resistance exercise session influences the hormonal and cytokine response patterns. Similar relative intensities may elicit not only higher exercise-induced fatigue but also an increased acute hormonal and cytokine response during the initial phase of a resistance training period [09095].
similar acute responses in serum cortisol concentration but increased responses in anabolic hormones of free testosterone and GH, as well as inflammation-responsive cytokine IL-6 and the anti-inflammatory cytokine IL-10, when the same relative load was used. This response was balanced by a higher release of pro-inflammatory cytokines IL-1beta and cytokine inhibitors (IL-1ra) when both the same relative and absolute load was used after training. This enhanced hormonal and cytokine response to strength exercise at a given relative exercise intensity after strength training occurred with greater accumulated fatigue and metabolic demand (i.e. blood lactate accumulation). The magnitude of metabolic demand or the fatigue experienced during the resistance exercise session influences the hormonal and cytokine response patterns. Similar relative intensities may elicit not only higher exercise-induced fatigue but also an increased acute hormonal and cytokine response during the initial phase of a resistance training period [09096].

Short-term effects on resistance training

The aim of one review is to highlight two emerging concepts for the elite athlete using the resistance-training model with short-term effects of testosterone (T) and cortisol (C) on the neuromuscular system; and the dose-response training role of these endogenous hormones. Exogenous evidence confirms that T and C can regulate long-term changes in muscle growth and performance, especially with resistance training. This evidence also confirms that changes in T or C concentrations can moderate or support neuromuscular performance through various short-term mechanisms (e.g. second messengers, lipid/protein pathways, neuronal activity, behaviour, cognition, motor-system function, muscle properties and energy metabolism). The possibility of dual T and C effects on the neuromuscular system offers a new paradigm for understanding resistance-training performance and adaptations. Endogenous evidence supports the short-term T and C effects on human performance. Several factors (e.g. workout design, nutrition, genetics, training status and type) can acutely modify T and/or C concentrations and thereby potentially influence resistance-training performance and the adaptive outcomes. This novel short-term pathway appears to be more prominent in athletes (vs non-athletes), possibly due to the training of the neuromuscular and endocrine systems. However, the exact contribution of these endogenous hormones to the training process is still unclear. Research also confirms a dose-response training role for basal changes in endogenous T and C, again, especially for elite athletes. Although full proof within the physiological range is lacking, this athlete model reconciles a proposed permissive role for endogenous hormones in untrained individuals. It is also clear that the steroid receptors (cell bound) mediate target tissue effects by adapting to exercise and training, but the response patterns of the membrane-bound receptors remain highly speculative. This information provides a new perspective for examining, interpreting and utilizing T and C within the elite sporting environment. For example, individual hormonal data may be used to better prescribe resistance exercise and training programmes or to assess the trainability of elite athletes. Possible strategies for acutely modifying the hormonal milieu and, thereafter, the performance/training outcomes were also identified. The limitations and challenges associated with the analysis and interpretation of hormonal research in sport (e.g. procedural issues, analytical methods, research design) were another discussion point [11083].

One study examined the effects of short-cycle sprints on power, strength, and salivary hormones in elite rugby players. Thirty male rugby players performed an upper-body power and lower-body strength (UPLS) and/or a lower-body power and upper-body strength (LPUS) workout using a crossover design (sprint vs. control). A 40-second upper-body or lower-body cycle sprint was performed before the UPLS and LPUS workouts, respectively, with the control sessions performed without the sprints. Bench throw (BT) power and box squat (BS) 1 repetition maximum (1RM) strength were assessed in the UPLS workout, and squat jump (SJ) power and bench press (BP) 1RM strength were assessed in the LPUS workout. Saliva
was collected across each workout and assayed for testosterone (Sal-T) and cortisol (Sal-C). The cycle sprints improved BS (2.6 ± 1.2 %) and BP (2.8 ± 1.0 %) 1RM but did not affect BT and SJ power. The lower-body cycle sprint produced a favorable environment for the BS by elevating Sal-T concentrations. The upper-body cycle sprint had no hormonal effect, but the workout differences (%) in testosterone and cortisol concentrations correlated to the BP, along with the testosterone/cortisol. In conclusion, the cycle sprints improved the BP and BS 1RM strength of elite rugby players but not power output in the current format. The improvements noted may be explained, in part, by the changes in absolute or relative hormone concentrations. These findings have practical implications for prescribing warm-up and training exercises [11084].

Effect of different types of training

In the last years, mainly 2 high-intensity-training (HIT) protocols became common: first, a Wingate-based "all-out" protocol and second, a 4×4 min protocol. However, no direct comparison between these protocols exists, and also a comparison with high-volume-training (HVT) is missing. Therefore, the aim of one study was to compare these 3 endurance training protocols on metabolic, hormonal, and psychological responses. Twelve subjects performed: 1) HVT (130 min at 55 % peak power output, PPO); 2) 4×4 min at 95 percent PPO; 3) 4×30 s all-out. Human growth hormone (hGH), testosterone, and cortisol were determined before (pre) and 0', 30', 60', 180' after each intervention. Metabolic stimuli and perturbations were characterized by lactate, blood gas (pH, BE, HCO$_3^-$, pO$_2$, PCO$_2$), and spirometric analysis. Furthermore, changes of the person's perceived physical state were determined. The 4×30 s training caused the highest increases in cortisol and hGH, followed by 4×4 min and HVT. Testosterone levels were significantly increased by all 3 exercise protocols. Metabolic stress was highest during and after 4×30 s, followed by 4×4 min and HVT. The 4×30 s training was also the most demanding intervention from an athlete's point of view. In conclusion, the results suggest that 4×30 s and 4×4 min promote anabolic processes more than HVT, due to higher increases of hGH, testosterone, and the T/C ratio. It can be speculated that the acute hormonal increase and the metabolic perturbations might play a positive role in optimizing training adaptation and in eliciting health benefits as it has been shown by previous long term training studies using similar exercise protocols [13199].

Effect of different short-time types of exercise

Hormonal responses to exercise could be used as a marker of overreaching. A short exercise protocol that induces robust hormonal elevations in a normal trained state should be able to highlight hormonal changes during overreaching. One study compared plasma and salivary cortisol and testosterone responses to 4 exercise trials at continuous cycle to fatigue at 75 percent of peak power output ($W_{max}$) (FAT), 30-minute cycle alternating 1-minute 60 percent and 1 minute 90 percent $W_{max}$ (60/90), 30-minute cycle alternating 1-minute 55 percent and 4-minute 80 percent $W_{max}$ (55/80), and squatting 8 sets of 10 repetitions at 10 repetition maximum (RESIST). Blood and saliva samples were collected pre-exercise and at 0, 10, 20, 30, 40, 50, and 60 minute postexercise. Pre- to postexercise plasma cortisol increased in all exercise trials, except 60/90. Increases in 55/80 remained above pre-exercise levels for the entire postexercise period. Salivary cortisol increased from pre- to postexercise in FAT and 55/80 trials only. Once elevated after 55/80, it remained so for the postexercise period. Plasma testosterone increased from pre- to postexercise in all trials except 55/80. Saliva testosterone increased from pre- to postexercise in all trials with the longest elevation occurring after 55/80. Area under the curve analysis indicated that the exercise response of salivary hormones was greater in all cycle trials (cortisol) and in the 60/90 and 55/80 trials (testosterone) compared with the other trials. The study indicates that
the 55/80 cycle protocol induces a prolonged salivary and plasma cortisol and salivary testosterone response compared with the other trials and so may be a useful diagnostic tool of overreaching [11085].

Explosive performances

The primary objective of one study was to analyze the relationship between testosterone levels and vertical jumping performance in elite men and women athletes. The secondary objective was to verify whether testosterone levels and vertical jumping performance were different in men and women athletes and if those measurements were different between different athletic groups. Seventy (22 women and 48 men) elite athletes in track and field (sprinters), handball, volleyball, and soccer competing at national and international levels participated in the study. After 10 hours of fasting and 1 day of rest, blood samples were drawn from the antecubital vein for determining testosterone levels. Vertical jumping tests consisted of countermovement jumps conducted on a resistive platform connected to a digital timer. Resting testosterone levels in women were 10 percent of those of the men. Countermovement jump performance was significantly different between women and men athletes, with women's jumping ability 86 percent of that of men. A significant positive relationship was identified between testosterone levels and vertical jump performance when all data were considered [06053].

Active recovery versus passive recovery

The aim of one study was to compare the effects of active (A) versus passive (P) recovery during high-intensity interval training on the acute hormonal and metabolic response. Twelve triathletes/cyclists performed four 4 min intervals on a cycle ergometer, either with A- or P-recovery between each bout. Testosterone, hGH, cortisol, VEGF, HGF and MIF were determined pre, 0', 30', 60' and 180' after both interventions. Metabolic perturbations were characterized by lactate, blood gas and spirometric analysis. A-recovery caused significant increases in circulating levels of cortisol, testosterone, T/C ratio, hGH, VEGF and HGF. Transient higher levels were found for cortisol, testosterone, hGH, VEGF, HGF and MIF after A-recovery compared to P-recovery, despite no differences in metabolic perturbations. A-recovery was more demanding from an athlete's point of view. Based on the data of testosterone, hGH and the T/C-ratio, as well as on the data of VEGF and HGF it appears that this kind of exercise protocol with A-recovery phases between the intervals may promote anabolic processes and may lead to pro-angiogenic conditions more than with P-recovery. These data support the findings that also the long term effects of both recovery modes seem to differ, and that both can induce specific adaptations [13200].

The exercise-induced metabolic stress can be influenced by the mode of recovery and is associated with acute hormonal responses. Therefore, it is hypothesized that active recovery between high intensity intervals reduces the metabolic stimulus and therefore the hormonal response compared to passive recovery. Twelve male cyclist/triathletes performed four 30s all-out intervals, either with active (A) or passive (P) recovery between each bout. Human growth hormone (hGH), testosterone and cortisol, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and macrophage migration inhibitory factor (MIF) were determined pre, 0', 30', 60' and 180' after both interventions. Metabolic stimuli and perturbations were characterized by lactate, blood gas (pH, BE, HCO₃⁻, PO₂, PCO₂), and spirometric analysis. Both interventions caused a transient increase in circulating levels of cortisol, testosterone, testosterone/cortisol-ratio, hGH, VEGF and HGF. Transient differences between A- and P-recovery were found only for testosterone and HGF directly after exercise, despite significant differences in metabolic disturbances (lactate, acid base status). Based on
the data of testosterone, hGH and the testosterone/cortisol-ratio, as well as on the data of VEGF and HGF it appears that this kind of exercise protocol may promote anabolic processes and may lead to pro-angiogenic conditions independent of the mode of recovery. However transient differences between A- and P-recovery were shown for testosterone and HGF. In contrast, cortisol and hGH, which are known to be sensitive for metabolic perturbations (e.g. pH) showed no differences. Therefore, it is proposed that if a certain threshold for metabolic perturbations is exceeded, a hormonal response is induced, which does not differ between A- and P-recovery [13201].

Influence of red color

One study examined the testosterone responses of men to an exercise bout simulating a competitive sporting effort in order to determine if the wearing of red-colored apparel influenced the hormonal response. Male subjects (n = 10) were placed into sets of matched-pairs and performed VO2max cycle ergometry exercise test to exhaustion to simulate the competitive effort. Each member of a pairing was randomly assigned to one of two treatment groups-the wearing of red-colored clothing, or the wearing of black-colored clothing. Blood samples were collected before exercise (REST), an immediate postexercise sample was collected at exhaustion (EXH), and a final sample was taken at 15 min into recovery (REC) from exercise. Blood was biochemically analyzed for total testosterone. In response to the exercise, performance characteristics (i.e. VO2max and maximal workload) of treatment groups did not differ significantly. A significant increase in the testosterone was observed in both treatment groups postexercise at EXH and at REC as compared to REST. However, no differences were observed between treatment groups in the before or postexercise hormonal concentrations. These findings suggest that the wearing of red-colored apparel had no affects on the testosterone responses to an exercise bout simulating a competition [06054].

Pre-game free testosterone concentrations and outcome

To assess the measures of salivary free-testosterone and cortisol concentrations across selected rugby union matches according to the game outcome 22 professional male rugby union players were studied across 6 games (3 wins and 3 losses). Hormone samples were taken 40 minutes prior to the game (pre) and 15 minutes after (post). The hormonal data was grouped and compared against competition outcomes. These competition outcomes included wins and losses and a game ranked performance score (1 to 6). Across the entire team, pre-game testosterone concentrations were significantly higher during winning games than losses. Analysis by playing position further revealed that, for the backs, pre-game testosterone concentrations and the T/C ratio were significantly greater before a win than a loss. Game ranked performance score (1 to 6) correlated to the team's pre-game testosterone concentrations. The backs also showed that pre-game testosterone and the T/C ratio correlated to game ranked performance. Analysis of the forwards' hormone concentrations did not distinguish between game outcomes nor did it correlate with game ranked performance. Game venue (home vs away) only affected post-game concentrations of testosterone and cortisol. It was concluded that monitoring game day concentrations of salivary free-testosterone may assist with identifying competitive readiness in rugby union matches. The link between pre-game T/C ratio and rugby players in the backs position suggest that monitoring weekly training loads and enhancing recovery modalities between games may also assist with favourable performance and outcome in rugby union matches [13196].

Pre-game testosterone level: home advantage
The home advantage is a robust phenomenon that occurs in the world of amateur and professional sport. Athletic teams have been shown to win significantly more games in their home venue as compared to their opponents' venue. Studies have suggested that the home advantage may be related to familiarity with the facility, increased crowd density and even pre-competition hormonal levels. The present study investigated pre-competition physiological and psychological states of elite hockey players in the home and away venues. Physiological measures included salivary cortisol and testosterone, which were assessed using enzyme immunoassays. In addition, pre-competition psychological states were assessed using the Competitive State Anxiety Inventory-2. Physiological measures indicated that the players had significantly higher pre-game testosterone when playing in their home venue as compared to their opponents' venue; however, this difference was not due to a pre-game rise in testosterone while competing at home. Furthermore, players showed a trend toward higher pre-game cortisol when playing in their home venue. Psychological measures indicated that players were more self-confident when playing in their home venue and also had higher somatic and cognitive anxiety when playing in their opponents' venue. The study supports the notion that there are differences in pre-competition hormonal and psychological states that may play a key role in the "home advantage" [06055].

Effect of testosterone on myoblasts

It was investigated the ability of testosterone (T) to restore differentiation in multiple population doubled (PD) murine myoblasts, previously shown to have a reduced differentiation in monolayer and bioengineered skeletal muscle cultures versus their parental controls (CON). Cells were exposed to low serum conditions in the presence or absence of T (100 nM) ± PI3K inhibitor (LY294002) for 72 h and 7 days (early and late muscle differentiation respectively). Morphological analyses were performed to determine myotube number, diameter (microm) and myonuclear accretion as indices of differentiation and myotube hypertrophy. Changes in gene expression for myogenin, mTOR and myostatin were also performed. Myotube diameter in CON and PD cells increased from 17.32 ±2.56 microm to 21.02 ± 1.89 microm and 14.58 ± 2.66 microm to 18.29 ± 3.08 microm, respectively after 72 h of T exposure. The increase was comparable in both PD (+25 %) and CON cells (+21 %) suggesting a similar intrinsic ability to respond to exogenous T administration. T treatment also significantly increased myonuclear accretion (% of myotubes expressing 5+ nuclei) in both cell types after 7 days exposure. Addition of PI3K inhibitor (LY294002) in the presence of T attenuated these effects in myotube morphology (in both cell types) suggesting a role for the PI3K pathway in T stimulated hypertrophy. Finally, PD myoblasts showed reduced responsiveness to T stimulated mRNA expression of mTOR vs. CON cells and T also reduced myostatin expression in PD myoblasts only. The present study demonstrates testosterone administration improves hypertrophy in myoblasts that basally display impaired differentiation and hypertrophic capacity versus their parental controls, the action of testosterone in this model was mediated by PI3K/Akt pathway [13202].

Testosterone levels after concussions

A 27-year-old man was admitted to an outpatient clinic with symptoms of loss at libido, erectile dysfunction and fatigue. He had been playing soccer from the age of 7, for the last 10 years as a high-level professional. During that time repeated mild head-trauma without loss of consciousness had occurred, mainly triggered by excessive header-training and occasional collisions. Serum levels of testosterone and luteinizing hormone were low. A gonadotropin releasing hormone loading test revealed significant gonadotropin responses, therefore pituitary gonadotropic insufficiency was unlikely. Further pituitary insufficiency of any other axis was also excluded by insulin hypoglycemia test. Magnetic resonance imaging
of the brain revealed no significant abnormalities of the hypothalamic-pituitary unit. Testosterone substitution, at first applied transdermally, then intramuscularly, was initiated after approval by the National Anti Doping Agency. Four months later most of the symptoms had regressed. It was concluded that pituitary deficiency in the course of craniocerebral trauma is frequent and may be transient or permanent, mostly affecting somatotropic or gonadotropic function. Hormonal imbalances may also be observed after mild but repeated trauma without loss of consciousness and should be considered in cases of isolated pituitary dysfunction, since such traumas may often occur in contacts sports such as boxing or intensive soccer play [13203].

Effect of soccer

The main aim of one study was to analyse the impact of an official match on hormonal and redox status, muscle damage and inflammation and neuromuscular function. Seven high-level male soccer players from the same team performed an official match and data were collected 72 h before, 24, 48 and 72 h post-match. Plasma testosterone/cortisol ratio (T/C), creatine kinase (CK), superoxide dismutase (SOD), glutathione peroxidase (GPX) and reductase (GR) activities, myoglobin (Mb), C-reactive protein (CRP), uric acid (UA), protein sulfhydryls (-SH), malondialdehyde (MDA) concentrations and total antioxidant status (TAS) were measured. Sprint, jump and change of direction performance, and maximal isokinetic knee extension and flexion were obtained as neuromuscular functional parameters. Cortisol increased and T/C decreased until 48 h recovery. Mb, CRP and -SH increased at 24 h recovery. CK, TAS, SOD and MDA increased up to 48 h recovery. GR increased and GPX decreased at 24 h recovery. Jump performance decreased 24 h post-match, but no significant alterations in sprint, change of direction and muscle strength were observed. In conclusion, an official match resulted in changes in plasma biomarkers until 48 h of recovery period, without major impact on performance [13198].

Effect of golf

The purpose of one investigation was to study the effects of 36 continuous holes of competitive golf on salivary testosterone, cortisol, and testosterone-to-cortisol ratio and their relation to performance in eight elite male collegiate golfers (age 20 years). Thirty-six holes of a 54-hole NCAA golf tournament were played on the first day of the competition. A saliva sample was taken 45 minutes prior to the round and immediately following each hole for a total of 37 samples per subject. Time matched baseline samples were collected on a different day to account for circadian variation. Six-hole areas under the curve (AUC) values were calculated for endocrine measures. Significant increases were noted for cortisol during competition, however, testosterone did not change during competition compared to baseline. Testosterone-to-cortisol (T/C) ratio was significantly lower throughout the competition compared to baseline measures. Thirty-six-hole AUC testosterone-to-cortisol ratio response was correlated to 36-hole score. There was a high correlation between pre-round testosterone, T/C ratio response, and 36-hole score. CSAI-2 somatic anxiety was correlated to pre-round cortisol and testosterone response. These results indicate a significant hormonal response during 10 hours of competitive golf. Good golf performance (low golf scores) in this competition was related to low T/C ratio [07088].

Overreaching

The purpose of one study was to study the effect of an 8-week Finnish military basic training period (BT) on physical fitness, body composition, mood state, and serum biochemical parameters among new conscripts; to determine the incidence of overreaching (OR); and to
evaluate whether initial levels or training responses differ between OR and noOR subjects. Fifty-seven males (20 years) were evaluated before and during BT. Overreaching subjects had to fulfill 3 of 5 criteria: decreased aerobic physical fitness (VO\(_{2\text{max}}\)), increased rating of perceived exertion (RPE) in 45-minute submaximal test at 70 percent of VO\(_{2\text{max}}\) or sick absence from these tests, increased somatic or emotional symptoms of OR, and high incidence of sick absence from daily service. VO\(_{2\text{max}}\) improved during the first 4 weeks of BT. During the second half of BT, a stagnation of increase in VO\(_{2\text{max}}\) was observed, basal serum sex hormone-binding globulin (SHBG) increased, and insulin-like growth factor-1 and cortisol decreased. Furthermore, submaximal exercise-induced increases in cortisol, maximum heart rate, and postexercise increase in blood lactate were blunted. Of 57 subjects, 33 percent were classified as OR. They had higher basal SHBG before and after 4 and 7 weeks of training and higher basal serum cortisol at the end of BT than noOR subjects. In addition, in contrast to noOR, OR subjects exhibited no increase in basal testosterone/cortisol ratio but a decrease in maximal La/RPE ratio during BT. As one-third of the conscripts were overreached, training after BT should involve recovery training to prevent overtraining syndrome from developing. The results confirm that serum SHBG, cortisol, and testosterone/cortisol and maximal La/RPE ratios could be useful tools to indicate whether training is too strenuous [11086].

**Older men**

To examine the relationship between different measures of testosterone and estradiol (E\(_2\)), muscle mass, muscle strength, and physical performance; and to test whether the association of sex hormone level with muscle strength and physical performance was independent of muscle mass. A cross-sectional survey on 1489 community-dwelling men older than 64 years of age had serum levels of testosterone and E\(_2\) measured by mass spectrometry, and sex hormone-binding globulin (SHBG) levels were measured by immunoradioassay. Muscle mass was examined by dual-energy X-ray absorptiometry and physical performance was assessed by hand-grip strength, gait speed, step length and chair-stand test. Appendicular skeletal mass (ASM) was positively associated with total testosterone, free testosterone, and total E\(_2\) but not with free E\(_2\). After adjustment for age, serum SHBG and relative ASM, both with total testosterone and free testosterone were significantly associated with grip strength, narrow-walk speed and the composite neuromuscular score. Higher total E\(_2\), but not free E\(_2\) was associated with lower grip strength after adjustment for age, FT, SHBG and relative ASM. Testosterone level was related to both muscle mass, to strength and to physical performance. Total E\(_2\) level, though related to muscle mass positively, affected muscle strength adversely in older men [11087].

**Females**

Physical exercise is known to strongly stimulate the endocrine system in both sexes. Among these hormones, androgens (e.g. testosterone, androstenedione, dehydroepiandrosterone) play key roles in the reproductive system, muscle growth and the prevention of bone loss. In female athletes, excessive physical exercise may lead to disorders, including delay in the onset of puberty, amenorrhoea and premature osteoporosis. The free and total fractions of circulating androgens vary in response to acute and chronic exercise/training (depending on the type), but the physiological role of these changes is not completely understood. Although it is commonly accepted that only the free fraction of steroids has a biological action, this hypothesis has recently been challenged. Indeed, a change in the total fraction of androgen concentration may have a significant impact on cells (inducing genomic or non-genomic signalling). The purpose of one review, therefore, was to visit the exercise-induced changes in androgen concentrations and emphasize their potential effects on female physiology.
Despite some discrepancies in the published studies (generally due to differences in the types and intensities of the exercises studied, in the hormonal status of the group of women investigated and in the methods for androgen determination), exercise is globally able to induce an increase in circulating androgens. This can be observed after both resistance and endurance acute exercises. For chronic exercise/training, the picture is definitely less clear and there are even circumstances where exercise leads to a decrease of circulating androgens. It was suggest that those changes have significant impact on female physiology and physical performance [11088].

The purpose of one study was to investigate the influence of a 14-week swimming training program on psychological, hormonal, and performance parameters of elite women swimmers. Ten Olympic and international-level elite women swimmers were evaluated 4 times along the experiment (i.e., in T1, T2, T3, and T4). On the first day at 8:00 am, before the blood collecting at rest for the determination of hormonal parameters, the athletes had their psychological parameters assessed by the profile of mood-state questionnaire. At 3:00 am, the swimmers had their anaerobic threshold assessed. On the second day at 3:00 am, the athletes had their alactic anaerobic performance measured. Vigor score and testosterone levels were significantly lower in T4 compared with T3. In addition, the rate between the peak blood lactate concentration and the median velocity obtained in the alactic anaerobic performance test increased in T4 compared with T3. For practical applications, the swimming coaches should not use a tapering with the present characteristics to avoid unexpected results [11089].

The association between androgens and competition in women has been understudied compared with men. The current study examined the link between testosterone and competition in elite female athletes, using a sample of female wrestlers that included athletes competing at both the national and international level. In a repeated-measures design, saliva samples were collected before and after wrestling bouts, with comparable samples of wins and losses, and subsequently analyzed for testosterone. Study results showed a 22 percent increase in circulating bioavailable testosterone from pre- to postbout, which was a significant difference. There was no significant difference in testosterone between win or loss outcomes. These findings showing a link between individual head-to-head competition and testosterone in women demonstrate that women's androgenic responses to environmental contexts are dynamic and may be an important factor to address in research on competitive performance [09101].

In a previous study, sprint training has been shown to increase muscle cross-sectional area in women but not in men [Eur J Appl Physiol Occup Physiol 1996; 74: 375]. It was hypothesized that sprint exercise induces a different hormonal response in women than in men. Such a difference may contribute to explaining the observed gender difference in training response. Metabolic and hormonal response to three 30-s sprints with 20-min rest between the sprints was studied in 18 physically active men and women. Significant accumulation of blood lactate and plasma ammonia after sprint exercise was greater in men. Serum insulin increased after sprint exercise more so in women than in men, while plasma glucose increased in men, but not in women. Serum growth hormone (GH) increased in both women and men reaching similar peak levels, but with different time courses. In women the peak serum GH level was observed after sprint 1, whereas in men the peak was observed after sprint 3. Serum testosterone tended to decrease in men and increase in women. Serum cortisol increased approx. 10-15 percent after sprint exercise, independent of gender. It was concluded that women elicited a greater response of serum GH and insulin to sprint exercise. This may contribute to explaining the earlier observed muscle hypertrophy in women in response to sprint training [09103].
**Effects in a young female**

A 14-year-old Caucasian girl was referred to the endocrine clinic for evaluation of voice deepening, facial hirsutism, and acne starting 2 years previously. She had been a competitive tennis player since age 7 years, practicing for 4-6 hours daily. Adrenal ultrasonography revealed a round left 4.6 × 5.3-cm adrenal mass. Laparoscopic left adrenalectomy was performed. The histologic findings were compatible with a benign adrenocortical tumor. Postoperatively, androgen levels dropped to within the normal range. Breast development proceeded normally, menarche occurred 2 months after tumor resection, and menses has been regular since then. Muscle strength of the dominant and nondominant upper and lower extremities was measured 1 month before surgery and 1 year later, using an isokinetic dynamometer (Biodex Systems II, Biodex, Shirley, NY, USA). There was no significant decrease in overall muscle strength after removal of the virilizing tumor and the marked drop in circulating androgens. In addition, the patient maintained her age category, number 1, national tennis ranking. The results suggest that even extremely high levels of tumor-related circulating androgens had no evident effect on muscle strength and competitive performance in a female adolescent tennis player. The lack of beneficial effect on performance in adolescents, combined with the potentially hazardous side effects of anabolic steroids, suggests that teenage athletes should avoid their use [11090].

**Adolecents**

One study investigated the effect of repeated bouts of short-term, high-intensity cycling exercise on the salivary cortisol, testosterone and immunoglobulin (A) concentrations of 15-16 year old boys. Seventeen apparently healthy schoolchildren (aged 15.5 ± 0.4 years) participated in this study. All participants completed 6 x 8 s sprints, interspersed with 30 s recovery intervals on a cycle ergometer. Using the passive drool method, salivary samples were taken before, and 5 min after, exercise. There were significant changes in both salivary testosterone and cortisol, 5 min after completing 6 x 8 s cycle sprints. No significant differences were recorded for immunoglobulin A. The increases in testosterone and cortisol reported confirm that repeated bouts of short-term, high-intensity exercise produces significant physiological hormonal responses in adolescent boys, but does not affect mucosal immune function [09097].

**Biking**

To determine whether cycling has an effect on serum PSA, gonadotropins, and uroflowmetric parameters a total of 34 healthy male athletes from the National Cycling Team and 24 healthy male student volunteers from University and medical staff were prospectively enrolled in a study. Blood samples for serum total prostate-specific antigen (tPSA), free PSA (fPSA), fPSA/tPSA, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone determinations were obtained before and after cyclists completed 300 km bicycle ride and with each cyclist seated without changing posture and with minimal movement for 10 minutes before blood collection. The cyclists also performed uroflowmetric and postvoid residual urine volume analysis before, and 1 hour after cycling course. Blood samples from the control group were drawn for serum hormones. They also underwent uroflowmetric and postvoid residual analysis. The athletes and the control group were well matched by age. There was no significant difference between the two groups in terms of serum tPSA, fPSA, f/t PSA values, FSH, LH, and testosterone levels and uroflowmetric parameters. The differences between pre- and postcycling values for tPSA, fPSA, f/t PSA, FSH, LH, and uroflowmetric parameters were not statistically significant. The postcycling serum testosterone level was significantly lower than precycling levels (mean, 6034 ng/dL vs
425 ng/dL. There was no correlation between body mass index values, postcycling serum FSH, LH levels, age, and testosterone levels [09098].

Response to marathon running

Exercise is known to be a powerful stimulus for the endocrine system. The hormonal response to exercise is dependent on several factors including the intensity, duration, mode of exercise (endurance versus resistance), and training status of the subject. The aim of one study was to determine the steroid hormonal response (immediately after a race and 1 week later) to endurance exercise under the real conditions of the classic Athens marathon in a group of well-trained, middle-aged, non-elite athletes. Blood samples were drawn 1 week before the race, directly after completion of the race, and 1 week later. Serum cortisol and prolactin showed distinct rises 1 h after the race and returned to baseline 1 week later. Androstenedione and dehydroepiandrosterone sulphate did not show any changes. Total testosterone as well as free testosterone dropped significantly 1 h after the race but returned to baseline 1 week later. In this particular group of non-elite, middle-aged marathon runners, the race resulted in an acute increase in serum cortisol and prolactin levels and in a concomitant decline in testosterone level. The aforementioned changes returned to baseline 1 week later [09099].

Effects on immune system

Intense exercise is known to cause temporary impairments in immune function. Few studies, however, have investigated the effects of intense competitive exercise on immunoendocrine variables in elite team sport athletes. The aim of one study was to evaluate the time course of changes in selected immunoendocrine and inflammatory markers following an international rugby union game. Blood samples were taken from players (n=10) on camp entry, the morning of the game (pre), immediately after (post) and 14 and 38 h into a passive recovery period. Players lost 1.4 ± 0.2 kg of body mass during the game (ambient conditions, 11 degrees C). An acute phase inflammatory response was observed as reflected through immediate significant increases in serum cortisol and IL-6 (post) followed by delayed increases in serum creatine kinase (CK; 14 h) activity and C-reactive protein (CRP; 38 h). Following a large decrease in serum testosterone to cortisol (T/C) ratio immediately post and 14 h after exercise, T/C values then significantly increased above those observed at camp entry 38 h into recovery. This rebound anabolic stimulus may represent a physiological requirement for recovery following intense tissue damage resulting from game collisions [09100].

Testosterone and age

To examine the association between aging and physical function in men by testing a theoretically based model of aging, hormones, body composition, strength, and physical function with data obtained from men enrolled in the Boston Area Community Health/Bone (BACH/Bone) a cross-sectional, observational survey was performed population-based. Testosterone, estradiol, sex hormone-binding globulin, lean and fat mass, grip strength, and summated index of physical function (derived from walk and chair stand tests). Measures of grip strength and physical function declined strongly with age. For instance, 10 years of aging was associated with a 0.49-point difference (scale 0-7) in physical function. Age differences in total testosterone and estradiol concentrations were smaller than age differences in their free fractions. Weak or nonsignificant age-adjusted correlations were
observed between hormones and measures of physical function, although path analysis revealed a positive association between testosterone and appendicular lean mass and a strong negative association between testosterone and total fat mass. Lean and fat mass, in turn, were strongly associated with grip strength and physical function, indicating the possibility that testosterone influences physical function via indirect associations with body composition. The authors concluded that age-related decline in serum testosterone concentration in men has a weak association with physical strength and functional outcomes through its associations with lean and fat mass [08123].

Aging athletes

A high prevalence of late-onset male hypogonadism has been observed in general population. Sport-participation influences the neuroendocrine system and may decrease serum testosterone. One preliminary study was designed to estimate the prevalence and the symptoms of undiagnosed testosterone deficiency in aging athletes. This observational survey was performed in 183 caucasian male athletes >50 years, in the setting of pre-participation screening. Pituitary-gonadal hormones and symptoms of hypogonadism were investigated. Serum total testosterone (TT), sex hormone binding globulin, luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin (PRL), free-T4, and thyroid stimulation hormone (TSH) were assayed, and free T, bioactive T, and the LH/TT ratio were calculated. The International Index of Erectile Dysfunction (IIEF-15) and the Center for Epidemiological Studies Depression Scale (CES-D) were administered. Hypogonadal athletes were compared with eugonadal athletes as controls. Prevalence and clinical symptoms of severe (TT < 8 nmol/L) or mild (8 nmol/L ≤ TT < 12 nmol/L) testosterone deficiency were investigated. The mean sample age was 62 ± 8 years (range 50-75). Severe or mild testosterone deficiency was observed in 12 and 18 percent, respectively, of overall athletes, with the highest prevalence in athletes >70 years (28 % and 25 %, respectively). TT did not correlate with age, training duration, or questionnaire scores. No differences were observed for nonspecific symptoms of hypogonadism, IIEF-15 and CES-D scores between eugonadal and severe hypogonadal athletes. It was concluded that independently of its etiology, a significant percentage of aging athletes had undiagnosed testosterone deficiency. In a relevant number of these cases, testosterone deficiency was not overtly symptomatic. The results suggest that sport-participation per se can influence the symptoms of hypogonadism. The history of clinical symptoms may be inaccurate to diagnose testosterone deficiency in aging athletes [10082].

In the HORMA (Hormonal Regulators of Muscle and Metabolism in Aging) Trial, supplemental testosterone and recombinant human growth hormone (rhGH) enhanced lean body mass, appendicular skeletal muscle mass, muscle performance, and physical function, but there was substantial interindividual variability in outcomes. One hundred and twelve men aged 65-90 years received testosterone gel (5 g/d vs 10 g/d via Leydig cell clamp) and rhGH (0 vs 3 vs 5 μg/kg/d) in a double-masked 2 × 3 factorial design for 16 weeks. Outcomes included lean tissue mass by dual energy x-ray absorptiometry, one-repetition maximum strength, Margaria stair power, and activity questionnaires. We used pathway analysis to determine the relationship between changes in hormone levels, muscle mass, strength, and function. Increases in total testosterone of 1046 ng/dL (95 % confidence interval 1040 to1051) and 898 ng/dL (95 % confidence interval 892 to 904) were necessary to achieve median increases in lean body mass of 1.5 kg and appendicular skeletal muscle mass of 0.8 kg, respectively, which were required to significantly enhance one-repetition maximum strength (≥ 30 %). Co-treatment with rhGH lowered the testosterone levels (quantified using liquid chromatography-tandem mass spectrometry) necessary to reach these lean mass thresholds. Changes in one-repetition maximum strength were associated with increases in stair climbing power. Pathway analysis supported the model that changes
in testosterone and insulin-like growth factor 1 levels are related to changes in lean body mass needed to enhance muscle performance and physical function. Testosterone's effects on physical activity were mediated through a different pathway because testosterone directly affected Physical Activity Score of the Elderly. To enhance muscle strength and physical function, threshold improvements in lean body mass and appendicular skeletal muscle mass are necessary and these can be achieved by targeting changes in testosterone levels. rhGH augments the effects of testosterone. To maximize functional improvements, the doses of anabolic hormones should be titrated to achieve target blood levels [10456].

**Effects of long flights**

Mild hypobaric hypoxia caused by pressurisation may contribute to alter rhythmicity after long-haul flights, independently of the number of time zones crossed. In this controlled crossover study, we assessed the effects of two levels of hypoxia, equivalent to 8000 ft and 12,000 ft above sea level, on the rhythm of plasma concentrations of three hormones: testosterone, LH, and FSH. A hypoxia-induced decrease in LH and FSH has often been reported during mountaineering while testosterone is considered a marker of fatigue. Sixteen healthy male volunteers, aged 23-39 years, spent 8 h in a hypobaric chamber (08:00-16:30), simulating conditions at 8000 ft. This was followed by an additional 8 h four weeks later, simulating conditions at 12,000 ft. Plasma hormones were assayed every 2 h over two 24-h cycles (control and hypoxic-exposure cycles). It was found no significant effects of hypoxia on the circadian profile of the gonadal axis hormones and, therefore, conclude that these hormones do not serve as valuable markers of post-flight alterations of the circadian system in human [06056].

**Influence of space flights**

Limited data suggest that testosterone is decreased during space flight, which could contribute to bone and muscle loss. The main objective was to assess testosterone and hormone status in long- and short-duration space flight and bed rest environments and to determine relationships with other physiological systems, including bone and muscle. Blood and urine samples were collected before, during, and after long-duration space flight. Samples were also collected before and after 12- to 14-d missions and from participants in 30- to 90-d bed rest studies. Space flight studies were conducted on the International Space Station and before and after Space Shuttle missions. Bed rest studies were conducted in a clinical research center setting. Data from Skylab missions are also presented. All of the participants were male, and they included 15 long-duration and nine short-duration mission crew members and 30 bed rest subjects. Serum total, free, and bioavailable testosterone were measured along with serum and urinary cortisol, serum dehydroepiandrosterone, dehydroepiandrosterone sulfate, and SHBG. Total, free, and bioavailable testosterone was not changed during long-duration space flight but was decreased on landing day after these flights and after short-duration space flight. There were no changes in other hormones measured. Testosterone concentrations dropped before and soon after bed rest, but bed rest itself had no effect on testosterone. Thus, there was no evidence for decrements in testosterone during long-duration space flight or bed rest [11443].

**Effects of diet on testosterone metabolites**

Longitudinal profiling of urinary steroids was investigated by using a gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) method. The carbon isotope ratio
of three urinary testosterone (T) metabolites: androsterone, etiocholanolone, 5beta-androstane-3alpha,17beta-diol (5beta-androstenediol) together with 16(5alpha)-androstene-3alpha-ol (androstenol) and 5beta-pregnanediol (5beta-pregnanediol) were measured in urine samples collected from three top-level athletes over 2 years. Throughout the study, the subjects were living in Switzerland and were residing every year for a month or two in an African country. $^{13}$C-enrichment larger than 2.5 per thousand was observed for one subject after a 2-month stay in Africa. The findings reveal that $^{13}$C-enrichment caused by a diet change might be reduced if the stay in Africa was shorter or if the urine sample was not collected within the days after return to Switzerland. The steroids of interest in each sample did not show significant isotopic fractionation that could lead to false positive results in antidoping testing. In contrast to the results obtained with the carbon isotopic ratio, profiling of urinary testosterone/epitestosterone (T/E) ratios was found to be unaffected by a diet change [06057].

**Influence of zinc**

There is fairly scarce information about the effects of zinc, an essential trace element, on performance. Studies concerned with the relation between zinc and exercise mostly concentrate on the distribution of this element in the body in response to exercise. The objective of one study was to explore how zinc supplementation affects testosterone levels and its relation with lactate in rats subjected to acute swimming exercise. Thirty adult male rats of Sprague-Dawley species were equally allocated to 3 groups. Group 1: Control. Group 2: Group subjected to 30-minute acute swimming exercise. Group 3: Group supplemented with intraperitoneal (i.p.) zinc (3 mg/kg day) for 4 weeks and subjected to 30-minute swimming exercise. Blood samples collected from all experimental animals by decapitation method were analyzed to determine free and total testosterone and lactate levels in the plasma. Group 3 had the highest free and total testosterone levels, followed by Group 1 and Group 2. The highest lactate levels were found in Group 2 and the levels in Group 3 were higher than those in Group 1. Results of the study demonstrate that zinc supplementation leads to a significant increase in testosterone levels and a significant decrease in lactate levels. In conclusion, physiological doses of zinc supplementation can be useful for performance [06058].

**In hypogonadal men**

Testosterone replacement therapy in hypogonadal men has been used for > 60 years. To describe serum and urinary hormones, androgens metabolites and testosterone/epitestosterone ratio profiles after testosterone administration in male hypogonadal volunteers, and, to evaluate their possible usefulness in detecting doping with testosterone in treated hypogonadal athletes a controlled open label design versus placebo study was performed. There were ten male volunteers affected by severe hypogonadism (serum testosterone < 2.31 ng/mL). Serum and urinary parameters were evaluated, by radioimmunoassay and gas chromatography-mass spectrometry, before and at different time points for seven/three weeks after a single administration of testosterone enanthate (250 mg) or placebo respectively. As partially known, testosterone administration increased, with great individual variability, urinary concentrations of glucuronide testosterone, androsterone, etiocholanolone, 5alphaandrostane-3alpha17betadiol, 5betaandrostane-3alpha17betadiol, testosterone/epitestosterone and testosterone/LH ratios; and decreased epitestosterone and 5alphaandrostane-3beta17betadiol/5betaandrostane-3alpha17betadiol ratio. Serum testosterone and dihydrotestosterone increased in all volunteers, and concentrations higher than the upper reference limits were observed in many volunteers until two weeks after
testosterone administration. Whereas the observed prolonged hyperandrogenism partially limited data interpretation, the reported characteristics of variation of urinary parameters might be used to suspect testosterone misuse in hypogonadal athletes treated with testosterone enanthate. In this sense, while the actual threshold for ratio of testosterone to epitestosterone ratio was confirmed of reduced usefulness, it was suggested a contemporary evaluation of whole urinary androgen metabolites profile and serum androgens, at specific time points after testosterone enanthate administration. Moreover, an adequate tailoring of treatment, to avoid transitory hyperandrogenism, is highly advisable [09104].

Testosterone has a steeply dose-dependent effect on muscle mass and strength irrespective of gonadal status. So, for reasons of fairness, people who engage in competitive sports should not administer exogenous testosterone raising their blood testosterone levels beyond the range of normal. There is a ban on exogenous anabolics for men and women in sports, but an exception has been made for men with androgen deficiency due to pituitary or testicular disease. Men who receive testosterone administration for the indication hypogonadism have an interest in the use of testosterone preparations generating blood testosterone levels within the normal range of healthy, eugonadal men. On the grounds of a positive correlation between blood testosterone concentrations muscle and volume/strength, they are best served with a parenteral testosterone preparation, rather than transdermal testosterone, but they should not run the risk of being excluded from competition because of supraphysiological testosterone levels. The latter is a realistic risk with the traditional parenteral testosterone esters. The new parenteral testosterone undecanoate preparation offers much better perspectives. Its pharmacokinetics have been investigated in detail and there is a fair degree of predictability of resulting blood testosterone levels with use of this preparation [08150].

**Testosterone level as an indicator of gender**

Based on DNA analysis of a historical case, the authors describe how a female athlete can be unknowingly confronted with the consequences of a disorder of sex development resulting in hyperandrogenism emerging early in her sports career. In such a situation, it is harmful and confusing to question sex and gender. Exposure to either a low or high level of endogenous testosterone from puberty is a decisive factor with respect to sexual dimorphism of physical performance. Yet, measurement of testosterone is not the means by which questions of an athlete's eligibility to compete with either women or men are resolved. It might be justifiable to use the circulating testosterone level as an endocrinological parameter, to try to arrive at an objective criterion in evaluating what separates women and men in sports competitions, which could prevent the initiation of complicated, lengthy and damaging sex and gender verification procedures. In 1949, the Dutch track athlete Foekje Dillema (1926–2007) came to prominence on the world athletic stage. She started to rival Fanny Blankers-Koen, the world-famous Dutch track athlete who won four gold medals during the 1948 Summer Olympics in London and was elected Female Athlete of the Century by the International Association of Athletics Federations (IAAF) in 1999. In contrast, Dillema's career was of short duration, with a dramatic ending. In 1950, she was expelled for life by the Royal Dutch Athletics Federation, due to the results of a 'sex test', for which details or results were never revealed and no records are available. Her 1950 national record of 24.1 s for the 200 m, which she took from Fanny Blankers-Koen, was erased, and only after her death 57 years later was she reinstated by the Royal Dutch Athletics Federation. The verification of the sex of athletes has been an issue for many decades. It should be noted that reports and reviews on this topic refer to gender verification, rather than sex verification. However, what counts in competitive sports is a person's sex characteristics. Trying to avoid the word sex,
given its charged nature, can only cause confusion. Herein, it was use the term sex for the biological and physiological characteristics that define men and women, as compared to gender and gender identity in reference to the socially and individually perceived sexual identity of an individual from birth to puberty and adulthood. Sex verification in 1950 was based solely on physical examination, predating hormone assays or sex chromosome analysis. Following discovery of the Barr body in female cells in 1949, it took some 12 years before it was known that this body represents an inactivated X chromosome; from the late 1960s its detection was used in sex verification tests in the context of sports competitions. Subsequent tests focused on the male-specific region of the Y chromosome, particularly the male sex determining SRY gene. However, opposition to sex verification for female athletes with laboratory-based genetic testing developed in the 1970s and 1980s, because these tests did not encompass the complexities of disorders of sex development (DSDs). Since the 2000 Summer Olympics, questioned sex and gender is evaluated on a case-by-case basis by a team of specialists in the areas of endocrinology, genetics, gynaecology and psychology. To broaden the perspective on sportswomen confronted with questioned sex characteristics, we have investigated the case of Foekje Dillema, with informed consent from her heirs, by means of DNA analysis of samples from worn clothing. Appreciating the nature of the samples tested, it was applied DNA methodology and lab quality standards used in human forensics. The DNA analysis indicates that Foekje Dillema had a 46,XX/46,XY mosaic condition with a rare origin, which was interpreted as leading to hyperandrogenism from her puberty [12111].

To have, or not to have, a Y chromosome is the primary decisive factor in human sexual differentiation, but there are exceptions. A prominent example is offered by 46,XY females who have the complete form of androgen insensitivity syndrome (cAIS), when the testes produce testosterone but the body is not able to respond to androgens (testosterone and its more powerful metabolite dihydrotestosterone) due to mutation of the X-encoded androgen receptor. Consequently, these individuals are born and raised as girls, and have a female gender identity. Action of testosterone through binding to the androgen receptor in the developing fetal brain is the predominant factor in programming human male gender identity, and the female gender identity of 46,XY cAIS women is explained by loss of this androgenic effect. In sports, 46,XY cAIS women can be expected to have a disadvantage compared to 46,XX women with a functional androgen receptor, the latter profiting from stimulation of muscle strength by a low level of circulating testosterone. A moderate pubertal and postpubertal excess of testosterone in a young woman can give extra muscle development and other signs of hyperandrogenism, but it would be a rude error to even suggest that this would affect her female gender identity. Competitive athletes exploit fortunate combinations of natural differences in physical and mental personal characteristics, including individual variation of the endogenous testosterone level. The World Anti-Doping Agency states that an athlete's sample will be found positive if the concentration of an endogenous androgenic steroid hormone is above the range normally found in humans, and is not likely consistent with normal endogenous production, unless the elevated concentration of the steroid hormone (or metabolites or markers) is attributable to a physiological or pathological condition. Strictly speaking, a female athlete is free to benefit from any endogenous source of androgen production. Some female athletes may benefit, probably to a small extent, from increased androgen production originating from a polycystic ovary. This is viewed as acceptable by the IAAF, who stated that conditions that may provide some advantages but nevertheless are acceptable include congenital adrenal hyperplasia, androgen-producing tumours and an ovulatory androgen excess associated with a polycystic ovary. According to these regulations, hyperandrogenism caused by ovotesticular DSD would be unacceptable only if sex and gender verification would provide evidence that the female athlete in fact is a man [12111].
Transdermal testosterone

The screening of testosterone (T) misuse for doping control is based on the urinary steroid profile, including T, its precursors and metabolites. Modifications of individual levels and ratio between those metabolites are indicators of T misuse. In the context of screening analysis, the most discriminant criterion known to date is based on the T glucuronide (TG) to epitestosterone glucuronide (EG) ratio (TG/EG). Following the World Anti-Doping Agency (WADA) recommendations, there is suspicion of T misuse when the ratio reaches 4 or beyond. While this marker remains very sensitive and specific, it suffers from large inter-individual variability, with important influence of enzyme polymorphisms. Moreover, use of low dose or topical administration forms makes the screening of endogenous steroids difficult while the detection window no longer suits the doping habit. As reference limits are estimated on the basis of population studies, which encompass inter-individual and inter-ethnic variability, new strategies including individual threshold monitoring and alternative biomarkers were proposed to detect T misuse. The purpose of this study was to evaluate the potential of ultra-high pressure liquid chromatography (UHPLC) coupled with a new generation high resolution quadrupole time-of-flight mass spectrometer (QTOF-MS) to investigate the steroid metabolism after transdermal and oral T administration. An approach was developed to quantify 12 targeted urinary steroids as direct glucuro- and sulfo-conjugated metabolites, allowing the conservation of the phase II metabolism information, reflecting genetic and environmental influences. The UHPLC-QTOF-MSE platform was applied to clinical study samples from 19 healthy male volunteers, having different genotypes for the UGT2B17 enzyme responsible for the glucuroconjugation of T. Based on reference population ranges, none of the traditional markers of T misuse could detect doping after topical administration of T, while the detection window was short after oral TU ingestion. The detection ability of the 12 targeted steroids was thus evaluated by using individual thresholds following both transdermal and oral administration. Other relevant biomarkers and minor metabolites were studied for complementary information to the steroid profile, including sulfoconjugated analytes and hydroxy forms of glucuroconjugated metabolites. While sulfoconjugated steroids may provide helpful screening information for individuals with homozygous UGT2B17 deletion, hydroxy-glucuroconjugated analytes could enhance the detection window of oral testosterone undecanoate (TU) doping [13190].

The legally defensible proof of the abuse of endogenous steroids in sports is currently based on carbon isotope ratio mass spectrometry (IRMS), i.e. a comparison between $^{13}$C/$^{12}$C ratios of diagnostic precursors and metabolites of testosterone. The application of this technique requires a chromatographic baseline separation of respective steroids prior to IRMS detection and hence laborious sample pre-processing of the urinary steroid extracts including clean up by solid-phase extraction and/or liquid chromatography. Consequently, an efficient pre-selection of suspicious control urine samples is essential for appropriate follow up confirmation by IRMS and effective doping control. Two single transdermal administration studies of testosterone (50 mg Testogel® and Testopatch® at 3.8 mg in 16 h, respectively) were conducted and resulting profiles of salivary testosterone and urinary steroid profiles and corresponding carbon isotope ratios were determined. Conventional doping control markers (testosterone/epitestosterone ratio, threshold concentrations of androsterone, etiocholanolone, or androstanediol) did not approach or exceed critical thresholds. In contrast to these moderate variations, the testosterone concentration in oral fluid increased from basal values (30-142 pg/mg) to peak concentrations above 1000 pg/mg. It is likely that this significant increase in oral fluid is due to a pulsatile elevation of free (protein unbound) circulating testosterone after transdermal administration and may be assumed to represent a more diagnostic marker for transdermal testosterone administration [13191].
The screening of testosterone (T) misuse for doping control is based on the urinary steroid profile, including T, its precursors and metabolites. Modifications of individual levels and ratio between those metabolites are indicators of T misuse. In the context of screening analysis, the most discriminant criterion known to date is based on the T glucuronide (TG) to epitestosterone glucuronide (EG) ratio (TG/EG). Following the World Anti-Doping Agency (WADA) recommendations, there is suspicion of T misuse when the ratio reaches 4 or beyond. While this marker remains very sensitive and specific, it suffers from large inter-individual variability, with important influence of enzyme polymorphisms. Moreover, use of low dose or topical administration forms makes the screening of endogenous steroids difficult while the detection window no longer suits the doping habit. As reference limits are estimated on the basis of population studies, which encompass inter-individual and inter-ethnic variability, new strategies including individual threshold monitoring and alternative biomarkers were proposed to detect T misuse. The purpose of this study was to evaluate the potential of ultra-high pressure liquid chromatography (UHPLC) coupled with a new generation high resolution quadrupole time-of-flight mass spectrometer (QTOF-MS) to investigate the steroid metabolism after transdermal and oral T administration. An approach was developed to quantify 12 targeted urinary steroids as direct glucuro- and sulfo-conjugated metabolites, allowing the conservation of the phase II metabolism information, reflecting genetic and environmental influences. The UHPLC-QTOF-MS(E) platform was applied to clinical study samples from 19 healthy male volunteers, having different genotypes for the UGT2B17 enzyme responsible for the glucuroconjugation of T. Based on reference population ranges, none of the traditional markers of T misuse could detect doping after topical administration of T, while the detection window was short after oral TU ingestion. The detection ability of the 12 targeted steroids was thus evaluated by using individual thresholds following both transdermal and oral administration. Other relevant biomarkers and minor metabolites were studied for complementary information to the steroid profile, including sulfoconjugated analytes and hydroxy forms of glucuroconjugated metabolites. While sulfoconjugated steroids may provide helpful screening information for individuals with homozygous UGT2B17 deletion, hydroxy-glucuroconjugated analytes could enhance the detection window of oral T undecanoate (TU) doping [13192].

Age-related hormonal decline is gradual and less recognized in men than in women. Symptoms are oftentimes ignored and non-specific. Fatigue, lack of concentration, mood swings, decreased sexual desire, erectile dysfunction, infertility, hair loss, reduced muscle and bone mass, and weight gain are a few of the symptoms of male hypogonadism. This disorder is linked to reduction in quality of life, and poorer health outcomes as it may increase the risk for cardiovascular disease, diabetes mellitus, metabolic syndrome, Alzheimer's disease and premature death. Different modalities of testosterone replacement therapy have evolved over 70 years, and sales continue to grow. Each preparation is differentiated by route of delivery, ease of use, cost and pharmacokinetics. Topical or transdermal testosterone replacement therapy, including patches and gels, are the most modern formulations on the market. These are more expensive treatments, but yield more physiological concentrations of testosterone. The International Society of Andrology, International Society for the Study of the Aging Male, European Association of Urology, European Academy of Andrology, American Society of Andrology (ISA/ISSAM/EAU/EAA/ASA), the Endocrine Society, and the American Association of Clinical Endocrinologists (AACE) have published clinical practice guidelines for hypogonadism diagnosis, treatment and monitoring that are more or less in general agreement with each other. The ISA/ISSAM/EAU/EAA/ASA proposes 230 ng/dL, the Endocrine Society recommends 300 ng/dL and the AACE suggests 200 ng/dL as the lower limit of serum total testosterone at which patients will benefit from testosterone replacement therapy (TRT). Total testosterone is the most widely used measurement for establishing biochemical hypogonadism. Free testosterone should be measured when total testosterone
is nondiagnostic, such as in chronic illness, obesity and alcoholism; however, parameters are lacking. The Endocrine Society and AACE recommend against TRT in patients with haematocrit >50 percent. ISA/ISSAM/EAU/EAA/ASA proposes dose adjustments and/or periodic phlebotomy to keep haematocrit <52–55 percent. TRT stimulates the bone marrow production of erythrocytes, resulting in increased haematocrit in some men, with the possibility of hyperviscosity side effects. Restoration of testosterone levels to the eugonadal range reverses signs and symptoms of hypogonadism, except for infertility, and may alleviate co-morbidities associated with hypogonadism. The risks of TRT are few, but in theory there have been concerns about worsening of benign prostatic hyperplasia (BPH), acceleration of pre-existing prostate cancer, exacerbation of sleep apnoea and congestive heart failure, increased risk for liver toxicity and tumour, gynecomastia, testicular atrophy and infertility, acne and oily skin and hair loss. Patient understanding of and compliance with both treatment and monitoring are of utmost importance to achieve clinical success with maximum benefit and minimum risk [12008].

Topical preparations

Commercially available testosterone replacement therapy (TRT) preparations have evolved from invasive to non-invasive treatments over the past 70 years. What initially began as subdermal testosterone pellet implants in 1940, evolved to intramuscular testosterone esters in 1954, to oral testosterone undecanoate in 1977, to transdermal delivery as scrotal patches in 1992, as non-scrotal patches in 1995, as topical gels in 2002, and as underarm roll-on solution in 2010. Other recent additions to TRT are the shortacting buccal testosterone and the long-acting intramuscular testosterone undecanoate, used extensively in Europe. Nasal sprays and oral preparations are currently under development. Choosing among these different preparations requires an understanding of their pharmacokinetics. The intramuscular testosterone esters are popular forms of TRT since they are inexpensive and require infrequent dosing. Testosterone propionate is administered every 2 to 3 days. Testosterone enanthate and cypionate are administered every 2 to 3 weeks, gain peak concentrations shortly after injection, and decline gradually after 7 to 15 days. New on the market is the longer-acting testosterone undecanoate (Nebido®) that is administered every 12-16 weeks, approximately 4 times a year. The drawbacks of intramuscular testosterone administration are the discomfort of injections, the resulting wide peak-to-trough fluctuations in serum testosterone levels, leading to instability in mood, libido and sexual function, and the inability to rapidly withdraw treatment if necessitated by adverse effects. The subdermal testosterone pellet implants are used commonly outside of the US. About 3 to 6 pellets of 200 mg unmodified testosterone are implanted subcutaneously every 4 to 6 months, providing stable physiological levels of testosterone. Downsides include discomfort and inconvenience from required minor surgery, extrusion of the pellets, and rarely, bleeding, infection and fibrosis at insertion site. The oral testosterone undecanoate, although a popular route of delivery, has disappeared from the market. It requires multiple, high daily doses due to low bioavailability (rapidly inactivated by first-pass hepatic metabolism). It carries the risks of gastrointestinal upset and hepatotoxicity, including cholestatic jaundice, hepatic tumours and vascular abnormalities of the liver. Buccal testosterone bypasses first-pass hepatic metabolism by entering the venous drainage from the mouth into the superior vena cava. The shortacting buccal testosterone (Striant®) is new to the market and contains testosterone 30 mg applied twice daily to a depression in the gums above the incisors. It restores physiological testosterone and dihydrotestosterone (DHT) levels within 4 hours after application, and steady-state concentrations are achieved within 24 hours. Side effects are gum irritation, which resolves over time, and low possibility of secondary transference via saliva [12008].
Transdermal preparations

Scrotal patches were the first transdermal TRT on the market but have now been replaced by non-scrotal patches and gels. In general, the gels cost more than the patches. According to Intercontinental Marketing Services Health 2009 data, global sales of testosterone therapies have grown to more than USD 1 billion per year, with sales of testosterone gels in the US accounting for USD 700 million. The major advantages of transdermal delivery include ease of use, avoidance of peaks and troughs found with other routes, and maintenance of relatively stable serum testosterone concentrations, leading to stability in energy, mood and libido. Topical preparations also appear to be associated with lesser risk of erythrocytosis and minimal effects on lipid profiles compared with intramuscular injections, and do not appear to be associated with hepatoxicity compared with oral forms containing methylated testosterone. The scrotal testosterone patch (Testoderm®), no longer available on the market, enables rapid absorption through the thin, highly vascularized scrotal skin. The scrotal skin has high 5alpha-reductase activity, which catalyzes the conversion of testosterone to DHT. It has been found that the scrotal testosterone patch delivers physiological levels of testosterone, but supraphysiological levels of DHT, which raised concerns regarding its long-term effects on the prostate gland. It also requires clipping of scrotal hair prior to nightly application, and use of brief-type underwear after application. The non-scrotal testosterone patch (Androderm®), still available in the market, comes in different patch sizes and doses (2, 2.5, 4 and 5 mg), and is worn nightly for 24 hours on the back, thigh, upper arm or abdomen. The 2 mg patch has a total contact surface area of 32 cm² with a 6.0 cm² central drug delivery reservoir containing testosterone 9.7 mg unique selling proportion (USP) for delivery of testosterone 2 mg per day, while the 4 mg patch has a total contact surface area of 39 cm² with a 12.0 cm² central drug delivery reservoir containing testosterone 19.5 mg USP for delivery of testosterone 4 mg per day. Testosterone USP is a white crystalline powder or crystals chemically described as 17X-hydroxyandrost-4-en-3-one. The adhesive side of the patch should be applied to a clean, dry area, and not over bony prominences or on a part of the body that may be subject to prolonged pressure. The recommended starting dose is one 4 mg/day system (not two 2 mg/day systems) applied nightly for 24 hours, delivering approximately 4 mg of testosterone per day. Skin rash (66 %) is a side effect of the patch, and necessitates discontinuation of therapy if quite severe. The rash may sometimes be prevented by topical corticosteroid pretreatment. Testosterone is continuously absorbed over a 24-hour dosing period through a non-rate-limiting microporous membrane with mean minimum testosterone concentration (Cmin) of 280 ng/dL, time-average testosterone concentration (Cave) of 517 ng/dL, maximum plasma testosterone concentration (Cmax) of 765 ng/dL, and time to Cmax (tmax) of 8 hours. An increase from 1 to 3 transdermal testosterone patches results in a linear increase in cumulative amounts of testosterone released. The use of three transdermal systems yields supraphysiological levels of testosterone, which is of possible benefit for patients who fail 1- and 2-patch regimens, particularly obese hypogonadal men. The optimal sites for this transdermal system are the back > thigh > upper arm > abdomen. The chest and the shin have lower and more variable rates of absorption. During steadystate, the average DHT:T and estradiol (E2):T ratios were approximately 1:10 and 1:200, respectively. Upon removal of the patch, serum testosterone concentrations decrease with an apparent half-life of 1.3 hours, and reach hypogonadal concentrations within 24 hours. There is no accumulation of testosterone during continuous treatment [12008].

Gels and solutions

The testosterone gels are widely available on the market. Skin rash (6 %) can also occur with gels, but is usually less severe and rarely results in discontinuation of therapy as it can the
patch. The primary concern with gels is potential for secondary exposure to another person, particularly to children, which in theory may result in inappropriate enlargement of genitalia, premature development of pubic hair, advanced bone age, increased libido, aggressive behaviour, and central or peripheral precocious puberty. Careful practices after every application, such as thoroughly washing hands and avoiding skin contact with another person, would avoid these consequences. Patients are also advised to avoid swimming or showering or washing the administration site for a minimum of 2 hours after application. Testim® is a clear-to-translucent gel that comes in 5 g and 10 g tubes, which contain 50 mg and 100 mg of testosterone, respectively. The recommended starting dose is 50 mg of testosterone (one 5 g tube) applied once daily in the morning to the shoulders and upper arms. It is generally well tolerated and is effective at raising testosterone levels, but requires a comparatively large weight of medication to be applied. It also has a musky odour that some patients do not like. Testogel/Androge® is a clear, odourless formulation that comes in a multi-dose pump. Each compression of the pump yields 1.25 g of gel, containing 12.5 mg of testosterone. The recommended starting dose is 50 mg of testosterone (four pump actuations) applied topically once daily in the morning to the shoulders, upper arms or stomach. Testim® has a more favourable pharmacokinetic profile than the testosterone patches (Andropatch® and Androderm®) and than another testosterone gel (Androgel®). This is likely attributable to the inclusion of pentadecalactone in the formulation, which provides increased emollient qualities and therefore is less drying to the skin, enabling higher testosterone absorption. Approximately 10 percent of the applied testosterone dose is absorbed across skin of average permeability, and provides continuous transdermal delivery of testosterone over a 24-hour period. Testosterone and DHT concentrations increase in parallel. In a 30-day efficacy trial response in sexual activity, desire and mood improved within the first week of Testim® use, with maximal response by the end of 2 weeks, and was maintained throughout the study. In a 90-day European trial, Testim® gel at two doses (50 and 100 mg/day, delivering 5 and 10 mg daily doses of testosterone, respectively) showed a significantly higher improvement of sexual function, motivation and desire and performance; a higher incidence of spontaneous erections; a higher reduction of percentage fat; and fewer adverse skin reactions than Andropatch® (two 2.5 mg patches, each delivering 12.2 mg of testosterone). These data are comparable to a 90-day US trial comparing Testim® gel (50 mg and 100 mg/day doses), Androderm® (two 2.5 mg patches), and a placebo gel. After 90 days, Testim® 100 mg/day showed significant improvements in sexual motivation, desire and performance, and a higher number of spontaneous erections than placebo. Both the Testim® 50mg and 100mg/day doses also showed significantly improved lean body mass, and reduced fat mass and fat percentage than the patch and the placebo. In a bioequivalence study comparing two commercially available testosterone gels, Testim® provided higher serum levels and greater bioavailability than Androgel®. TheCmax and the 24-hour systemic availability (area under the plasma concentration-time curve; AUC0–24) estimates for Testim® of total testosterone, DHT and free testosterone were 30 percent and 30, 19 and 11 percent, and 38 and 47 percent higher than for Androgel®, respectively. In a 180-day trial, serum testosterone concentrations increased to physiological range within first day of treatment with Androgel® 1 percent testosterone gel applied to arms, shoulders or abdomen. These levels were maintained in 87 percent of the study patients for the entire duration of the study. In another trial, gel application to four separate sites produced slightly higher, but not statistically significant, concentrations than repeated application to only one site. This suggested that the surface area of gel application had only a modest impact on the pharmacokinetic parameters of testosterone concentration. Androgel® has a newer, more concentrated, 1.62 percent low-volume formulation that allows the amount of applied gel required to be reduced, with each compression of the pump yielding 1.25 g of gel, containing 20.25 mg of testosterone. The recommended starting dose for this newer 1.62 percent formulation is 41 mg of testosterone (two pump actuations) applied topically once daily in the morning to the shoulders and upper arms, and titrated between 1.25 and 5.0 g a day. The
newer Androgel® 1.62 percent formulation has increased viscosity, reduced volume of application and increased skin permeation compared with the 1 percent gels, and is found to be safe, efficacious and well tolerated. More than 77 percent of hypogonadal men achieved normal serum testosterone levels as reported in a 12-month trial. In a cross-over trial, pharmacokinetics and relative bioavailability were compared following application of 5 g (81 mg of testosterone dose) daily of Androgel® 1.62 percent testosterone gel for 7 days to the abdomen, the upper arms/shoulders, or alternating abdomen and upper arms/shoulders. On both days 1 and 7, mean serum testosterone, DHT and E2 concentrations were higher for the upper arms/shoulders group than for the abdomen group. The alternating sites group yielded concentrations similar to the abdomen group on day 1 (both applied to the abdomen), and to the upper arms/shoulders group on day 7 (both applied to the upper arms/shoulders). Approximately 30-40 percent lower testosterone bioavailability was observed when applied to the abdomen than to the upper arms/shoulders, but both sites still yielded eugonadal serum testosterone levels (300 to 1000 ng/dL). Steady-state testosterone concentrations were achieved by day 2 on either site. After administration of the last dose, serum testosterone concentrations return to hypogonadal levels within 48 hours after abdominal application, and 72 hours after application to upper arms/shoulders. Skin washing as early as 2 hours after gel application had little impact on bioavailability and was effective in reducing residual testosterone on the skin to prevent secondary transference. Recently developed 2 percent testosterone gels are marketed as Tostran®, Fortigel®, Itnogen®, Tostrex®, and Fortesta®. Patients find this 2 percent concentration a favourable TRT because it is low in volume, and is easy to apply and titrate. Fortesta®, for example, is a clear, odourless gel that also comes in a metered-dose pump. Each pump compression delivers 0.5 g of gel, containing 10 mg of testosterone. It is applied to the front and inner thighs, and not the upper body, with a recommended starting dose of 40 mg of testosterone (four pump actuations) once daily in the morning, and titrated between 10 and 70 mg per day in 10 mg increments, as determined by the serum testosterone concentration. It is generally well tolerated, with the most common side effect being mild to moderate transient skin reactions, without serious adverse reactions. In a 90-day trial, once-daily topical application of 2 percent testosterone gel restored normal levels of testosterone in more than 75 percent of hypogonadal patients, with a low risk of supraphysiological testosterone levels. It provides steady levels throughout the day, with mean $C_{\text{ave}}$ of 438.6 ng/dL, and $C_{\text{max}}$ of 827.6 ng/dL at day 90, and more than 80 percent of subjects only required 60 mg testosterone or less. Axiron® is the newest option for topical TRT, and is actually the first pharmaceutical product to be applied underarm. It is a fragrance-free testosterone solution that comes in a metered-dose pump with a silicone applicator (rather than using hands), with each pump depression delivering 1.5 mL of solution containing 30 mg of testosterone. The recommended starting dose is 60 mg of testosterone (one pump actuation to each axilla, no shaving required) once every morning, and adjusted to a minimum of 30 mg and maximum of 120 mg daily, as judged by the serum testosterone concentration from a single blood draw 2 to 8 hours after applying the testosterone solution and at least 14 days after starting treatment or following dose adjustment. After application of the solution to the axillae, ethanol and isopropanol evaporate, leaving testosterone and octisalate. The octisalate is a thickening agent that increases the permeability of testosterone, which becomes absorbed through the skin to the systemic circulation over time. In a 120-day efficacy trial, 84 percent of hypogonadal men achieved eugonadal testosterone concentrations (300 to 1050 ng/dL). Steady-state serum testosterone, free testosterone and DHT were attained by day 15 of daily dosing of the standard initial dose of 60 mg of testosterone. Significant improvements in mood, sexual desire and activity, and general physical and mental health were noted. Adverse events reported include mild skin irritation, erythema and oedema. Deodorant and antiperspirants do not interfere with the efficacy of Axiron® [12008].
Male hypogonadism is a significant and growing problem that can be successfully treated with testosterone replacement therapy. A new formulation of testosterone gel (1.62%) was developed with increased viscosity, reduced volume of application, and increased skin permeation compared with other currently available testosterone gels. It was evaluated the efficacy and safety of titrated doses of 1.62 percent testosterone gel after daily application to the skin of hypogonadal men for 182 days. It was a multicenter, randomized, double-blind, placebo-controlled study in hypogonadal men (234 active; 40 placebo), 18 to 80 years of age with average serum total testosterone concentrations <300 ng/dL and prostate-specific antigen <2.5 ng/mL. Topical testosterone gel (1.62%), 1.25 g, 2.5 g, 3.75 g, and 5.0 g, or placebo gel was applied once daily to either upper arms/shoulders or abdomen. Dose adjustments were made on days 14, 28, and 42. The percentage of subjects with serum total testosterone average concentrations within the normal range of 300-1,000 ng/dL on study days 14, 56, 112, and 182. Following titration, significantly more subjects receiving active treatment had testosterone C values (range 82% to 83%) within the eugonadal range compared with placebo (range 29% to 37%) on all study days. The 1.62 percent gel was safe and well tolerated. It was concluded that, treatment with 1.62 percent testosterone gel was safe and efficacious, resulting in an acceptable percentage of hypogonadal males achieving eugonadal serum testosterone levels.

Current approaches to diagnosing testosterone deficiency do not consider the physiological consequences of various testosterone levels or whether deficiencies of testosterone, estradiol, or both account for clinical manifestations. It was provided 198 healthy men 20 to 50 years of age with goserelin acetate (to suppress endogenous testosterone and estradiol) and randomly assigned them to receive a placebo gel or 1.25 g, 2.5 g, 5 g, or 10 g of testosterone gel daily for 16 weeks. Another 202 healthy men received goserelin acetate, placebo gel or testosterone gel, and anastrozole (to suppress the conversion of testosterone to estradiol). Changes in the percentage of body fat and in lean mass were the primary outcomes. Subcutaneous- and intraabdominal-fat areas, thigh-muscle area and strength, and sexual function were also assessed. The percentage of body fat increased in groups receiving placebo or 1.25 g or 2.5 g of testosterone daily without anastrozole (mean testosterone level, 44 ± 13 ng per deciliter, 191 ± 78 ng per deciliter, and 337 ± 173 ng per deciliter, respectively). Lean mass and thigh-muscle area decreased in men receiving placebo and in those receiving 1.25 g of testosterone daily without anastrozole. Leg-press strength fell only with placebo administration. In general, sexual desire declined as the testosterone dose was reduced. The amount of testosterone required to maintain lean mass, fat mass, strength, and sexual function varied widely in men. Androgen deficiency accounted for decreases in lean mass, muscle size, and strength; estrogen deficiency primarily accounted for increases in body fat; and both contributed to the decline in sexual function. The findings support changes in the approach to evaluation and management of hypogonadism in men.

Effect of postexercise ethanol ingestion

Alcohol (ethanol) and resistance exercise can independently affect circulating bioavailable testosterone concentration. The purpose of one study was to examine the testosterone bioavailability and the anabolic endocrine milieu in response to acute ethanol ingestion after a bout of heavy resistance exercise. Eight resistance-trained men (25 years) completed two identical acute heavy resistance exercise tests (AHRET: six sets of 10 repetitions of Smith machine squats) separated by 1 week. Post-AHRET, participants consumed either 1.09 g of grain ethanol per kilogram lean mass (EtOH condition) or no ethanol (placebo condition). Blood samples were collected immediately before exercise (PRE), immediately after exercise.
(IP), and every 20 min postexercise for 300 min. Samples after IP were pooled into phases (20-40 min, 60-120 min, and 140-300 min after exercise) and analyzed for total testosterone (TT) and free testosterone (FT), sex hormone-binding globulin (SHBG), cortisol, and estradiol. Peak blood ethanol concentration (0.088 ± 0.015 g/dL) was achieved 60-90 min postexercise. TT and FT were elevated significantly at IP for both conditions. At 140-300 min postexercise, TT, FT, and free androgen index were significantly higher for EtOH (TT: 22.5 ± 12.5 nmol·L; FT: 40.5 ± 7.6 pmol·L) than for placebo (TT: 13.9 ± 6.8 nmol·L; FT: 22.7 ± 10.0 pmol·L). No differences between conditions were noted for SHBG, cortisol, or estradiol. It was concluded that postexercise ethanol ingestion affects the hormonal milieu including testosterone concentration and bioavailability during recovery from resistance exercise [13204].

Effect on cocaine's vascular effects

Neural plasticity has been observed in the bed nucleus of the stria terminalis (BNST) following exposure to both cocaine and androgenic-anabolic steroids. Here we investigated the involvement of the BNST on changes in cardiovascular function and baroreflex activity following either single or combined administration of cocaine and testosterone for 10 consecutive days in rats. Single administration of testosterone increased values of arterial pressure, evoked rest bradycardia and reduced baroreflex-mediated bradycardia. These effects of testosterone were not affected by BNST inactivation caused by local bilateral microinjections of the nonselective synaptic blocker CoCl2. The single administration of cocaine as well as the combined treatment with testosterone and cocaine increased both bradycardiac and tachycardiac responses of the baroreflex. Cocaine-evoked baroreflex changes were totally reversed after BNST inactivation. However, BNST inhibition in animals subjected to combined treatment with cocaine and testosterone reversed only the increase in reflex tachycardia, whereas facilitation of reflex bradycardia was not affected by local BNST treatment with CoCl2. In conclusion, the present study provides the first direct evidence that the BNST play a role in cardiovascular changes associated with drug abuse. Our findings suggest that alterations in cardiovascular function following subchronic exposure to cocaine are mediated by neural plasticity in the BNST. The single treatment with cocaine and the combined administration of testosterone and cocaine had similar effects on baroreflex activity, however the association with testosterone inhibited cocaine-induced changes in the BNST control of reflex bradycardia. Testosterone-induced cardiovascular changes seem to be independent of the BNST [13205].

Interaction with NSAIDs

Testosterone and epitestosterone are secreted mainly as glucuronide metabolites and the urinary ratio of testosterone glucuronide to epitestosterone glucuronide, often called T/E, serves as a marker for possible anabolic steroids abuse by athletes. UDP-glucuronosyltransferase (UGT) 2B17 is the most important catalyst of testosterone glucuronidation. The T/E might be affected by drugs that interact with UGT2B17, or other enzymes that contribute to testosterone glucuronidation. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used by sportsmen and we have examined the effect of two NSAIDs, diclofenac and ibuprofen, on testosterone and epitestosterone glucuronidation in human liver microsomes. In parallel, we have studied the inhibitory effect of these NSAIDs on recombinant UGT2B17 and UGT2B15, as well as other human hepatic UGTs that revealed low but detectable testosterone glucuronidation activity, namely UGT1A3, UGT1A4, UGT1A9 and UGT2B7. Both diclofenac and ibuprofen inhibited testosterone glucuronidation.
in microsomes, as well as UGT2B15 and UGT2B17. Interestingly, UGT2B15 was more sensitive than UGT2B17 to the two drugs, particularly to ibuprofen. Human liver microsomes lacking functional UGT2B17 exhibited significantly higher sensitivity to ibuprofen, suggesting that UGT2B15 plays a major role in the residual testosterone glucuronidation activity in UGT2B17-deficient individuals. Nonetheless, a minor contribution of other UGTs, particularly UGT1A9, to testosterone glucuronidation in such individuals cannot be ruled out at this stage. The epitestosterone glucuronidation activity of human liver microsomes was largely insensitive to ibuprofen and diclofenac. Taken together, the results highlight potential interactions between NSAIDs and androgen glucuronidation with possible implications for the validity of doping tests [09105].

**Influence on gastric ulcer precursors**

In one study, it was demonstrated that Helicobacter pylori absorbs a steroid prehormone (pregnenolone) and two androgens (dehydroepiandrosterone and epiaンドロステロン), glucosylates these steroids, and utilizes glucosyl-steroid hormone compounds as the membrane lipid components. The only common structure among the steroid prehormone and the two androgens is a 3beta-OH in the steroid framework. The results indicate that the 3beta-OH in the steroid hormones is a crucial conformation required for steroid glucosylation by H. pylori. In addition, it was found that H. pylori absorbs and holds estrogens possessing 3-OH (estrone and estradiol) into the membrane. The effective absorption of estrogen into the membrane appeared to be controlled by the number of hydroxyl groups modifying the steroid framework. In contrast, H. pylori induced neither membrane absorption nor glucosylation of the other steroid hormones possessing 3=O (progesterone, androstenedione and testosterone) or 3alpha-OH (androsterone). These results indicate that H. pylori selectively absorbs 3beta-OH and 3-OH steroid hormones, and utilizes only 3beta-OH steroid hormones as the materials for glucosylation [09106].

**Laboratory techniques**

The metabolic effect of multiple oral testosterone undecanoate (TU) doses over 4 weeks was assessed in seven voluntary men. The protocol was designed to detect accumulation of the substance by choosing the appropriate spot urines collections time and to study the urinary clearance of the substance after weeks of treatment. Urines were analysed by a new GC/C/isotope ratio mass spectrometry (IRMS) method to establish the delta 13C-values of testosterone metabolites (androsterone and etiocholanolone) together with an endogenous reference compound (16(5alpha)-androsten-3alpha-ol). The significant differences in inter-individual metabolism following TU intake was illustrated by large variations in delta 13C-values of both T metabolites (maximum Deltadelta 13C-values = 5.5 per thousand), as well as by very stable longitudinal T/E profiles and carbon isotopic ratios in the first hours following administration. According to T/E ratios and delta(13)C-values, the washout period after 80 mg TU intake was less than 48 h for all subjects and no accumulation phenomenon was observed upon chronic oral administration [06062].

Testosterone abuse is conventionally assessed by the urinary testosterone/epitestosterone (T/E) ratio, levels above 4.0 being considered suspicious. A deletion polymorphism in the gene coding for UGT2B17 is strongly associated with reduced testosterone glucuronide (TG) levels in urine. Many of the individuals devoid of the gene would not reach a T/E ratio of 4.0 after testosterone intake. Future test programs will most likely shift from population based- to individual-based T/E cut-off ratios using Bayesian inference. A longitudinal analysis is
dependent on an individual's true negative baseline T/E ratio. The aim was to investigate whether it is possible to increase the sensitivity and specificity of the T/E test by addition of UGT2B17 genotype information in a Bayesian framework. A single intramuscular dose of 500mg testosterone enanthate was given to 55 healthy male volunteers with either two, one or no allele (ins/ins, ins/del or del/del) of the UGT2B17 gene. Urinary excretion of TG and the T/E ratio was measured during 15 days. The Bayesian analysis was conducted to calculate the individual T/E cut-off ratio. When adding the genotype information, the program returned lower individual cut-off ratios in all del/del subjects increasing the sensitivity of the test considerably. It will be difficult, if not impossible, to discriminate between a true negative baseline T/E value and a false negative one without knowledge of the UGT2B17 genotype. UGT2B17 genotype information is crucial, both to decide which initial cut-off ratio to use for an individual, and for increasing the sensitivity of the Bayesian analysis [10079].

The determination of the carbon isotope ratio in androgen metabolites has been previously shown to be a reliable, direct method to detect testosterone misuse in the context of antidoping testing. Here, it was examine the variability in the $^{13}$C/$^{12}$C ratios in urinary steroids in a widely heterogeneous cohort of professional soccer players residing in different countries (Argentina, Italy, Japan, South-Africa, Switzerland and Uganda). Carbon isotope ratios of selected androgens in urine specimens were determined using gas chromatography/combustion/isotope ratio mass spectrometry (GC-C-IRMS). Urinary steroids in Italian and Swiss populations were found to be enriched in $^{13}$C relative to other groups, reflecting higher consumption of C3 plants in these two countries. Importantly, detection criteria based on the difference in the carbon isotope ratio of androsterone and pregnanediol for each population were found to be well below the established threshold value for positive cases. The results obtained with the tested diet groups highlight the importance of adapting the criteria if one wishes to increase the sensitivity of exogenous testosterone detection. In addition, confirmatory tests might be rendered more efficient by combining isotope ratio mass spectrometry with refined interpretation criteria for positivity and subject-based profiling of steroids [09107].

Carbon isotope ratio of androgens in urine specimens is routinely determined to exclude an abuse of testosterone or testosterone prohormones by athletes. Increasing application of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) in the last years for target and systematic investigations on samples has resulted in the demand for rapid sample throughput as well as high selectivity in the extraction process particularly in the case of conspicuous samples. For that purpose, it was presented herein the complimentary use of an SPE-based assay and an HPLC fractionation method as a two-stage strategy for the isolation of testosterone metabolites and endogenous reference compounds prior to GC/C/IRMS analyses. Assays validation demonstrated acceptable performance in terms of intermediate precision (range: 0.1-0.4 per thousand) and Bland-Altman analyses revealed no significant bias (0.2 per thousand) [09108].

It is necessary to develop a sensitive and simple method to monitor testosterone and its epimer epitestosterone. An off-line immunoaffinity extraction followed by capillary electrophoresis for simultaneous determination of testosterone and epitestosterone has been described in this paper. Anti-epitestosterone monoclonal antibody which is specific to both testosterone and epitestosterone had been prepared and immobilized on a Sepharose 4B stationary phase. The immunoaffinity column was used for sample cleanup, extraction and preconcentration. After elution and reconstitution, testosterone and epitestosterone in the sample were separated and quantified by micellar electrokinetic chromatography. The immunoaffinity column was evaluated in different parameters such as the retention mechanism, selectivity, binding capacity, elution protocol, and reusability. It was satisfactory to apply this method to analyze testosterone and epitestosterone in spiked urine sample with
Athletes have increasingly used testosterone and other endogenous anabolic steroids that cannot be detected by conventional gas chromatography-mass spectrometry. This led to gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS), which measures the relative amount of $^{13}$C in urinary steroids. Because exogenous testosterone is relatively low in $^{13}$C content, this study will determine if consuming a diet low in $^{13}$C plants, such as soy, can be confused with a GC/C/IRMS-positive test for exogenous testosterone. A cross-sectional study in which 22 vegetarians known to consume a diet depleted of $^{13}$C isotope were compared with a geographic control group of 14 subjects consuming a normal diet. Comparisons were made with respect to dietary analysis, isoflavones, and urinary steroid measurements using GC-C-IRMS. The delta$^{13}$C values for 2 major metabolites of testosterone (androsterone and etiocholanolone) were significantly lower for the vegetarians than the controls. The vegetarians excreted a median of 23 micromol/d of total isoflavones compared with 2.7 micromol/d for the control group, which was a significant difference. The carbon isotope ratios of urinary testosterone metabolites of vegetarians consuming a diet that is markedly depleted of $^{13}$C content were lower than that of control subjects, but not low enough to result in World Anti-Doping Agency criteria for a positive IRMS analysis [09110].

The metabolism of testosterone is revisited. Four previously unreported metabolites were detected in urine after hydrolysis with KOH using a liquid chromatography-tandem mass spectrometry method and precursor ion scan mode. The metabolites were characterized by a product ion scan obtained with accurate mass measurements. Androsta-4,6-dien-3,17-dione, androsta-1,4-dien-3,17-dione, 17-hydroxy-androsta-4,6-dien-3-one and 15-androsten-3,17-dione were proposed as feasible structures for these metabolites on the basis of the mass spectrometry data. The proposed structures were confirmed by analysis of synthetic reference compounds. Only 15-androsten-3,17-dione could not be confirmed, owing to the lack of a commercially available standard. That all four compounds are testosterone metabolites was confirmed by the qualitative analysis of several urine samples collected before and after administration of testosterone undecanoate. The metabolite androsta-1,4-dien-3,17-dione has a structure analogous to that of the exogenous anabolic steroid boldenone. Specific transitions for boldenone and its metabolite 17β-hydroxy-5β-androst-1-en-3-one were also monitored. Both compounds were also detected after KOH treatment, suggesting that this metabolic pathway is involved in the endogenous detection of boldenone previously reported by several authors [10457].

A sensitive and rapid liquid chromatographic (LC) method for the simultaneous determination of testosterone (T) and epitestosterone (E) in human urine samples has been developed and elaborated. The ratio of the both steroids (T/E) in human urine is a widely used as doping control indicator. A sample pretreatment by solid-phase extraction (SPE) after hydrolysis using 36% hydrochloric acid for determination of total level of T has been applied. Unconjugated (free) forms of the both androgens were determined without hydrolysis steps, what makes novelty of the method, because simplifies the proposed procedure. In turn, the measurements of urinary free T and E provided the diagnostic information for excess adrenal production of steroids. The proposed LC assay was evaluated by analyzing a series of urine samples containing T, E and methytestosterone (MT) as internal standard at the range of concentration 2-300 ng/mL of both analyzed hormones. The proposed method was fully validated for specificity, linearity, limits of detection and quantitation, precision and trueness according to the current requirements concerning analytical methods. Interestingly, the developed LC method allows to obtain a sensitive enhancement with respect to UV detection with the quantitation limit for T and E equaled 2 ng/mL. The method was selective and reliable for identity and enables to detect changes of endogenous levels of T and E in urine independently of fluctuations characteristic for both analyzed endogenous hormone level in
It was presented a powerful 3D sensing platforms using novel disc-ring microelectrode array devices and exploit them for the competitive immunosensing of testosterone. Each device contains a microelectrode array that consists of a large number of individual microdiscs and is used as the substrate for immune-functionalization and assay performance. One micrometer above it, a second microelectrode array, this time consisting of microrings, is used as the working electrode for electrochemical monitoring. The physical separation of these two functions allows the incorporation of relatively thick biocomponent layers during immunofunctionalization of the microdiscs without negatively affecting electrochemical detection at the rings. Moreover, it permits electrochemical activation of the latter immediately before substrate addition and hence enables optimal electrode performance. The optimized assay showed a linear range between 0.01 and 10 ng/mL and a limit of detection of 12.5 pg/mL testosterone with detection times of 45 min [11078].

The detection of an intact ester of testosterone in plasma is leading towards unequivocal proof of the administration of exogenous testosterone. In the current study, a sensitive screening method for the detection of nine testosterone esters in human plasma was developed. By preparing oxime derivatives of intact testosterone esters, the sensitivity of the assay was increased. Furthermore, the method included liquid-liquid extraction (LLE) as sample clean-up, as well as online separation of the target analytes from the derivatization solution. The analysis was performed by liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS). The method developed herein is simple and rapid, and was validated according to World Anti-Doping Agency (WADA) guidelines [13216].

**Testosterone versus epitestosterone**

Ordinarily, testosterone and epitestosterone are produced in equal amounts, and testosterone cannot be converted to epitestosterone. Accordingly, use of exogenous testosterone should increase the relative amount of testosterone versus epitestosterone (T/E ratio). The first tests at the 1983 Pan American Games used a 6:1 T/E ratio as the cut-off for a positive test. Fifteen athletes tested positive; additional athletes withdrew from competition and were not tested. Athletes soon recognized that taking epitestosterone along with testosterone could prevent a positive drug test. For this reason, epitestosterone is included on WADA’s list of prohibited substances. In 2005, WADA reduced the upper limit for an allowable T/E ratio to 4:1. Epitestosterone changes, or more importantly, changes in the T/E ratio as a function of winning or losing a competition are unstudied. The mechanisms of epitestosterone production, synthesis, and binding are still not well characterized. Knowing that the T/E ratio is a critical metric of doping and that competition alters testosterone concentrations, should anti-doping agencies be concerned that the T/E ratio may be subject to systematic fluctuations as a function of exertion, competition, victory and defeat? Knowing that testosterone concentrations can change up to 100 percent post-competition, false positives in doping assessment based on the T/E ratio are plausible. This is especially true for winners, who tend to experience testosterone increases from both winning and exertion and are most likely to be tested post-competition [12100].

A series of molecularly imprinted polymers have been prepared and investigated as stationary phases in high performance liquid chromatography for the separation of testosterone and epitestosterone using non-polar mobile phases. The polymers were imprinted using 5alpha-dihydrotestosterone as template, and all retain testosterone more strongly than its 17alpha-OH epimer. The best polymer was prepared using trifluoromethylacrylic acid as functional monomer (interacting with the template via hydrogen
bonds), divinylbenzene as “inert” cross-linker, and chloroform as porogen. It also included a steroid-based cross-linker, which may interact with the template via van der Waals interactions to lend additional “shape selectivity”. A 250×4.6 mm column packed with this polymer gave baseline resolution of testosterone and epitestosterone (15 microg each) in under 20 min. Preparation of the steroid based cross-linker included the selective reduction of 5α-dihydrötestosterone (17beta-hydroxy-5alpha-androstan-3-one) to the 3alpha,17beta-diol using K-selectride [11079].

A thin sheet of polydimethylsiloxane membrane was used as an extraction phase for solid-phase microextraction. Compared with fiber or rod solid-phase microextraction geometries, the thin film exhibited much higher extraction capacity without sacrificing extraction time due to its higher area-to-volume ratio. The analytical method involved direct extraction of unconjugated testosterone (T) and epitestosterone (ET) followed by separation on a C18 column and detection by selected reaction monitoring in positive ionization mode. The limit of detection was 1 ng/l for both T and ET. After method validation, free (unconjugated) T and ET were extracted and quantified in real samples. Since T and ET are extensively metabolized, the proposed method was also applied to extract the steroids after enzymatic deconjugation of urinary-excreted steroid glucuronides. The proposed method allows quantification of both conjugated and unconjugated steroids, and revealed that there was a change in the ratio of T to ET after enzymatic deconjugation, indicating different rates of metabolism [11080].

**TLC-densitometry method**

Anabolic androgenic steroids (AAS) are widely misused for the enhancement of performance in sports. Several drugs are available that contain different combinations or individual steroids in different dosage form. This paper describes a TLC densitometric method for simultaneous determination of four AAS of testosterone derivatives including testosterone propionate (TP), testosterone phenyl propionate (TPP), testosterone isocaproate (TI) and testosterone deaconate (TD) in their pharmaceutical products. Separation was carried out on Al based TLC plates, pre-coated with silica gel 60F-254 using hexane and ethyl acetate (8.5:1.5, v/v). Spots at Rf 0.31 ± 0.01, 0.34 ± 0.01, 0.40 ± 0.01 and 0.45 ± 0.02 were recognized as TPP, TP, TI and TD, respectively. Quantitative analysis was done by densitometric measurements at λmax 251 nm for all derivatives. The developed method was validated as per ICH guidelines. Method was found linear over the concentration range of 200-1200 ng/spot with the correlation coefficient of 0.995, 0.993, 0.995 and 0.996 for TP, TPP, TI, TD, respectively. Limit of detection for all derivatives were in the range of 16.7-22.3 ng/spot while limit of quantitation were found to be in the range of 55.7-70.9 ng/spot. It was concluded that the developed TLC method can be applied for the simultaneous routine analysis of testosterone derivatives in their individual and combined pharmaceutical formulations [12112].

**Mobility spectrometry separations**

UPLC-ion mobility spectrometry separations combined with mass spectrometry (UPLC-IM-MS) and tandem mass spectrometry (UPLC-IM-MS/MS) have been investigated for the simultaneous determination of testosterone and epitestosterone glucuronides in urine. The glucuronide epimers of testosterone and epitestosterone were separated by ion mobility spectrometry prior to mass analysis on the basis of differences in their collision cross sections, which have been measured in nitrogen. Combining ion mobility separation with UPLC/MS enhances the analysis of these low-abundance steroids in urine by selective interrogation of specific retention time, mass-to-charge and mobility regions. Detection limits
for the UPLC-IM-MS/MS analysis of TG and ETG were 9.9 ng/mL and 98 ng/mL respectively, equivalent to 0.7 ng/mL and 7.4 ng/mL in urine, with linear dynamic ranges corresponding to 0.7-108 ng/mL and 7.4-147 ng/mL in urine [11446].

Analysis of the transcriptome

The abuse of anabolic steroid hormones in human sports and animal husbandry is an ubiquitous problem and therefore a tight control program in both areas is very important. Within these control programs, hormone residues are detected by immunoassays or chromatographical methods in combination with mass spectrometry. With these methods, all known substances can be detected; yet new xenobiotic growth promoters and new ways of application are difficult to detect. Therefore it is important to develop new sensitive screening methods to enable an efficient control for misused anabolic substances. The detection of their physiological action is a promising approach. Anabolic steroid hormones directly influence the expression of specific genes and thus the analysis of the transcriptome of different target tissues and matrices is of great interest. One review described our recent efforts made concerning the analysis of gene expression changes in different tissues, different species and under different anabolic treatments [11447].

Experimental

Declines in skeletal muscle size and strength, often seen with chronic wasting diseases, prolonged or high-dose glucocorticoid therapy, and the natural aging process in mammals, are usually associated with reduced physical activity and testosterone levels. However, it is not clear whether the decline in testosterone and activity are causally related. Using a mouse model, it was found that removal of endogenous testosterone by orchidectomy results in an almost complete cessation in voluntary wheel running but only a small decline in muscle mass. Testosterone replacement restored running behavior and muscle mass to normal levels. Orchidectomy also suppressed the IGF-I/Akt pathway, activated the atrophy-inducing E3 ligases MuRF1 and MAFBx, and suppressed several energy metabolism pathways, and all of these effects were reversed by testosterone replacement. The study also delineated a distinct, previously unidentified set of genes that is inversely regulated by orchidectomy and testosterone treatment. These data demonstrate the necessity of testosterone for both speed and endurance of voluntary wheel running in mice and suggest a potential mechanism for declined activity in humans where androgens are deficient [11096].
One of the most frequently misused steroid precursors (prohormones) is 19-norandrostenedione (4-estrene-3,17-dione), which is, after oral administration, readily metabolised to nor testosterone, also known as nandrolone (Durabolin®). In one study it was characterised molecular mechanisms of its action determined its tissue specific androgenic and anabolic potency after subcutaneous administration and investigated potential adverse effects. Receptor binding tests demonstrate that norandrostenedione binds with high selectivity to the androgen receptor (AR). The potency of norandrostenedione to transactivate androgen receptor dependent reporter gene expression was 10 times lower as compared to dihydrotestosterone (DHT). In vivo experiments in orchiectomised rats demonstrated that subcutaneous treatment with norandrostenedione resulted only in a stimulation of the weight of the levator ani muscle; the prostate and seminal vesicle weights remained completely unaffected. Like testosterone, administration of norandrostenedione resulted in a stimulation of androgen receptor and myostatin mRNA expression in the gastrocnemius muscle. Norandrostenedione did not affect prostate proliferation, the liver weight and the expression of the tyrosine aminotransferase gene (TAT) in the liver. Summarizing these data it is obvious that norandrostenedione, if administrated subcutaneous and in contrast to its metabolite nandrolone, highly selectively stimulates the growth of the skeletal muscle but has only weak androgenic properties [08157].

19-Norandrosterone (19-NA) as its glucuronide derivative is the target metabolite in antidoping testing to reveal an abuse of nandrolone or nandrolone prohormone. To provide further evidence of a doping with these steroids, the sulfoconjugate form of 19-norandrosterone in human urine might be monitored as well. In one study, the profiling of sulfate and glucuronide derivatives of 19-norandrosterone together with 19-noretiocholanolone (19-NE) were assessed in the spot urines of 8 male subjects, collected after administration of 19-nor-4-androstenedione (100 mg). An LC/MS/MS assay was employed for the direct quantification of sulfoconjugates, whereas a standard GC/MS method was applied for the assessment of glucuroconjugates in urine specimens. Although the 19-NA glucuronide derivative was always the most prominent at the excretion peak, inter-individual variability of the excretion patterns was observed for both conjugate forms of 19-NA and 19-NE. The ratio between the glucuro- and sulfoconjugate derivatives of 19-NA and 19-NE could not discriminate the endogenous versus the exogenous origin of the parent compound. However, after ingestion of 100 mg 19-nor-4-androstenedione, it was observed in the urine specimens that the sulfate conjugates of 19-NA was detectable over a longer period of time with respect to the other metabolites [08158].

The detection of 19-norandrosterone (19-NA) in a competitor's urine sample is taken as prima facie evidence of administration of nandrolone or other 19-norsteroid but a potential problem is that administration of norethisterone, a progestogen used for menstrual disorders and for hormonal contraception, also results in the excretion of 19-NA that can exceed the laboratory reporting threshold of 2 ng/mL. The contribution of norethisterone to urinary 19-NA with and without 19 norandrostenedione, a known norethisterone tablet impurity, requires evaluation. Preparations containing, either <2 ng or 1 mug 19-norandrostenedione impurity per 5 mg of norethisterone, administered to female volunteers (n=10) in doses comparable to those used for menstrual disorders (5 mg three times daily for 10 days), resulted in maximal 19-NA concentrations of 51 and 63 ng/mL, respectively. The maximal concentration of 19-NA, 2 h post-administration of a single 1 mug dose of 19-norandrostenedione, was 2.4 ng/mL. These results prove unequivocally that norethisterone is metabolized to 19-NA and that there is only a minor contribution from the impurity 19-norandrostenedione. Administration to women (n=30) of a single contraceptive tablet containing norethisterone (1
mg) with one of the highest proportions of the impurity 19-norandrostenedione (approximately 0.5 mug, 0.05 %, w/w) resulted in a urinary 19-NA concentration of 9.1 ng/mL, with a maximum concentration ratio of 19-NA to the norethisterone metabolite 3alpha,5beta-tetrahydroxynorethisterone of 0.36. These data should remove the need for time-consuming follow-up investigations to consider whether doping with 19-norandrogens has occurred [08159].

19-Norandrosterone (19-NA) is the principal urinary metabolite of the anabolic steroid nandrolone and its prohormones. The administration of these 19-nor androgens is prohibited in sport by the WADA but, even so, adverse findings for 19-NA continue to be commonly reported. Little is known about the urinary concentrations of 19-NA that can occur in women who are not using anabolic steroids, including those using oral contraceptives containing the 19-nor progestogen norethisterone. In 2004, WADA lowered the reporting threshold for 19-NA for females from 5 to 2 ng/mL. The lack of any substantial data on 19-NA excretion in women prompted this large-scale investigation. In this investigation, single untimed urines collected from 1202 female volunteers, 38 of whom were taking norethisterone containing contraceptives, were analysed for 19-NA. None of the women was a competitive athlete and pregnancy had been excluded by a urinary test for human chorionic gonadotropin (hCG). Only one sample exceeded the 19-NA reporting threshold having a concentration of 4.1 ng/mL. This sample was from a user of a norethisterone-containing contraceptive [08160].

The finding of measurable amounts of 19-norandrostenedione in norethisterone tablets prompted us to develop an assay to quantify this steroid. 19-Norandrostenedione is an anabolic steroid whose use in sport is prohibited by WADA. The assay was developed using isotope dilution and liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the quantification of 19-norandrostenedione in norethisterone formulations, with [3,4-(13)C(2)]-19-norandrostenedione as the internal standard. The results showed amounts up to 1.01 ± 0.01 mug per tablet in those containing 5 mg of norethisterone or norethisterone acetate (0.02 %, w/w) and up to 0.5 ± 0.01 mug per tablet (0.05 %, w/w) in oral contraceptive tablets containing 0.35-1.5 mg of norethisterone or norethisterone acetate. No tablet tested exceeded the British Pharmacopoeia limit of 0.1 percent for this impurity [08161].

The administration of 19-norsteroids such as 19-nortestosterone (nandrolone), 19-norandrostene-3,17-dione and 19-norandrostene-3,17-diol (delta-4 and -5 isomers) has been shown to lead mainly to the excretion of 19-norandrosterone (NA), 19-noretioclanolone (NE), and 19-norepiandrosterone (NEA). The latter is found exclusively as its sulfocojugate while the others are usually excreted as their glucuronide derivative. The sulfate derivatives, generally persistent, may be prevalent at the end of the excretion period. After intramuscular administration of the long-lasting preparations of nandrolone, the metabolites may be detected for months, but metabolites formed after oral ingestion are excreted massively the first hours and remain detectable for only a few days [09111].

The abuse of anabolic androgenic steroids (AASs) is not only a problem in the world of sports but is associated with the polydrug use of nonathletes. Investigations of the neurochemical effects of AAS have focused in part on the monoaminergic systems, involving, among other things, the development of dependence. It has previously been shown that pretreatment with nandrolone decanoate attenuates dose-dependently the increase in extracellular dopamine (DA) concentration evoked by amphetamine and 3,4-methylenedioxyxymethamphetamine in the nucleus accumbens (NAC). The aim of this study was to investigate whether the nandrolone pre-exposure modulates the acute neurochemical and behavioral effects of cocaine in rats and whether the effects are long lasting. DA, 5-hydroxytryptamine (5-HT), and their metabolites were measured from samples collected from the NAc by microdialysis. The behavior of the animals was recorded. The present study
demonstrates that five injections of nandrolone (5 and 20 mg/kg) inhibited cocaine-evoked DA and 5-HT outflow in the NAc, locomotor activity (LMA), and stereotyped behavior in experimental animals, and that these effects are seen even after elimination of nandrolone from bloodstream. Given that accumbal outflow of DA and 5-HT, as well as LMA and stereotyped behavior, is related to gratification of stimulant drugs, this study suggests that nandrolone, at the doses tested, has a significant effect on the pleasurable properties of cocaine. Furthermore, because neurochemical and behavioral responses were still attenuated after a fairly long recovery period, it seems that nandrolone may induce long-lasting changes in the brains of rat [10083].

Nandrolone or nortestosterone, an anabolic-androgenic steroid, has been prohibited by doping control regulations for more than 30 years. Although its main metabolism in the human body was already known at that time, and detection of its misuse by gas or liquid chromatographic separation with mass spectrometric detection is straightforward, many interesting aspects regarding this doping agent have appeared since. Over the years, nandrolone preparations have kept their position among the prohibited substances that are most frequently detected in WADA-accredited laboratories. Their forms of application range from injectable fatty acid esters to orally administered nandrolone prohormones. The long detection window for nandrolone ester preparations and the appearance of orally available nandrolone precursors have changed the pattern of misuse. At the same time, more refined analytical methods with lowered detection limits led to new insights into the pharmacology of nandrolone and revelation of its natural production in the body. Possible contamination of nutritional supplements with nandrolone precursors, interference of nandrolone metabolism by other drugs and rarely occurring critical changes during storage of urine samples have to be taken into consideration when interpreting an analytical finding. A set of strict identification criteria, including a threshold limit, is applied to judge correctly an analytical finding of nandrolone metabolites. The possible influence of interfering drugs, urine storage or natural production is taken into account by applying appropriate rules and regulations [10084].

It was investigated the effects of the menstrual cycle, oral contraception and physical training on exhaustive exercise-induced changes in the excretion of nandrolone metabolites. i.e.19-norandrosterone (19-NA), and 19-noretiocholanolone (19-NE), in young women. Twenty-eight women were allocated to an untrained group (n=16) or a trained group (n=12), depending on their physical training background. The untrained group was composed of nine oral contraceptive users (OC+) and seven eumenorrheic women (OC-), while the trained group was entirely composed of OC+ subjects. Three laboratory sessions were conducted in a randomized order: a prolonged exercise test, a short-term exercise test and a control session. Urine specimens were collected before and 30, 60 and 90 min after the exercise test and at the same times of the day during the control session. Urinary concentrations of nandrolone metabolites were determined by gas chromatography coupled to mass spectrometry. Urinary concentrations of 19-NA and 19-NE ranged from undetectable levels to 1.14 and 0.47 ng/mL, respectively. Nandrolone excretion was not affected by the menstrual cycle phase (early follicular vs mid-luteal), prior physical training, oral contraception or acute physical exercise. Therefore, a urinary concentration of 2 ng/mL of 19-NA appears to be fair as the upper acceptable limit in doping control tests for female athletes [10085].

One study describes development and subsequent validation of a reversed phase high performance liquid chromatographic (RP-HPLC) method for the estimation of nandrolone phenylpropionate, an anabolic steroid, in bulk drug, in conventional parenteral dosage formulation and in prepared nanoparticle dosage form. The chromatographic system consisted of a Luna Phenomenex, CN (250 mm x 4.6 mm, 5 microm) column, an isocratic mobile phase comprising 10 mM phosphate buffer and acetonitrile (50:50, v/v) and UV
detection at 240 nm. Nandrolone phenylpropionate was eluted about 6.3 min with no interfering peaks of excipients used for the preparation of dosage forms. The method was linear over the range from 0.050 to 25 microg/mL in raw drug. The intra-day and inter-day precision values were in the range of 0.22-0.61 percent and 0.44-0.88 percent, respectively. Limits of detection and quantitation were 0.010 microg/mL and 0.050 microg/mL, respectively. The results were validated according to International Conference on Harmonization guidelines in parenteral and prepared nanoparticle formulation. The validated HPLC method is simple, sensitive, precise, accurate and reproducible [11097].

Nandrolone is an androgenic-anabolic steroid (AAS) with diverse medical applications but taken indiscriminately by some to rapidly increase muscle mass. The aim of this study was to evaluate the genotoxic and clastogenic potential of nandrolone (Deca-durabolin®) in vivo in different cells of mice, using the comet assay and micronucleus test, respectively. The animals received subcutaneous injection of the three doses of the steroid (1.0, 2.5 and 5.0 mg kg(-1) body weight). Cytotoxicity was assessed by scoring 200 consecutive total polychromatic (PCE) and normochromatic (NCE) erythrocytes (PCE-NCE ratio). The results showed a significant dose-related increase in the frequency of DNA damage in leukocytes, liver, bone marrow, brain and testicle cells at the three tested doses and a significant increase of the micronucleated polychromatic erythrocytes at all tested doses. Under our experimental conditions, the nandrolone steroid hormone showed genotoxic and clastogenic effects when administered subcutaneously to mice [11098].

Nandrolone or 19-nortestosterone (19-NT) is an anabolic androgenic steroid (AAS) produced first for androgen replacement therapy as a testosterone analog. Due to its higher potency than testosterone itself it has been misused for doping purposes and forbidden in sports in 1976. Since then, and despite the appearance of other doping products, 19-NT preparations have remained one of the most abused substances in sports as well as in the society. Nandrolone is mainly metabolized in the liver into 19-norandrosterone prior to glucuronidation and excretion through urine over an extended period of time. 19-NT is mainly metabolized into 19-norandrosterone (19-NA) prior to glucuronidation and excretion via urine. Whereas 19-NT is known to be detectable in the serum for only 2-5 weeks after injection, urinary 19-NA in its glucuronidated form is detectable up to 1 year after injection of 19-NT and this excretion is prone to very large inter-individual variation. Several UGTs (i.e. UGT2B7, UGT2B15, and UGT2B17) are thought to be the major enzymes responsible for conjugation of androgens in human. An in vitro study using recombinant enzymes expressed in insect cells showed that UGT1A4 and UGT2B7 are the two main enzymes responsible of 19-norandrosterone glucuronidation. However, the identity of the enzyme involved in nandrolone metabolism in vivo together with their relative contribution and regulation remain unknown. Inhibition assays using human liver microsomes (HLM) incubated with 19-norandrosterone and selective inhibitors confirmed that UGT2B7 and UGT2B15 are involved in 19-norandrosterone glucuronidation, since the presence of the specific UGT2B7 and UGT2B15 inhibitors gemfibrozil and valproic acid inhibited the 19-norandrosterone glucuronidation by 35 and 45 percent, respectively. HLM were genotyped for UGT2B15 D85Y, UGT2B7 H268Y, and the UGT2B17 deletion polymorphism. The glucuronidation activity on 19-norandrosterone was significantly higher in UGT2B15 DD than in the other UGT2B15 genotypes. Moreover, human liver cancer HepG2 cells were exposed to androgens to determine if the transcriptional activity of the genes of interest was affected. Only UGT2B7 mRNA expression was significantly increased (1.8-folds) after incubation with nandrolone decanoate. These results show that the UGT2B7 and UGT2B15 are involved in 19-norandrosterone glucuronidation and that the UGT2B15 polymorphism (D85Y) is the only UGT genetic variation that influences the glucuronidation activity. This could partly explain the inter-individual variation in 19-norandrosterone excretion. Several genetic polymorphisms in UGTs have been reported to have an impact on their ability to metabolize androgens. A
frequent polymorphism in UGT2B7 gene leading to a histidine to tyrosine substitution in codon 268 (H268Y) has shown variable functional impact with different substrates. The results indicate that androgens induce enzymes involved in its own elimination, which may further contribute to the inter-individual variability in disposition of 19-NA after the administration of nandrolone in healthy volunteers [13220].

**Physiology**

*Effects on mitochondria*

Respiratory failure in patients with COPD may be caused by insufficient force production or insufficient endurance capacity of the respiratory muscles. Anabolic steroids may improve respiratory muscle function in COPD. The effect of anabolic steroids on mitochondrial function in the diaphragm in emphysema is unknown. In an emphysematous male hamster model, we investigated whether administration of the anabolic steroid nandrolone decanoate (ND) altered the activity of mitochondrial respiratory chain complexes in the diaphragm. The bodyweight of hamsters treated with ND was decreased after treatment compared with initial values, and serum testosterone levels were significantly lower in hamsters treated with ND than in control hamsters. No difference in the activity of mitochondrial respiratory chain complexes in the diaphragm between normal and emphysematous hamsters was observed. Treatment with ND did not change the activity of mitochondrial respiratory chain complexes in the diaphragm of both normal and emphysematous hamsters. In emphysematous hamsters, administration of ND decreased the activity of succinate:cytochrome C oxidoreductase compared with ND treatment in normal hamsters. It was concluded that anabolic steroids have negative effects on the activity of succinate:cytochrome C oxidoreductase and anabolic status in this emphysematous hamster model [06103].

*Effect on dynorphin A in the brain*

The misuse of anabolic androgenic steroids (AAS) seems to produce profound effects on the central nervous system, leading to aggressive behavior and increased sensitivity to other drugs of abuse. The present study addresses the effect on the enzymatic transformation, here called dynorphin converting enzyme-like activity. The formation of the mu/delta opioid peptide receptor-prefering Leu-enkephalin-Arg(6) from the kappa opioid peptide receptor-prefering dynorphin A was measured in rats treated with nandrolone decanoate. Significant variations in enzymatic transformation were observed in several brain regions. An altered receptor activation profile in these regions may be one contributory factor behind AAS-induced personality changes [06104].

**Metabolism**

19-Nortestosterone-derived anabolic steroids show promising anabolic:androgenic dissociation. Almost simultaneously with the development of prohormones of testosterone, 19-nor analogues were developed as precursors of 19-nortestosterone and these substances are also prohibited in sports. In doping control, 19-norandrosterone, the major urinary metabolite of 19-nortestosterone is used as the primary indicator for the misuse of these substances, although it can also naturally occur in urine in small concentrations or as an artefact by demethylation of androsterone. Moreover, several oral contraceptives including norethisterone and lynestrenol are metabolised to norandrosterone and could give rise to inadvertent positive doping cases. To avoid false positive doping tests, due to a possibly endogenous origin of norandrosterone or the use of oral contraceptives, several
precautions have been taken by WADA, including the inclusion of a threshold value (2 ng/mL) and the obligation for laboratories to test for pregnancy and for the presence of tetrahydronoretisterone when norandrosterone is found in urine of women [06004].

**Norandrostenedione and norandrostenediol**

In the 1990s, 19-norandrost-4-ene-3,17-dione, 19-norandrost-5-ene-3,17-dione, 19 norandrost-4-ene-3beta,17beta-diol and 19-norandrost-5-ene-3beta,17beta-diol became available as over-the-counter anabolic steroids in the US under the DSHEA. Several studies on the in vivo and in vitro metabolism of these steroids have been performed. So far, all excretion studies with norandrostenedione and norandrostenediol have also identified 19-norandrosterone as the major urinary metabolite [06004].

Prohormones such as 19-norandrostenediol (estr-4-ene-3beta,17beta-diol) have been added to the list of prohibited substances of the World Anti-Doping Agency because they are metabolized to the common nandrolone metabolites norandrosterone and noretiocholanolone. So far, no studies on the metabolism and in vivo conversion of 19-norandrostenediol after oral or sublingual administration have been reported nor have had quantified data on resulting plasma nandrolone levels. In the present study, an open-label crossover trial with eight healthy male volunteers was conducted. After application of capsules or sublingual tablets of 19-norandrostenediol plasma concentrations of 19-norandrostenediol, nandrolone as well as major metabolites (19-norandrosterone and 19-noretiocholanolone) were determined using a validated assay based on gas chromatography/mass spectrometry. The administration of 100-mg capsules of 19-norandrostenediol yielded maximum plasma total concentrations (i.e., conjugated plus unconjugated compounds) of 1.1 ± 0.7 ng/mL for 19-norandrostenediol, 4.0 ± 2.6 ng/mL for nandrolone, 154.8 ± 130.8 ng/mL for 19-norandrosterone, and 37.7 ± 6.9 ng/mL for 19-noretiocholanolone. The use of 25-mg sublingual tablets resulted in 3.3 ± 1.0 ng/mL for 19-norandrostenediol, 11.0 ± 6.4 ng/mL for nandrolone, 106.3 ± 40.1 ng/mL for 19-norandrosterone, and 28.5 ± 20.8 ng/mL for 19-noretiocholanolone. Most interestingly, the pharmacologically active unconjugated nandrolone was determined after administration of sublingual tablets (up to 5.7 ng/mL) in contrast to capsule applications. These results demonstrate the importance of prohibiting prohormones such as 19-norandrostenediol, in particular, since plasma concentrations of nandrolone between 0.3 to 1.2 ng/mL have been reported to influence endocrinological parameters [06105].

**Norethandrolone and ethylestrenol**

The metabolism of norethandrolone (17alpha-ethyl-17alpha-hydroxy-estr-4-en-3-one) has been investigated in man. 17alpha-ethyl-5alpha-estran-3alpha,17beta-diol, 17alpha-ethyl-5beta-estran-3alpha,17beta-diol and 17alpha-ethyl-5beta-estran-3alpha,17beta,21-triol were identified as the major urinary metabolites. In man, ethylestrenol is metabolized to norethandrolone. However, contrarily to earlier findings, de-ethynylation of both norethandrolone and ethylestrenol can occur and small amounts of 19-norandrosterone (higher than the threshold concentration of 2.0 ng/ml) can be detected in urine after administration of these steroids [06004].

**Norethisterone and lynestrenol**

Norethisterone acetate and lynestrenol are progestins used as oral contraceptives in women and are prodrugs of norethisterone. Norethisterone is metabolized in vivo to 5alpha- and 5beta-dihydronorethisterone and tetrahydronorethisterone. In urine, tetrahydronorethisterone is used as a marker for the use of norethisterone acetate. In addition, norandrosterone and other metabolites can also be detected in concentrations exceeding the doping threshold concentration. Administration of lynestrenol in therapeutic doses can also result in the detection of norandrosterone in concentrations higher than 2 ng/ml. However the precautions
taken by WADA [59] for norethisterone allow for avoiding false positive doping tests due to the use of this type of oral contraceptives [06004].

**Methyl-19-nortestosterone**

Several substances including 18-methyl-19-nortestosterone, 7alpha-methyl-19-nortestosterone and 17alpha-methyl-19-nortestosterone have been described in the literature and/or are available via the internet. Also 7alpha,11beta-dimethyl-19-nortestosterone has also been reported as a highly potent anabolic androgenic steroid. 7alpha-Methyl-19-nortestosterone, also referred to as MENT, is a potent androgen with a low oral activity, and promoted as a long-acting contraceptive in men in clinical trials. Three metabolites of 7alpha-methyl-19-nortestosterone have been reported, including 7alpha-methyl-5(10)-estrene-3alpha-ol-17-one and — analogous to the 19-nortestosterone metabolism — 7alpha-methyl-5beta-estran-3alpha-ol-17-one and 7alpha-methyl-5beta-estran-3alpha-ol-17-one. 18-Methyl-19-nortestosterone was listed on the 2005 WADA list of prohibited substances (13-ethyl-17alpha-hydroxy-4-ene-3-one), but not on the 2006 list. In humans 18-methyl-19-nortestosterone is metabolized to 18-methyl-norandrostosterone (18-methyl-5beta-estran-3alpha-ol-17-one) and 18-methylnoretiocholanolone (18-methyl-5beta-estran-3alpha-ol-17-one). No data on the metabolism of 17alpha-methyl-19-nortestosterone and 7alpha,11alpha-dimethyl-19-nortestosterone is available [06004].

**Tibolone**

Tibolone ((7alpha,17alpha)-17-hydroxy-7-methyl-19-norpregn-5(10)-en-20-yn-3-one) is a steroid structurally related to norsteroids, commonly used in hormone replacement therapy during menopause and prohibited in sports. In vitro and in vivo studies have shown that the major metabolite of tibolone is 3alpha-hydroxy-tibolone. Other metabolites include 3beta-hydroxy-tibolone and delta4-tibolone. All metabolites, as well as the parent compound, are excreted as sulphates [06004].

**Protein disulphide isomerase (PDI)**

Protein disulphide isomerase (PDI) in the endoplasmic reticulum catalyzes the rearrangement of disulphide bridges during folding of secreted proteins. It binds various molecules that inhibit its activity. But here, it was looked for molecules that would potentiate its activity. PDI reductase activity was measured in vitro using di-eosin-oxidized glutathione as substrate. Its classical inhibitor bacitracin was found to exert a biphasic effect: stimulatory at low concentrations (10^{-6} M) and inhibitory only at higher concentrations (10^{-3}-10^{-3} M). The weak estrogenic molecule bisphenol A was found to exert a weak inhibitory effect on PDI reductase activity relative to the strong oestrogens, ethynylestradiol, and diethylstilbestrol. Like 19-nortestosterone, fluoxetine was found to exert a potentiating effect on PDI reductase activity and their potentiating effects could be reversed by increasing concentrations of oestrogens. In conclusion, this paper provides the first identification of potentiators of PDI activity that are potential pharmaceuticals against pathologies affecting protein folding such as Alzheimer's disease [11449].

**Diagnostic metabolites**

Quantities of various anabolic steroids have been found in dietary supplements without their presence being disclosed on the label. The aim of this study was to quantify the excretion patterns of the diagnostic metabolites, 19-norandrostosterone (19-NA), and 19-noretioccholanolone (19-NE) after ingestion of small doses of 19-nor-4-androstene-3,17-dione
(19-norandrostenedione). Eleven males and nine females entered the laboratory in the morning after an overnight fast. An initial urine sample was collected, and volunteers then ingested 500 mL of water containing 5 g of creatine monohydrate and 1.0, 2.5, or 5.0 microg of 19-norandrostenedione. The volume of each urine void was measured, and an aliquot was taken. Baseline urinary 19-NA concentrations were 0.19 ± 0.14 ng/mL. Ingestion of the supplement resulted in peak mean urinary 19-NA concentrations of 0.68 ± 0.36, 1.56 ± 0.86, and 3.89 ± 3.11 ng/mL in the 1.0-, 2.5-, or 5.0-microg trials, respectively. Under current WADA regulations, ingestion of the 1.0-microg dose produced 0 positive doping tests, 5 subjects (20 %) tested positive in the 2.5-microg trial, and 15 subjects (75 %) had urinary 19-NA concentrations exceeding 2 ng/mL after ingesting creatine containing 5.0 microg of the steroid. The recovery of the ingested dose was highly variable between individuals, with values ranging from 11 to 84 percent. Ingestion of trace amounts of 19-norandrostenedione can result in transient elevations of urinary 19-NA and 19-NE concentrations. The addition of as little as 2.5 microg of 19-norandrostenedione to a supplement (0.00005 % contamination) appears sufficient to result in a doping violation in some individuals [09112].

One study examined the influence of a supplement matrix on the excretion pattern of nandrolone metabolites in response to ingestion of a trace amount of 19-norandrostenedione. Ten male and nine female volunteers were recruited. On two occasions subjects entered the laboratory in the morning following an overnight fast. After an initial urine collection, subjects ingested either 500 mL of plain water or a commercially-available energy bar: 10 mug of 19-orandrostenedione was added to each. The volume of each urine sample passed over the next 24 h was measured and an aliquot retained for analysis. All samples were analysed for the metabolites 19-norandrosterone (19-NA) and 19-noretiocohanolone (19-NE) by GCMS. The total volume of urine passed was significantly higher in the water trial (than in the bar trial. Baseline urinary 19-NA concentrations were all below the limit of quantification for the assay. Peak urinary 19-NA was lower in the water trial than in the bar trial. The time elapsed between ingestion of the supplement and the peak urinary 19-NA concentration was longer following ingestion of the bar than on the water trial. There was no difference in the total recovery of 19-NA+19-NE between the liquid and solid supplements. It was concluded that peak 19-NA concentrations were higher, and occurred later, when the 19-norandrostenedione was added to a solid supplement. This may be due to a slower rate of absorption, and/or a reduced diuresis, resulting in a longer period for the metabolites to accumulate in the urine [09113].

Effects of the menstrual cycle

It was investigated the effects of the menstrual cycle, oral contraception and physical training on exhaustive exercise-induced changes in the excretion of nandroloane metabolites (19-norandrosterone, and 19-noretiocholanolone) in young women. Twenty-eight women were allocated to an untrained group (n=16) or a trained group (n=12), depending on their physical training background. The untrained group was composed of nine oral contraceptive users (OC+) and seven eumenorrheic women (OC-), while the trained group was entirely composed of OC+ subjects. Three laboratory sessions were conducted in a randomized order: a prolonged exercise test, a short-term exercise test and a control session. Urine specimens were collected before and 30, 60 and 90 min after the exercise test and at the same times of the day during the control session. Urinary concentrations of nandrolone metabolites were determined by gas chromatography coupled to mass spectrometry. Urinary concentrations of 19-NA and 19-NE ranged from undetectable levels to 1.14 and 0.47 ng/mL, respectively. Nandrolone excretion was not affected by the menstrual cycle phase (early follicular vs mid-luteal), prior physical training, oral contraception or acute physical exercise.
Therefore, a urinary concentration of 2 ng/mL of 19-norandrosterone appears to be fair as the upper acceptable limit in doping control tests for female athletes [09114].

**Influence on hypothalamic-pituitary-adrenal axis**

Elite athletes, body builders and adolescents misuse anabolic-androgenic steroids (AAS) in order to increase muscle mass or to enhance physical endurance and braveness. The high doses misused are associated with numerous adverse effects. The purpose of one study was to evaluate the impact of chronic supratherapeutic AAS treatment on circulating hormones and gene expression in peripheral tissues related to such adverse effects. Quantitative real-time PCR was used to measure expression levels of in total 37 genes (including peptide hormones, cell membrane receptors, nuclear receptors, steroid synthesising enzymes and other enzymes) in the pituitary, testes, adrenals, adipose tissue, kidneys and liver of male Sprague-Dawley rats after 14-day administration of the AAS nandrolone decanoate, 3 or 15 mg/kg. Plasma glucose and levels of adrenocorticotropic hormone (ACTH), adiponectin, corticosterone, ghrelin, insulin and leptin were also measured. It was found several expected effects on the hypothalamic-pituitary-gonadal axis, while the treatment also caused a number of other not previously identified changes in circulating factors and gene transcription levels such as the dose-dependent reduction of the beta3-adrenergic receptor in adipose tissue, reduction of both circulating and mRNA levels of adiponectin, up-regulation of both hydroxyethylglutaryl-CoA-reductase, the rate-limiting enzyme in de novo synthesis of cholesterol, and the receptor for ACTH in the adrenals. The results provide evidence for wide ranging effects of AAS on the, adipose tissue and substrates of the renal control of blood pressure [09115].

**Effect on muscles**

Vascular endothelial growth factor (VEGF) is a key compound for induction of angiogenesis in both physiological and pathological conditions. The aim of one study was to investigate the effect of androgenic-anabolic steroids (AAS) administration on VEGF mRNA expression in the rat soleus muscle after jumping training. Wistar rats were grouped into: sedentary; nandrolone decanoate-treated sedentary; trained without AAS, and trained and treated with AAS. Exercised groups performed a 7-weeks water-jumping program. Animals killed immediately after the last exercise bout showed significantly increased VEGF mRNA expression; however, the AAS treatment completely inhibited this effect. These results suggest that the AAS may be strongly prejudicial to muscle remodeling and performance at least partially due to an impaired angiogenesis [09116].

**Effect of training**

Exercise is a potent stimulus for release of growth hormone (GH), cortisol, testosterone and prolactin, and prolonged exercise inhibits insulin secretion. These responses seem to be specific to the type of exercise but this has been poorly characterised primarily because they have not been compared during exercise performed by the same individuals. It was investigated hormone responses to resistance, sprint and endurance exercise in young men using a repeated measures design in which each subject served as their own control. Eight healthy non-obese young adults (18-25 years) were studied on four occasions in random order: 30-s cycle ergometer sprint (Sprint), 30-min resistance exercise bout (Resistance), 30-min cycle at 70 % VO_{2max} (Endurance), and seated rest in the laboratory (Rest). Cortisol, GH, testosterone, prolactin, insulin and glucose concentrations were measured for 60 min after
the four different interventions. Endurance and sprint exercise significantly increased GH, cortisol, prolactin and testosterone. Sprint exercise also increased insulin concentrations, whereas this decreased in response to endurance exercise. Resistance exercise significantly increased only testosterone and glucose. Sprint exercise elicited the largest response per unit of work, but the smallest response relative to mean work rate in all hormones. In conclusion, the nature and magnitude of the hormone response were influenced by exercise type, perhaps reflecting the roles of these hormones in regulating metabolism during and after resistance, sprint and endurance exercise [13221].

**Effect of winning or losing**

One study assessed the responses of salivary-free testosterone (T) and cortisol (C) concentrations across selected training workouts and their association with the subsequent competition outcomes in professional rugby league. Thirteen rugby league players were assessed for salivary-free T and C concentrations across 5 training workouts performed 3-4 days before a competitive game. The game outcomes included wins and losses and game-ranked performance (1-5) based on the number of points scored, the points differential, and a coach rating. Data were pooled across the winning (n=3) and losing (n=2) outcomes. Pooled free T concentrations (absolute and relative changes) were significantly elevated across those workouts that preceded winning games, but not the losses, and the relative (percent) T changes were significantly higher before winning (31 %) than before losing (3 %). Both outcomes were associated with workout decreases in pooled free C concentrations and the relative C changes were not significantly different between wins (-23 %) and losses (-26 %). In conclusion, the free T responses to selected training workouts showed some association with subsequent winning (being elevated) and losing (no change) during a limited number of competitive games in professional rugby league. Speculatively, the free T responses to a midweek workout might provide an early sign of team readiness to compete or to recovery state, thereby providing a novel format for implementing training or management strategies to improve the competition outcomes [13222].

**Effect on healing of muscle injuries**

Although testosterone administration elicits well-documented anabolic effects on skeletal muscle mass, the enhancement of muscle regeneration after injury has not been widely examined. The purpose of one study was to determine whether anabolic steroid administration improves skeletal muscle regeneration from bupivacaine-induced injury. Male C57BL/6 mice were castrated 2 weeks before muscle injury induced by an intramuscular bupivacaine injection into the tibialis anterior (TA) muscle. Control mice received an intramuscular PBS injection. Anabolic steroid (nandrolone decanoate, ND), 6 mg/kg, or sesame seed oil was administered at the time of initial injury and continued every 7 days for the study's duration. Mice were randomly assigned to one of four treatment groups for 5, 14, or 42 days of recovery, as follows: 1) control (uninjured); 2) ND only (uninjured + ND); 3) bupivacaine only (injured); or 4) bupivacaine + ND (injured + ND). TA morphology, protein, and gene expression were analyzed at 14 and 42 days after injury; protein expression was analyzed at 5 days after injury. After 14 days of recovery, the injury and injury + ND treatments induced small-diameter myofiber incidence and also decreased mean myofiber area. The increase in small-myofiber incidence was 65% greater in injury + ND muscle compared with injury alone. At 14 days, injury + ND induced a fivefold increase in muscle IGF-I mRNA expression, which was greater than injury alone. Muscle Akt activity and glycogen synthetase kinase-3beta activity were also induced by injury + ND at 14 days of recovery, but not by injury alone. ND had a main effect for increasing muscle MyoD and cyclin D1 mRNA expression at 14 days. After 42 days of recovery, injury + ND increased large-diameter myofiber incidence compared with injury only. Nandrolone decanoate (ND) administration can enhance castrated mouse muscle regeneration during the recovery from
bupivacaine-induced injury [09117].

One study analyzed the effect of nandrolone decanoate (ND) on muscle repair and the expression of myogenic regulatory factors following cryoinjury in rat skeletal muscle. Adult male Wistar rats were randomly divided into 4 groups: control group, sham group, cryoinjured group treated with ND and non-injured group treated with ND. Treatment consisted of subcutaneous injections of ND (5 mg/kg) twice a week. After sacrifice, the tibialis anterior muscle was removed for the isolation of total RNA and analysis of myogenic regulatory factors using real-time PCR as well as morphological analysis using the hematoxylin-eosin assay. There was a significant increase in MyoD mRNA after 7 days and in myogenin mRNA after 21 days in the cryoinjured ND group in comparison to other groups in the same period. The morphological analysis revealed no edema or myonecrosis after 7 days as well as no edema or inflammatory infiltrate after 14 days in the cryoinjured ND group. In conclusion the anabolic steroid nandrolone decanoate can modulate the muscle repair process in rats following cryoinjury by influencing the expression of regulatory myogenic factors and phases of muscle repair [13230].

Positive effect on nerve regeneration

Recovery from peripheral nerve repair is frequently incomplete. Hence drugs that enhance nerve regeneration are needed clinically. In 40 rats, a 40-mm segment of the left median nerve was removed and interposed between the stumps of a sectioned right median nerve. Starting 7 days after nerve grafting and continuing over a 6-month period, we administered nandrolone at a dose of 5 mg/kg/week to half the rats (n = 20). All rats were assessed behaviorally for grasp function and nociceptive recovery for up to 6 months. At final assessment, reinnervated muscles were tested electrophysiologically and weighed. Results were compared between rats that had received versus not received nandrolone and versus 20 nongrafted controls. Rats in the nandrolone group recovered finger flexion faster. At 90 days postsurgery, they had recovered 42 percent of normal grasp strength versus just 11 percent in rats grafted but not treated with nandrolone. At 180 days, the average values for grasp strength recovery in the nandrolone and no-nandrolone groups were 40 and 33 percent of normal values for controls, respectively. At 180 days, finger flexor muscle twitch strength was 16 percent higher in treated versus nontreated rats. Thresholds for nociception were not detected in either group 90 days after nerve grafting. At 180 days, nociceptive thresholds were significantly lower in the nandrolone group. It was concluded that nandrolone decanoate improved functional recovery in a model of deficient reinnervation [13231].

Side effects

It was reported the case of a 50-year-old body-builder Caucasian man with a long-standing abuse of nandrolone and erythropoietin that developed a ventricular septal defect following acute myocardial infarction. This mechanical complication led to cardiogenic shock ultimately treated with the implantation of a circulatory support by means of extracorporeal membrane oxygenation. The patient subsequently underwent orthotopic heart transplantation. The association of intense isometric exercise, abuse of erythropoietin and nandrolone is likely to have predisposed to coronary thrombus formation and acute myocardial infarction, as the patient presented no traditional cardiovascular risk factors [09118].

Effect on male fertility
High doses of anabolic-androgenic steroids (AAS) are used by some athletes to increase muscle mass, that is often associated with male infertility. The aim of one study was to investigate the possible cause/s of male infertility using a rat model by analysing sperm quality, including its protamine content and DNA integrity, as well as pregnancy rate. Five groups of male Wistar rats were treated for 10 weeks as follows: nandrolone decanoate (10 mg/kg per week) (ND); running exercise (50 min per day, 5 days a week) (EX); Combination of ND and exercise (ND-EX); nandrolone decanoate solvent (Sham); and control without any injection or exercise (CO). Deterioration in sperm quantity was observed in all test groups. The frequency of fertile rats was decreased in the ND-EX and ND groups. Chromomycin-A3 staining showed a protamine deficiency in the epididymal spermatozoa in the ND-EX rats. Chromatin analysis indicated an abnormal maturation of the sperm nuclei in all test groups compared with the controls. TUNEL analyses showed a highly significant increase in apoptosis in the EX, ND, and ND-EX groups. The data show that a combination of exercise and high doses of nandrolone decanoate negatively influences the DNA integrity and protamine content resulting in lower sperm quality and reduced pregnancy rate [13223].

Cardiotoxic effects

The association between synthetic androgen (SA) abuse and sudden cardiac death is often cited in literature; in this respect, to date there are little data on the effects of chronic administration of supraphysiological doses of SA on tonic cardiac autonomic control. In one study it was shown that chronic treatment with a high dose of nandrolone decanoate (ND) induces cardiac parasympathetic dysfunction and disturbances in ventricular depolarization in both sedentary and exercised rats. One study shows, unequivocally, that the blockade of the renin-angiotensin system (RAS), and particularly of angiotensin II type 1 receptor (AT1R) by losartan, prevents QT prolongation and that the administration of chronic, supraphysiological doses of ND induces parasympathetic autonomic dysfunction. This positive feedback of the pharmacological blockade of the receptor has been tested on an experimental basis, both on groups of sedentary rats and on groups of rats forced to exercise; in both cases, QT prolongation and parasympathetic autonomic dysfunction have been prevented, thus demonstrating that exercise does not alter the effects of the receptor blockade. This article is inscribed in the interesting line of research that moves from the assertion that the mechanisms by which supraphysiological doses of SA cause cardiac depolarization disturbances and autonomic dysfunction have not yet been clearly established. More generally, it is extremely important and very opportune to see how and why the use of chronic supraphysiological doses of SA induces cardiovascular disorders up to determine sudden cardiac death in healthy young athletes. Animal model studies have been conducted to evaluate the impact of SA supraphysiological doses on the cardiovascular system and on myocardial injury and to understand the pathogenesis of ventricular remodelling and dysfunction, of ventricular arrhythmias and of sudden cardiac death associated with SA-abuse. Taken together, these studies support the hypothesis that supraphysiological doses of SA induce adrenergic overstimulation and myocardial injury in addition to cardiac dysautonomia. In fact, the electrical and histological remodelling induced by SA may create a substrate causing electrical disturbances and sudden cardiac death. Some authors suggested that nandrolone could cause the release of intracellular calcium and thereby explain the pro-arrhythmic effects observed in rats. In a recent experimental study, researchers treated rats with supraphysiological doses of ND in association with exercise, and they observed an increase in heart collagen concentration associated with the activation of the cardiac RAS. These pathophysiological changes undoubtedly provide an anatomo-pathological substrate that may explain the increased propensity to the generation and continuation of malignant cardiac arrhythmias. An explanation, placed at the base of these pathogenic mechanisms, resides in the alteration of the sympathetic autonomic activity modulated by the RAS. It is well known that many deleterious actions on the cardiovascular
system are connected to the function of the AT1R. The stimulation of AT1R is involved in modulating cell growth and proliferation of vascular smooth muscle cells, cardiomyocytes and endothelial cells, thus being at the basis of endothelial dysfunction, atherosclerotic vascular phenomena, congestive heart failure and, finally, myocardial infarction. All the pathological phenomena listed above are combined and are determined by the AT1R mediation which underlies the pathogenesis of left ventricular remodelling, of myocardium hypertrophy and fibrosis and of cardiac apoptosis. Regarding fibrosis, many experimental data appear to show that the RAS plays an important role in the development of myocardial fibrosis and of left ventricle hypertrophy. Conversely, the blocking of AT1R determines the regression of the phenomena of ventricular hypertrophy. Recently, the same research group presented the results about heart rate variability analysis in human and animal model, showing a SA-induced cardiac autonomic imbalance, with reduction in parasympathetic cardiac modulation and increase in sympathetic cardiac modulation. These data are in accord with post-mortem histopathological data from SA users, because the presence of contraction band necrosis in the myocardium is associated with adrenergic overstimulation. One study group has over the years shown that major cardiac abnormalities, found experimentally in the hearts of rats treated with high doses of SA, are due to overstimulation of adrenergic mechanisms. Moreover, the study group has investigated the role of cytokines and the interactions of TNF-alpha in the induction of cardiac apoptotic mechanism, which represents a line of research worthy of attention. Again, the findings based on data from experimental studies in animals, support the hypothesis that the combined effects of strenuous exercise and of the abuse of SA, stimulating the sympathetic nervous system, may predispose to myocardial injury and consequent heart failure mediated by oxidative stress. These cardiovascular effects of SA are mediated by genomics (intracellular androgen receptor – nuclear transcription – gene expression) and non-genomic mechanisms. The sympathetic modulatory role of the RAS is quite recently reiterated in literature: during physical inactivity vasomotor sympathetic tone is inhibited, while sympathetic activation is enhanced as a result of physical stress. Certainly, experimental studies have the duty to determine the influence of physical activity in causing cardiac dysfunction. In this study, this has been carried out by the authors with great care, and the conclusions are that cardiac autonomic dysfunction and repolarisation disturbances also occur in exercised rats, which were administered supraphysiological doses of SA. From the data presented, it can be realized that to date considerable research has led to the identification of a growing number of SA-adverse effects due to abuse in healthy athletes. However, it should be kept in mind that, although substantial progress has been made in identifying novel mechanism of damage of SA-abuse, great efforts are still needed to elucidate their pathophysiological mechanisms [13225].

Harmful effects on brain axons

Anabolic-androgenic steroids (AAS) are used in the medical treatment of many disorders. Erythropoietin (EPO) is a hematopoietic cytokine that has anti-apoptotic, anti-oxidative, and anti-inflammatory effects. The aim of one study was to investigate the neuroprotective effects of EPO in the hippocampus, parietal cortex and prefrontal cortex, in brain damage due to nandrolone decanoate. 35 Wistar male rats were randomly divided into: (1) control group, (2) sham group, (3) nandrolone decanoate group (ND, intramuscular, 10 mg/(kg week), 8 weeks), (4) ND+low dose EPO treated group (ND+L-EPO) and (5) ND+high dose EPO treated group (ND+H-EPO). EPO was administrated by intraperitoneal injection at a dose of 100 U/(kg day) for L-EPO treatment and at a dose of 500 U/(kg day) for H-EPO treatment during 8 weeks. The number of neurons of CA1, CA2, CA3 and dentate gyrus of hippocampus, parietal cortex and prefrontal cortex were significantly less in the ND group compared with the control group. Treatment with H-EPO significantly preserved the number of neurons in hippocampus when compared with ND administrated. Besides, H-EPO
treatment decreased the number of TUNEL-positive and active caspase-3 positive cells and MDA levels and increased GPx levels when compared to ND group. In conclusion, abuse of AAS causes reduction in the number of neurons in hippocampus, parietal cortex and prefrontal cortex regions and increases oxidative damage and therefore H-EPO may be useful as a neuroprotective agent in brain injury [13226].

**Harmful effects on learning capacity**

Chronic exposure to the anabolic androgenic steroids (AAS) nandrolone decanoate (ND) in supra-physiological doses is associated with learning and memory impairments. Given the well-known beneficial effects of voluntary exercise on cognitive functions, we examined whether voluntary exercise would improve the cognitive deficits induced by chronic administration of ND. We also investigated the effects of ND and voluntary exercise on hippocampal BDNF levels. The rats were randomly distributed into 4 experimental groups: the vehicle-sedentary group, the ND-sedentary group, the vehicle-exercise group, and the ND-exercise group. The vehicle-exercise and the ND-exercise groups were allowed to freely exercise in a running wheel for 15 days. The vehicle-sedentary and the ND-sedentary groups were kept sedentary for the same period. Vehicle or ND injections were started 14 days prior to the voluntary exercise and continued throughout the 15 days of voluntary exercise. After the 15-day period, the rats were trained and tested on a water maze spatial task using four trials per day for 5 consecutive days followed by a probe trial two days later. Exercise significantly improved performance during both the training and retention of the water maze task, and enhanced hippocampal BDNF. ND impaired spatial learning and memory, and this effect was not rescued by exercise. ND also potentiated the exercise-induced increase in hippocampal BDNF levels. These results seem to indicate that voluntary exercise is unable to improve the disruption of cognitive functions by chronic ND. Moreover, increased levels of BDNF may play a role in ND-induced impairments in learning and memory. The harmful effects of ND and other AAS on learning and memory should be taken into account when athletes decide to use AAS for performance or body image improvement [13229].

**Effect on hypertension**

The aims of one study were to evaluate the effects of nandrolone (ND) on cardiac inflammatory cytokines, ACE activity, troponin I, and the sensitivity of the Bezold-Jarisch reflex (BJR). Male Wistar rats were administered either ND (20 mg/kg; DECA) or vehicle (control animals; CONT) for 4 weeks. BJR was analyzed by measuring the bradycardia and hypotension responses elicited by serotonin administration (2-32 μg/kg). Mean arterial pressure (MAP) was assessed and myocyte hypertrophy was determined by the heart weight/body weight ratio and by morphometric analysis. Matrix collagen deposition was assessed by histological analysis of the picrosirius red-stained samples. Mesenteric vascular reactivity was performed and central venous pressure (CVP) evaluated. Cardiac inflammatory cytokine levels and angiotensin-converting enzyme (ACE) activity were studied as well the biomarker of cardiac lesion, troponin I. DECA group showed enhancement of matrix type I collagen deposition and cardiac ACE activity compared with the CONT. Interleukin (IL)-10 was reduced and pro-inflammatory cytokines (TNF-alpha and IL-6) were increased in the DECA group compared with CONT. Cardiac injury was observed in the DECA group shown by the reduction in cardiac troponin I compared with the CONT group. Animals in the DECA group also developed myocyte hypertrophy and reduction of BJR sensitivity. The MAP of animals treated with ND reached hypertensive levels. No changes in CVP and vascular reactivity were observed in both experimental groups. It was concluded that high doses of ND elicit cardiotoxic effects with cardiac remodelling and injury. Cardiac
changes reduce the BJR sensitivity. Together, these abnormalities contributed to the development of hypertension in animals in the DECA group [13224].

**Effect on peripheral nerve injury**

Suboptimal recovery following repair of major peripheral nerves has been partially attributed to denervation atrophy. Administration of anabolic steroids in conjunction with neurotization may improve functional recovery of chronically denervated muscle. The purpose of one study was to evaluate the effect of the administration of nandrolone on muscle recovery following prolonged denervation in a rat model. Eight groups of female Sprague-Dawley rats (15 rats per group, 120 in all) were divided into 3- or 6-month denervated hind limb and sham surgery groups and, then, nandrolone treatment groups and sham treatment groups. Evaluation of treatment effects included nerve conduction, force of contraction, comparative morphology, histology (of muscle fibers), protein electrophoresis (for muscle fiber grouping), and immunohistochemical evaluation. Although a positive trend was noted, neither reinnervated nor normal muscle showed a statistically significant increase in peak muscle force following nandrolone treatment. Indirect measures, including muscle mass (weight and diameter), muscle cell size, muscle fiber type, and satellite cell counts, all failed to support significant anabolic effect. Thus, there does not seem to be a functional benefit from nandrolone treatment following reinnervation of either mild or moderately atrophic muscle (related to prolonged denervation) in a rodent model [13227].

**Effect on growth hormone**

Growth hormone (GH) and anabolic androgenic steroids (AAS) are commonly used in sports communities. Several studies have suggested an association between GH and AAS. It was investigated the impact of GH in rats treated with nandrolone decanoate (ND). Male Wistar rats received ND (15 mg/kg) every third day during three weeks and were subsequently treated with recombinant human GH (1.0 IU/kg) for ten consecutive days. Plasma samples were collected and peripheral organs (i.e. heart, liver, testis and thymus) were dissected and weighed. Concentration of thirteen endogenous steroids was measured in the rat plasma samples using high specificity LC-MS/MS methods. Seven steroids were detected and quantified, and concentrations of estrone, testosterone, and androstenedione were significantly different among the groups, while concentrations of pregnenolone, DHEA, 17-hydroxyprogesterone and corticosterone were not altered. Administration of rhGH alone altered the plasma steroid distribution, and the results demonstrated significantly increased concentrations of plasma estrone as well as decreased concentrations of testosterone and androstenedione in the ND-treated rats. Administration of rhGH to ND-pretreated rats did not reverse the alteration of the steroid distribution induced by ND. Administration of ND decreased the weight of the thymus, and addition of rhGH did not reverse this reduction. However, rhGH administration induced an enlargement of thymus. Taken together, the plasma steroid profile differed in the four groups, i.e. control, AAS, rhGH and the combination of AAS and rhGH treatment [13228].

**Interaction with receptors**

Interest in anabolic steroids has been renewed in the last decade with the discovery of tissue-selective androgen receptor modulators exhibiting high myotropic and small androgenic activity. An explanation put forward by us in 1982 for the mechanism of the
preferential myotropic effect of nandrolone (19-nortestosterone) exploits the fundamental difference between the 5alpha-reductase concentrations in skeletal muscle and androgenic target tissue. In androgenic tissue, testosterone is converted to the more potent 5alpha-dihydrotestosterone whereas nandrolone is converted to a less potent derivative. As 5alpha-reduction is negligible in skeletal muscle, this explains why nandrolone shows a greater myotropic to androgenic ratio when compared with testosterone. Anabolic steroids that do not undergo 5alpha-reduction exert myotropic-androgenic dissociation because their effect in androgenic tissues is not amplified by 5alpha-reduction. Tissue selectivity by receptor modulators may be achieved by inducing specific conformational changes of the androgen receptor that affect its interaction with transcriptional coregulators. Anabolic activity is mediated by the stimulation of ribosomal RNA synthesis therefore regulation of this synthesis by anabolic steroids would deserve detailed studies [09119].

Effect of small doses on stress response

Androgenic-anabolic steroid (AAS) misuse has been associated with depression. It has been proposed that stress has a role in depression and that serotonin is involved in both endocrine responses to stress and depressive physiopathology. Although reports demonstrate that AAS chronic administration modifies components of stress-responsive hypothalamic-pituitary-adrenal axis (HPAA), no study has evaluated AAS effect on the response to stressful stimuli. We studied the effects of the subchronic administration (once a day for 14 days in rats) of a supratherapeutical dose of nandrolone decanoate (ND) on HPAA and cortical serotoninergic system response to acute restraint stress (RS). Acute RS produced the following effects: increase in CORT (in blood) and ACTH (both in blood and in pituitary corticotropes), GR depletion in hippocampus and hypothalamus cytosol and GR translocation in hippocampus nuclear fraction, cortical serotonin re-uptake stimulation and hippocampus cytosolic ERK2 activation. ND by itself, i.e. in non-stressed rats, did not modify these parameters, except for a decrease of plasma CORT and ACTH levels and an increase in hippocampus cytosolic phospho-ERK1/2. On the contrary, in stressed rats ND affected stress-induced plasma ACTH increase and prevented all other above reported stress effects, except the increase in pituitary ACTH positive cell density. The results show that the prolonged administration of a supratherapeutical dose of ND in rats, albeit did not affect in a notable way HPAA and serotonin transporter activity in the absence of stress, may deregulate the stress-induced hormonal cascade which plays a crucial role in depressive psychopathology [12162].

Aortic adaptations to exercise

In one study it was investigated the interaction between exercise-induced mitochondrial adaptation of large vessels and the effects of chronic anabolic androgenic steroids (AASs). Four groups of Sprague-Dawley rats were studied: (i) sedentary, (ii) sedentary + nandrolone-treated, (iii) aerobic exercise trained, and (iv) trained + nandrolone-treated. Aerobic training increased the levels of aortic endothelial nitric oxide synthase (eNOS) and heme oxygenase-1 (HO-1) in accordance with improved acetylcholine-induced vascular relaxation. These beneficial effects were associated with induction of mitochondrial complexes I and V, increased mitochondrial DNA copy number, and greater expression of transcription factors involved in mitochondrial biogenesis/fusion. It was also observed enhanced mitochondrial autophagy pathway activity, including increased conversion of LC3-I to LC3-II and greater expression of beclin1 and autophagy-related protein-7 (ATG7). The levels of thiobarbituric acid-reactive substances and protein carbonyls remained unchanged, whereas significant increases in catalase and mitochondrial manganese superoxide dismutase (MnSOD) levels
were observed in the aortas of trained animals, when compared with sedentary controls. Nandrolone increased oxidative stress biomarkers and inhibited exercise-induced increases of eNOS, HO-1, catalase, and MnSOD expression. In addition, it also attenuated elevated peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1alpha) and mitofusin-2 expression, and further up-regulated LC3II conversion, beclin1, ATG7, and dynamin-related protein-1 expression. These results demonstrate that nandrolone attenuates aortic adaptations to exercise by regulating mitochondrial dynamic remodelling, including down-regulation of mitochondrial biogenesis and intensive autophagy [13232].

**Nandrolone-mediated testosterone reduction during alcohol intoxication**

Human studies have indicated that the use of anabolic androgenic steroids may be associated with the abuse of alcohol and other drugs. Also, experimental animal research has indicated that chronic nandrolone administration subsequently increases voluntary alcohol drinking. The aim of one study was to test a hypothesis that alcohol-induced testosterone elevation, especially associated with stress conditions derived by nandrolone treatment, could be the underlying factor in causing increased alcohol drinking. Male alcohol-prefering AA and low drinking Wistar rats were randomly divided into control and nandrolone decanoate treated (15 mg/kg for 14 days) groups. Basal serum testosterone and corticosterone were determined before the first nandrolone treatment, after 7days of treatment, and after an additional (7/day) washout period, during which also the acute effect of alcohol (1.5 g/kg) on steroid hormones was determined. Hereafter followed a (5/week) voluntary alcohol consumption period, during the last 2 weeks of which the rats were treated again with nandrolone. Both normal and reversed dark- versus light-cycle experimental designs were used. Contrary to the hypothesis, nandrolone treatment decreased voluntary alcohol consumption in both AA and Wistar rats. Also, instead of stress causation, elevated basal testosterone and lowered basal corticosterone levels were observed after nandrolone treatment in both AA rats and Wistars. During acute alcohol intoxication the frequency of testosterone decreases was higher in the nandrolone-treated groups compared with control AA and Wistar rats. Present data support the hypothesis that nandrolone-treatment mediated attenuation of alcohol intake in both AA and Wistar rats may be the result of negative reinforcement caused by alcohol-mediated testosterone reduction [13233].

**Impact of nandrolone on biosynthesis of steroids**

Growth hormone (GH) and anabolic androgenic steroids (AAS) are commonly used in sports communities. Several studies have suggested an association between GH and AAS. It was investigated the impact of GH in rats treated with nandrolone decanoate (ND). Male Wistar rats received ND (15 mg/kg) every third day during three weeks and were subsequently treated with recombinant human GH (1.0 IU/kg) for ten consecutive days. Plasma samples were collected and peripheral organs (i.e. heart, liver, testis and thymus) were dissected and weighed. Concentration of thirteen endogenous steroids was measured in the rat plasma samples using high specificity LC-MS/MS methods. Seven steroids were detected and quantified, and concentrations of estrone, testosterone, and androstenedione were significantly different among the groups, while concentrations of pregnenolone, DHEA, 17-hydroxyprogesterone and corticosterone were not altered. Administration of rhGH alone altered the plasma steroid distribution, and the results demonstrated significantly increased concentrations of plasma estrone as well as decreased concentrations of testosterone and androstenedione in the ND-treated rats. Administration of rhGH to ND-pretreated rats did not reverse the alteration of the steroid distribution induced by ND. Administration of ND decreased the weight of the thymus, and addition of rhGH did not reverse this reduction.

721
However, rhGH administration induced an enlargement of thymus. Taken together, the plasma steroid profile differed in the four groups, i.e. control, AAS, rhGH and the combination of AAS and rhGH treatment [13234].

Effects of nandrolone on recovery of denervated muscle

Suboptimal recovery following repair of major peripheral nerves has been partially attributed to denervation atrophy. Administration of anabolic steroids in conjunction with neurotization may improve functional recovery of chronically denervated muscle. The purpose of one study was to evaluate the effect of the administration of nandrolone on muscle recovery following prolonged denervation in a rat model. Eight groups of female Sprague-Dawley rats (15 rats per group, 120 in all) were divided into 3- or 6-month denervated hind limb and sham surgery groups and, then, nandrolone treatment groups and sham treatment groups. Evaluation of treatment effects included nerve conduction, force of contraction, comparative morphology, histology (of muscle fibers), protein electrophoresis (for muscle fiber grouping), and immunohistochemical evaluation. Although a positive trend was noted, neither reinnervated nor normal muscle showed a statistically significant increase in peak muscle force following nandrolone treatment. Indirect measures, including muscle mass (weight and diameter), muscle cell size, muscle fiber type, and satellite cell counts, all failed to support significant anabolic effect. It was concluded that there does not seem to be a functional benefit from nandrolone treatment following reinnervation of either mild or moderately atrophic muscle (related to prolonged denervation) in a rodent model [13235].

Genotoxic effects

To evaluate the impact potential of nandrolone decanoate on DNA damage in multiple organs of Wistar rats by means of single-cell gel (comet) assay and micronucleus test. A total of 15 animals were distributed into three groups of five animals each as follows: control group was animal not exposed to nandrolone decanoate; experimental group was animals exposed to nandrolone decanoate for 24 h at 5 mg/kg subcutaneously; and experimental group was animals exposed to nandrolone decanoate for 24 h at 15 mg/kg subcutaneously. Significant statistical differences were noted in peripheral blood, liver, and heart cells exposed to nandrolone decanoate at the two doses evaluated. A clear dose-response relationship was observed between groups. Kidney cells showed genetic damage at only the highest dose (15 mg/kg) used. However, micronucleus data did not show remarkable differences among groups. In conclusion, the present study indicates that nandrolone decanoate induces genetic damage in rat blood, liver, heart, and kidney cells as shown by single-cell gel (comet) assay results [13236].

Nandrolone is an androgenic-anabolic steroid (AAS) with diverse medical applications but taken indiscriminately by some to rapidly increase muscle mass. The aim of one study was to evaluate the genotoxic and clastogenic potential of nandrolone (Deca-Durabolin®) in vivo in different cells of mice, using the comet assay and micronucleus test, respectively. The animals received subcutaneous injection of the three doses of the steroid (1.0, 2.5 and 5.0 mg/kg body weight). Cytotoxicity was assessed by scoring 200 consecutive total polychromatic (PCE) and normochromatic (NCE) erythrocytes (PCE-NCE ratio). The results showed a significant dose-related increase in the frequency of DNA damage in leukocytes, liver, bone marrow, brain and testicle cells at the three tested doses and a significant increase of the micronucleated polychromatic erythrocytes at all tested doses. Under our
experimental conditions, the nandrolone steroid hormone showed genotoxic and clastogenic effects when administered subcutaneously to mice [12163].

**Upregulation of aromatase expression**

Several doping agents, such as anabolic androgenic steroids (AAS) and peptide hormones like insulin-like growth factor-I (IGF-I), are employed without considering the potential deleterious effects that they can cause. In addition, androgens are used in postmenopausal women as replacement therapy. However, there are no clear guidelines regarding the optimal therapeutic doses of androgens or long-term safety data. In this study we aimed to determine if two commonly used AAS, nandrolone and stanozolol, alone or in combination with IGF-I, could activate signaling involved in breast cancer cell proliferation. Using a human breast cancer cell line, MCF-7, as an experimental model we found that both nandrolone and stanozolol caused a dose-dependent induction of aromatase expression and, consequently, estradiol production. Moreover, when nandrolone and stanozolol were combined with IGF-I, higher induction in aromatase expression was observed. This increase involved phosphatidylinositol 3-kinase (PI3K)/AKT and phospholipase C (PLC)/protein kinase C (PKC), which are part of IGF-I transductional pathways. Specifically, both AAS were able to activate membrane rapid signaling involving IGF-I receptor, extracellular regulated protein kinases 1/2 (ERK1/2) and AKT, after binding to estrogen receptor (ER), as confirmed by the ability of the ER antagonist ICI182, 780 to block such activation. The estrogenic activity of nandrolone and stanozolol was further confirmed by their capacity to induce the expression of the ER-regulated gene, CCND1 encoding for the cell cycle regulator cyclin D1, which represents a key protein for the control of breast cancer cell proliferation. In fact, when nandrolone and stanozolol were combined with IGF-I, they increased cell proliferation to levels higher than those elicited by the single factors. Taken together these data clearly indicate that the use of high doses of AAS, as occurs in doping practice, may increase the risk of breast cancer. This potential risk is higher when AAS are used in association with IGF-I. This might be the first report directly associating AAS with this type of cancer [12164].

**Effect of small doses on stress response**

Androgenic-anabolic steroid (AAS) misuse has been associated with depression. It has been proposed that stress has a role in depression and that serotonin is involved in both endocrine responses to stress and depressive physiopathology. Although reports demonstrate that AAS chronic administration modifies components of stress-responsive hypothalamic-pituitary-adrenal axis (HPAA), no study has evaluated AAS effect on the response to stressful stimuli. We studied the effects of the subchronic administration (once a day for 14 days in rats) of a supratherapeutical dose of nandrolone decanoate (ND) on HPAA and cortical serotoninergic system response to acute restraint stress (RS). Acute RS produced the following effects: increase in CORT (in blood) and ACTH (both in blood and in pituitary corticotropes), GR depletion in hippocampus and hypothalamus cytosol and GR translocation in hippocampus nuclear fraction, cortical serotonin re-uptake stimulation and hippocampus cytosolic ERK2 activation. ND by itself, i.e. in non-stressed rats, did not modify these parameters, except for a decrease of plasma CORT and ACTH levels and an increase in hippocampus cytosolic phospho-ERK1/2. On the contrary, in stressed rats ND affected stress-induced plasma ACTH increase and prevented all other above reported stress effects, except the increase in pituitary ACTH positive cell density. The results show that the prolonged administration of a supratherapeutical dose of ND in rats, albeit did not affect in a notable way HPAA and
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Endogenous or exogenous origin of nandrolone

On the one hand, 19-norandrosterone (NA) is the most abundant metabolite of the synthetic anabolic steroid 19-nortestosterone and related prohormones. On the other hand, small
amounts are biosynthesized by pregnant women and further evidence exists for physiological origin of this compound. The World Anti-Doping Agency (WADA) formerly introduced threshold concentrations of 2 or 5 ng of NA per ml of urine to discriminate 19-nortestosterone abuse from biosynthetic origin. Recent findings showed however, that formation of NA resulting in concentrations in the range of the threshold levels might be due to demethylation of androsterone in urine, and the WADA 2006 Prohibited List has defined NA as endogenous steroid. To elucidate the endogenous or exogenous origin of NA, $^{13}$C/$^{12}$C-analysis is the method of choice since synthetic 19-nortestosterone is derived from C(3)-plants by partial synthesis and shows delta$^{13}$C(VPDB)-values of around -28 per thousand. Endogenous steroids are less depleted in $^{13}$C due to a dietary mixture of C(3)- and C(4)-plants. An extensive cleanup based on two high performance liquid chromatography cleanup steps was applied to quality control and doping control samples, which contained NA in concentrations down to 2 ng per ml of urine. $^{13}$C/$^{12}$C-ratios of NA, androsterone and etiocholanolone were measured by gas chromatography/combustion/isotope ratio mass spectrometry. By comparing delta$^{12}$C(VPDB)-values of androsterone as endogenous reference compound with NA, the origin of NA in doping control samples was determined as either endogenous or exogenous [06107].

**Significance of 19-norandrosterone in athletes' urine**

Nandrolone and other 19-norsteroid potent anabolic steroids have been prohibited in sports for 30 years. The detection of the main urinary metabolite – 19-norandrosterone – in amounts greater than 2 ng/ml constitutes an adverse analytical finding. The presence in nutritional sport supplements of steroids not listed on the label has undoubtedly resulted in positive tests, but inadvertent consumption of meat containing residues of hormonal treatment should not realistically cause apprehension. Although highly improbable, athletes should prudenty avoid meals composed of pig offal in the hours preceding the test since the consumption of edible parts of a non-castrated pig, containing 19-nortestosterone, has been shown to results in the excretion of 19-norandrosterone in the following hours. Norsteroid metabolites are formed during pregnancy and excreted as minor metabolites of norethisterone, and minute amounts have been identified in some male and female samples when using more sensitive techniques of detection. Whereas exercise does not seem to be a significant factor in 19-norandrosterone excretion, some rare urine samples were found to be a suitable medium for in situ 19-demethylation of urinary metabolites [06106].

**Laboratory techniques**

Nandrolone, an anabolic steroid, is used for the treatment of several diseases and is available in various pharmaceutical formulations. The most widely used pharmaceutical formulation is Deca-Durabolin®, but other products, such as Keratyl eye drops solution, are also currently administered. Nandrolone is one of the most abused anabolic steroid in sports. Analyses for this anabolic steroid according to the World Anti-Doping Agency (WADA) protocol are based on the identification of the nandrolone two main urinary metabolites which, in humans, are glucuronides of 19-norandrosterone and 19-noretiocholanolone. A positive cut off limit of 2 ng/mL has been set by the anti-doping code for the first metabolite, 19-norandrosterone. In this preliminary study, an eye drops solution (Keratyl®) containing a therapeutic dose of a nandrolone sodium sulphate was administered to several male volunteers during 3 days and urines were collected during 3 weeks. Surprisingly, contrary to all expectations, the urinary concentrations measured in urines reached 450 ng/mL and 70 ng/mL for norandrosterone and noretiocholanolone, respectively. Moreover, concentration...
levels near to 2 ng/mL were found, more than 2 weeks after the last administration, depending on individual metabolism. Inter-variability as well as intra-variability of nandrolone excretion kinetic, regarding this particular administration mode, were also evaluated. Quantification of nandrolone metabolites was performed by GC–MS. The method was previously validated in terms of specificity, precision, linearity, LOD, LOQ, robustness, accuracy and the expanded uncertainty was also evaluated [07089].

**Liquid chromatography/tandem mass spectrometry**

A confirmatory method for the simultaneous determination of nandrolone (alpha and beta) and trenbolone (alpha and beta) in urine samples by liquid chromatography electrospray mass spectrometry (LC-MS-MS) was developed. After an enzymatic deconjugation, the urine was subjected to a one-step cleanup on a commercially available immunoaffinity chromatography cartridge. The analytes were detected by liquid chromatography-positive ion electrospray tandem mass spectrometry using deuterium labelled internal standards. The analytical procedure was applicable to bovine and swine urine samples. The procedure was validated as a quantitative confirmatory method according to the Commission Decision 2002/657/EC criteria. The results obtained showed that the method was suitable for statutory residues testing regarding the following performance characteristics: instrumental linearity, specificity, precision (repeatability and intra-laboratory reproducibility), recovery, decision limit (CCalpha), detection capability (CCbeta) and ruggedness. The decision limits (CCalpha) obtained, were between 0.54 and 0.60 μg/L; the recovery was above 64 percent for all the analytes. Repeatability was between 1.6 percent and 5.7 percent and within-laboratory reproducibility between 1.6 percent and 6.0 percent for all the steroids [09121].

19-Norandrosterone sulfate (19-NAS) is the sulfoconjugated form of 19-norandrosterone (19-NA), the major metabolite of the steroid nandrolone. A sensitive and accurate liquid chromatography/tandem mass spectrometry (LC-MS/MS) assay was developed for the direct measurement of 19-NAS in human urine samples. The method involved a quaternary amine SPE protocol and subsequently injection of the extract onto an analytical column (Uptisphere ODB, 150 mm x 3.0 mm, 5 microm) for chromatographic separation and mass spectrometry detection in negative electrospray ionisation mode. The sulfoconjugate of 19-NA was identified in urine by comparison of mass spectra and retention time with a reference substance. The limit of detection (LOD) and lowest limit of quantification (LLOQ) of 19-NAS were of 40 pg/mL and 200 pg/mL, respectively. For a nominal concentration of 2 ng/mL, recovery (94 %), intra-day precision (2.7 %), intra-assay precision (6.6 %) and inter-assay precision (14.3 %) were determined. Finally, this analytical method was applied for quantifying the concentration of 19-NAS in doping samples, using calibration curves (0.2-20 ng/mL) and the standard-addition method. The results show the feasibility of applying this LC-MS/MS assay as a complementary tool to detect misuse of nandrolone or nandrolone precursors [07090].

Determination of nandrolone metabolites in human urine: comparison between liquid chromatography/tandem mass spectrometry and gas chromatography/mass spectrometry. Nandrolone (19-nortestosterone) is an androgenic anabolic steroid illegally used as a growth-promoting agent in animal breeding and as a performance enhancer in athletics. Therefore, its use was officially banned in 1974 by the Medical Commission of the International Olympic Committee (IOC). Following nandrolone administration, the main metabolites in humans are 19-norandrosterone, 19-norethiocolanolone and 19-norepiandrosterone, and their presence in urine is the basis of detecting its abuse. One work was undertaken to determine, in human urine, nandrolone metabolites (phase I and phase II) by developing and comparing multiresidue liquid chromatography/tandem mass spectrometry (LC/MS/MS) and gas chromatography/mass spectrometry (GC/MS) methods. A double extraction by solid-phase
extraction (SPE) was necessary for the complete elimination of the interfering compounds. The proposed methods were also tested on a real positive sample, and they allow us to determine the conjugated/free fractions ratio reducing the risk of false positive or misleading results and they should allow laboratories involved in doping control analysis to monitor the illegal use of steroids. The advantages of LC/MS/MS over GC/MS (which is the technique mainly used) include the elimination of the hydrolysis and derivatization steps: it is known that during enzymatic hydrolysis several steroids can be converted into related compounds and deconjugation is not always 100 percent effective. The validation parameters for the two methods were similar (limit of quantification (LOQ) <1 ng/mL and percentage coefficient of variance (CV %) <16.4), and both were able to confirm unambiguously all the analytes, thus confirming the validity of both techniques [10344].

19-Norandrosterone (19-NA) is the major metabolite of the steroid nandrolone, one of the most commonly abused anabolic androgenic agents. 19-NA exists mainly as the glucuronide form in human urine. A candidate reference measurement procedure for 19-NA in urine involving isotope dilution coupled with liquid chromatography/tandem mass spectrometry (LC/MS/MS) has been developed and critically evaluated. The 19-NA glucuronide was enzymatically hydrolyzed, and the 19-NA along with its internal standard (deuterated 19-NA) was extracted from urine using liquid-liquid extraction prior to reversed-phase LC/MS/MS. The accuracy of the measurement of 19-NA was evaluated by a recovery study of added 19-NA. The recovery of the added 19-NA ranged from 99.1 to 101.4 percent. This method was applied to the determination of 19-NA in urine samples fortified with 19-NA glucuronide at three different concentrations (equivalent to 1, 2, and 10 ng/mL 19-NA). Excellent reproducibility was obtained with within-set coefficients of variation (CVs) ranging from 0.2 to 1.2 percent, and between-set CVs ranging from 0.1 to 0.5 percent. Excellent linearity was also obtained with correlation coefficients of all linear regression lines (measured intensity ratios vs mass ratios) ranging from 0.9997 to 0.9999. The detection limit for 19-NA at a signal-to-noise ratio of approximately 3 was 16 pg. The mean results of 19-NA yielded from hydrolysis of 19-NA glucuronide compared well with the theoretical values (calculated from the conversion of 19-NA glucuronide to 19-NA) with absolute relative differences ranging from 0.2 to 1.4 percent. This candidate reference measurement procedure for 19-NA in urine, which demonstrates good accuracy and precision and low susceptibility to interferences, can be used to provide an accuracy base to which routine methods for 19-NA can be compared and that will serve as a standard of higher order for measurement traceability [06108].

**Cyclic, differential pulse and square-wave voltammetry**

The electrochemical behaviour of nandrolone was investigated by cyclic, differential pulse and square-wave voltammetry in phosphate buffer system at fullerene-C60-modified electrode. The modified electrode shows an excellent electrocatalytic activity towards the oxidation of nandrolone resulting in a marked lowering in the peak potential and considerable improvement of the peak current as compared to the electrochemical activity at the bare glassy carbon electrode. The oxidation process is shown to be irreversible and diffusion-controlled. A linear range of 50 microM to 0.1 nM is obtained along with a detection limit and sensitivity of 0.42 nM and 0.358 nA/nM, respectively, in square-wave voltammetric technique. The effect of interferents, stability and reproducibility of the proposed method were also studied. The described method was successfully employed for the determination of nandrolone in human serum and urine samples. A cross-validation of observed results by GC-MS indicates that the results are in good agreement with each other [07091].

**Gas chromatography-tandem mass spectrometry**

727
A rapid sample treatment procedure for the gas chromatography-tandem mass spectrometry (GC-MS) determination of 19-nortestosterone (19-NT) in animal tissues has been developed. In our optimized procedures, enzymatic hydrolysis with beta-glucuronidase from Escherichia coli was performed in an acetate buffer (pH 5.2, 0.2 mol/L). Next, the homogenate was mixed with methanol and heated at 60 °C for 15 min, then placed in an ice-bath at -18 °C for 2 h. After liquid-liquid extraction with n-hexane, the analytes were subjected to a normal-phase solid phase extraction (SPE) C₁₈ cartridge for clean-up. The dried organic extracts were derivatized with heptafluorobutyric anhydride (HFBA), and then the products were injected into GC-MS. Using electron impact mass spectrometry (EI-MS) with positive chemical ionization (PCI), four diagnostic ions (m/z 666, 453, 318, and 306) were determined. A standard calibration curve over the concentration range of 1-20 ng/g was reached, and the detection limit was 0.3 ng. When applied to spiked samples collected from bovine and ovine, the recoveries ranged from 63 to 101 percent with relative standard deviation (RSD) between 2.7 and 8.9 percent. The procedure is a highly efficient, sensitive, and more economical method which offers considerable potential to resolve cases of suspected nandrolone doping in husbandry animals [11099].

**Experimental**

The abuse of anabolic androgenic steroids (AASs) is not only a problem in the world of sports but is associated with the polydrug use of nonathletes. Investigations of the neurochemical effects of AAS have focused in part on the monoaminergic systems, involving, among other things, the development of dependence. It has previously been shown that pretreatment with nandrolone decanoate attenuates dose-dependently the increase in extracellular dopamine (DA) concentration evoked by amphetamine and 3,4-methylenedioxyamphetamine in the nucleus accumbens (NAc). The aim of the study was to investigate whether the nandrolone pre-exposure modulates the acute neurochemical and behavioral effects of cocaine in rats and whether the effects are long lasting. DA, 5-hydroxytryptamine (5-HT), and their metabolites were measured from samples collected from the NAc by microdialysis. The behavior of the animals was recorded. The study demonstrated that five injections of nandrolone (5 and 20 mg/kg) inhibited cocaine-evoked DA and 5-HT outflow in the NAc, locomotor activity (LMA), and stereotyped behavior in experimental animals, and that these effects are seen even after elimination of nandrolone from bloodstream. Given that accumualt outflow of DA and 5-HT, as well as LMA and stereotyped behavior, is related to gratification of stimulant drugs, the study thus suggests that nandrolone, at the doses tested, has a significant effect on the pleasurable properties of cocaine. Furthermore, because neurochemical and behavioral responses were still attenuated after a fairly long recovery period, it seems that nandrolone may induce long-lasting changes in the brains of rat [10086].

One of the most frequently misused steroid precursors (prohormones) is 19-norandrostenedione (estr-4-ene-3,17-dione). Recently it has been shown that norandrostenedione stimulates skeletal muscle growth after s.c. administration in a highly selective manner but exhibits only weak androgenic activity in rats. Because most abusers take norandrostenedione orally, the aim of this study was to compare the anabolic and androgenic potency of norandrostenedione between s.c. and oral application. Orchiectomised rats were treated with norandrostenedione either s.c. (1 mg/kg body weight/day) or orally (0.1, 1 and 10 mg/kg body weight/day). The tissue weights of the levator ani, the seminal vesicle and the prostate were analysed to determine the anabolic and androgenic activity. Heart and liver wet weights were examined to identify side effects. Serum concentrations of norandrostenedione and its metabolite nandrolone were
determined. GCMC analysis revealed that free and glucuronidated norandrostenedione and nandrolone were detectable in the serum after oral and s.c. administration and that norandrostenedione was converted to nandrolone in comparable amounts independent of the route of administration. In agreement to a previous study s.c. application of norandrostenedione stimulates skeletal muscle growth but has only weak androgenic effects. In contrast, after oral administration of norandrostenedione neither stimulation of the prostate nor the levator ani could be observed in the doses administered in this study. Interestingly, and in contrast to s.c. treatment, oral administration of norandrostenedione resulted in a dose-dependent decrease of body weight. In summary, oral administration of norandrostenedione, at least in the rat, seems to be a very ineffective strategy for stimulating skeletal muscle mass increases but may be associated with side effects [09122].

Anabolic-androgenic steroids are used at high doses by athletes for improving athletic ability, physical appearance and muscle mass. Unfortunately, the abuse of these agents has significantly increased. It has been established that exercise and high doses of anabolic-androgenic steroids may influence the hypothalamic-pituitary-gonadal axis, which can in turn affect testicular apoptosis. However, the effect of the combination of exercise and high dose of anabolic-androgenic steroids on testicular apoptosis is not known. It was investigated the combined effects of exercise and high doses of nandrolone decanoate on apoptosis in the spermatogenic cell lineage. Five groups of male Wistar strain albino rats were treated as follows for 8 weeks: solvent of nandrolone decanoate (peanut oil) as a vehicle (Sham); nandrolone decanoate (10 mg/kg/weekly) (nandrolone decanoate); exercise (1 hr/day, 5 days a week) (exercise); nandrolone decanoate (10 mg/kg/weekly) and exercise (1 hr/day, 5 days a week) (nandrolone decanoate exercise); and sedentary control without any injection or exercise (control). Apoptosis in the male germ line was characterized by TUNEL, caspase-3 assay and transmission electron microscopy. The weights of the testis and accessory sex organs, as well as sperm parameters significantly decreased in the experimental groups relative to the sham and control groups. Germ cell apoptosis and a significant decrease in the number of germ cell layers in nandrolone decanoate exercise-treated testes were observed. Exercise training seems to increase the extent of apoptotic changes caused by supraphysiological dose of nandrolone decanoate in rats, which in turn affects fertility [09123].

There is still controversy about the effect of anabolic steroid on connective tissue. One study examined the hypothesis that the local use of nandrolone decanoate, an anabolic steroid on rotator cuff, facilitates the healing process when used in combination with surgical repair. Forty-eight male rabbits were divided in four groups with anabolic steroids (Nandrolone Decanoate 10 mg/kg) and immobilization as variables. The groups were the following: first group, nonsteroid use-immobilization; second group, nonsteroid use-nonimmobilization; third group, steroid use-immobilization; fourth group steroid use-nonimmobilization. Every rabbit underwent a rotator cuff incision and reconstruction. Fifteen days later the tendons were sent for biomechanical and histological evaluation. It was shown that groups that did not receive anabolic steroids showed better healing and more tendon strength in comparison to groups that received anabolic steroids. Microscopic examination of specimens from the groups without the use of anabolic steroid showed extensive fibroblastic activity whereas the specimens from those groups with anabolic steroid use showed focal fibroblastic reaction and inflammation. Immobilization provided better results in the groups with anabolic steroid use but it did not influence healing in groups without steroids. It was concluded that the effect of local nandrolone decanoate use on a rotator cuff tear is detrimental, acting as a healing inhibitor [10346].
DEHYDROEPIANDROSTERONE (DHEA)

Testosterone precursors, also called prohormones, are precursors in the endogenous production of testosterone. Androstenedione (Andro), dehydroepiandrosterone (DHEA), and androstenediol are the three testosterone precursors that are marketed heavily. The efficacy and safety of these prohormones were not well established, but they were believed to have the same androgenic effects on building muscle mass and strength as anabolic-androgenic steroids (AASs). The theory is to increase the body’s endogenous production of testosterone by increasing the concentration of testosterone precursors exogenously. The short- and long-term side effects also were not well known, but theoretically they may cause the same adverse side effects as AASs [07009].

In 1994, the Dietary Supplement Health and Education Act allowed for the US marketing and sale of “natural” dietary supplements without the US Food and Drug Administration (FDA) regulation for guaranteeing the purity and safety of these substances. The passage of the Dietary Health and Education Act of 1994 allowed for these precursors to be sold over the counter as “natural” dietary supplements without regulation. In 1996, androstenedione and DHEA became available in the United States market as over-the-counter nutritional supplements. Dosages as high as androstenedione,100 to 300 mg per day, and DHEA,150 mg per day, were recommended. These prohormones became an attractive performance-enhancing alternative to using illegal AASs, which were banned by most major sports organizations. They were classified as natural substances and were not regulated by the FDA. They became popular supplements among athletes, and, thus, became readily available to the adolescent population [07009].

Dehydroepiandrosterone (DHEA) and its sulfated derivative (DHEA-S) are the most abundant circulating steroid hormones in humans. The circulation of large amounts of dehydroepiandrosterone and its sulfated derivative) suggests a physiological role in human physiology. In the central nervous system, DHEA is considered a neurosteroid with a wide range of functions. Large amounts of DHEA and DHEA-S are produced during fetal development, but after birth, this production falls sharply and remains low for several years, after which the synthesis resumes and the levels peak during the second decade of life. By the third decade, an age-dependent decline in DHEA and DHEA-S levels ensues and progresses with advancing age. Clinical studies have shown that DHEA improved endothelial function in hypercholesterolemic men and on cutaneous vascular and brachial artery reactivity in postmenopausal women, inhibited vascular inflammation, and reduced risk factors for ischemic heart disease (IHD) in men. Reduced levels of DHEA-S in men correlated with congestive heart failure (CHF). DHEA reduced vascular risk markers and therefore may be protective against vascular and cardiovascular diseases (CVDs). Some studies have suggested that DHEA is associated with reduced mortality in men and women, improves physical function in frail women, and ameliorates sexual dysfunction in both genders. Thus the data available in the literature point to a multitude of physiological effects of DHEA including

- improved endothelial function
- improved cellular immunity
- attenuation of the inflammatory process
- amelioration of atherosclerosis
- neuroprotection against ischemia
- increased bone density
- improved physical strength
- improved sexual function
The pleiotropic effects of DHEA on the metabolism and function of many tissues and organs cannot be explained only on the basis that DHEA is a precursor of other sex steroids such as testosterone and estradiol but must take into consideration the direct effect of DHEA itself and other unique 7alpha and 7beta-hydroxylated derivatives of DHEA. Specific receptors for DHEA and its derivatives have been characterized. The recent findings that DHEA activates specific biochemical pathways and alters cellular function via binding to a membrane receptor have provided novel molecular mechanisms of action for this hormone. In addition, the discovery that other DHEA metabolites with unique cellular function via interaction with receptor molecules has increased renewed interest in DHEA action. The modulatory effects of DHEA on the immune system may contribute to an attenuation of immunosenescence as well as positive results in the treatment of autoimmune diseases. In the latter, some of its effects may be a consequence of the antagonization of corticosteroid-induced side effects and direct modulation of inflammatory substances (e.g. Th1–Th2 cells; IL-2; IL-6; TNFalpha). A number of preclinical and clinical studies demonstrated that DHEA prevents or reduces the progression of atherosclerosis DHEA mediates its action via multiple signaling pathways involving specific membrane receptors and via transformation into androgen and estrogen derivatives (e.g. androgens, estrogens, 7alpha and 7beta DHEA, and 7alpha and 7beta epiandrosterone derivatives) acting through their specific receptors. These pathways include: nitric oxide synthase activation, modulation of gamma-amino butyric acid receptors, N-methyl D-aspartate receptors sigma receptors (Sigma-1), differential expression of inflammatory factors, adhesion molecules and reactive oxygen species, among others. Clinical and epidemiological studies suggested that low DHEA levels might be associated with ischemic heart disease, endothelial dysfunction, atherosclerosis, bone loss, inflammatory diseases, and sexual dysfunction. Most importantly, no significant adverse or negative side effects of DHEA were reported in clinical studies of men and women. It was concluded that DHEA modulates endothelial function, reduces inflammation, improves insulin sensitivity, blood flow, cellular immunity, body composition, bone metabolism, sexual function, and physical strength in frailty and provides neuroprotection, improves cognitive function, and memory enhancement. DHEA possesses pleiotropic effects and reduced levels of DHEA and DHEA-S may be associated with a host of pathologies; however, the clinical efficacy of DHEA supplementation in ameliorating patho-physiological symptoms remains to be evaluated [11451].

Dehydroepiandrosterone (DHEA) mediates its action via multiple signalling pathways involving specific membrane receptors and via transformation into androgen and oestrogen derivatives (e.g. androgens, oestrogens, 7alpha and 7beta DHEA, and 7alpha and 7beta epiandrosterone derivatives) acting through their specific receptors and is associated with ischaemic heart disease, endothelial dysfunction, atherosclerosis [12125].

It was studied blood serum levels of neurosteroids, dehydroepiandrosterone and its sulfate, in individuals with personality disorders convicted of serious violent crimes. The data were compared with that of a group of mentally and physically healthy persons convicted of acquisitive crimes, and with that of the control group. Significant increase in DHEA in both groups of convicts in comparison with the control was shown. The level of dehydroepiandrosterone sulfate remained unchanged. Increased dehydroepiandrosterone level in the convicted individuals with personality disorders is probably more associated with detention stress than directly with psychopathology or criminal aggression [12174].

**Physiology**
Dehydroepiandrosterone (DHEA) is secreted by the zona reticularis of the adrenal cortex and is converted into potent sex steroids in peripheral target cells. As oral DHEA administration can lead to dose-dependent increases in circulating androgens, which may reach high supraphysiologic levels in women, it has been included in the list of prohibited substances by the World Anti-Doping Agency. However, evidence for an ergogenic activity of DHEA is still largely nonexistent. Randomized trials in elderly subjects with an age-dependent decrease in DHEA have provided little or no evidence for enhanced physical performance after long-term administration of DHEA, 50 mg/d, and smaller short-term studies in healthy male athletes using higher doses were completely negative. Thus the widely perceived performance-enhancing activity of DHEA is still more myth than reality. However, because studies in female athletes are still lacking, an ergogenic activity of high-dose DHEA in this population cannot be excluded but is expected to be associated with adverse events like hirsutism, acne, and alopecia [10094].

Testosterone precursors are involved in the endogenous production of testosterone. DHEA is produced naturally in the adrenal glands and gonads. DHEA is converted to androstenedione or androstenediol in the steroid synthesis pathway. Androstenedione and androstenediol are converted to testosterone in the testes and any tissue cells that contain androgen or estrogen receptors. Adipose, bone, muscle, breast, prostate, liver, brain, and skin can be affected. The conversion of androstenedione and androstenediol to testosterone is regulated by the enzymes 17beta-hydroxysteroid dehydrogenase and 3beta-hydroxysteroid dehydrogenase, respectively. The increased circulating levels of androstenedione and testosterone can be aromatized to estrone and estradiol. Gonadal production of testosterone and estrogen is regulated by a negative-feedback system. The peripheral conversion of androgens and estrogens depends upon the quantity of circulating steroid precursors and not on any physiologic regulation system. These precursors bind poorly to androgen receptors and have few inherent androgenic-anabolic properties. The theory is that by increasing the circulating concentration of steroid precursors, the body’s endogenous production of testosterone increases and promotes anabolic effects in peripheral tissues. It has been found that low to moderate doses of these precursors do not increase testosterone levels significantly; however, at high doses, testosterone levels can be increased significantly. Therefore, the increased testosterone levels may build muscle mass, increase strength, and improve athletic performance [07009].

Metabolism

The synthesis and metabolism of DHEA involves a host of enzymes with different tissue distributions. The synthesis of DHEA requires the activity of the enzyme 20alpha, 22 desmolase (CYP11A1), a cholesterol side-chain cleaving enzyme, which converts cholesterol into pregnenolone. Loss of activity of this enzyme is incompatible with life. The other enzyme is 17alpha-hydroxylase/17, 20 lyase (CYP17), which converts pregnenolone to DHEA by first hydroxylating pregnenolone at the 17alpha position followed by cleavage of the C17, C20 side chain. Deficiency in this enzyme has serious health implications. DHEA is produced mainly by the adrenal cortex and to a limited extent by the testes as well as the ovaries, and is rapidly sulfated by sulfotransferases into DHEA-S. The latter is more stable with longer half-life and its levels remain stable throughout the day. Furthermore, DHEA-S levels are not altered significantly by the menstrual cycle. However, DHEA-S can be rapidly hydrolyzed back to DHEA by sulfatases, in response to metabolic demand. DHEA is also transformed via 3beta-hydroxysteroid dehydrogenases (3β-HSD) into androstenedione. The latter can be transformed by the 17beta-hydroxysteroid dehydrogenases (17β-HSD) into testosterone or via the aromatase into estrone. Alternatively, andione can be transformed into 5alpha-androstenedione (5α-adione) via the 5α-reductase enzyme system, and this
derivative may be transformed into 5alpha-dihydrotestosterone (5alpha-DHT). Testosterone may be converted into 5alpha-DHT via the 5alpha-reductase or into estradiol via the aromatase enzyme. Animals have lower systemic levels of DHEA, but the CNS concentration is approximately 600 times higher [11451].

**Molecular and cellular mechanism of DHEA**

The most controversial aspect of DHEA action in human physiology is that no specific nuclear receptor protein had been identified or characterized for DHEA. It is well recognized that glucocorticoids (e.g. cortisol), mineralocorticoids (e.g. aldosterone), androgens (e.g., testosterone and 5alpha-DHT), progestins (e.g. progesterone), and estrogens (e.g. estradiol) elicit their physiological functions via binding to specific nuclear receptors (genomic mechanism). Recent studies have documented that most steroid hormones, including androgens, estrogens, glucocorticoids, and progestins, also elicit physiological responses by nongenomic activation of distinct subpopulations of non-nuclear receptors. Inhibition of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (known as Akt) abolished the effect of DHEA on nuclear factor kB (NF-kB) activation, suggesting that DHEA mediated its function via activation of NF-kB via the PI3K/AKT pathway. It was postulated that DHEA-S directly activates recombinant PKC-beta in a cell-free assay. The authors suggested that PKC-beta acts as an intracellular receptor for DHEA-S in human neutrophils. In addition to its function via direct DHEA receptors, androgen and estrogen metabolites of DHEA also exert biological functions. It has been shown that growth of prostatic epithelial cells and prostate-specific antigen expression were induced by DHEA via stimulation of NF-kB DNA-binding activity, and these functions were blocked by an androgen receptor antagonist bicalutamide as well as androgen receptor small inhibitory RNAs. DHEA binding specifically and with high affinity to membrane receptors and eliciting a host of biological responses strongly supports the existence of a molecular and cellular mechanism of action for this steroid hormone. [11451].

**Effects of DHEA on body composition, bone metabolism, and skin**

The effect of DHEA on body composition and bone metabolism has been investigated in a number of clinical studies. DHEA treatment for 1 year in aging men resulted in significant reduction in body fat mass and increased muscle strength when compared with baseline and placebo. Furthermore, DHEA treatment resulted in weight loss in obese patients. Short-term studies in men and women using DHEA daily for 3-4 months, however, did not reveal any significant changes in body composition. However, 6 months of treatment with DHEA, in healthy, nonobese men aged 50-65, led to reduced fat mass by 6 percent. In the area of muscle function, knee extension/flexion strength, and lumbar back strength increased significantly by 15 percent and 14 percent, respectively. DHEA also resulted in significant decreases in abdominal visceral fat by 7 percent and abdominal subcutaneous fat by 6 percent as measured by magnetic resonance imagery (MRI) and significantly reduced the insulin area under the curve during an oral glucose tolerance test and significantly increased the insulin sensitivity index. DHEA reduced triglyceride and low density lipoprotein (LDL) levels and improved HDL levels but not total cholesterol levels in subjects aged 65-82 years. In elderly men with low baseline DHEA-S, treatment with DHEA significantly improved bone mineral density (BMD) for total body and lumbar spine after 6 months, and DHEA improved BMD in patients with osteoporosis. The protective effect of DHEA on the bone is partly mediated by conversion of DHEA to androgens and estrogens in human osteoblasts. DHEA appears to also have positive effects on skin structure and function. DHEA improves skin hydration and brightness, increases sebum production, and possibly prevents wrinkles. Several studies have attempted to provide insight into the molecular bases for such observations and have demonstrated that topical DHEA increases pro-collagen production in
postmenopausal women as well as both young and elderly men. DHEA has also been reported to attenuate, via conversion to estrogen and estrogen receptors, age-related impairment to wound healing [11451].

**Role of DHEA in sexual function**

The Massachusetts Male Aging Study presented an inverse correlation of the serum levels of DHEA-S and the incidence of erectile dysfunction (ED). Serum levels of DHEA-S in patients with ED were lower than in healthy volunteers under 60 years of age. Furthermore, DHEA treatment in men was associated with higher mean scores for all five domains of the International Index of Erectile Function. It has been demonstrated an association between DHEA-S and sexual dysfunction in 348 men. DHEA treatment improved sexual interest with improvement in desire, arousal, activity, interest, fantasy and drive, and relationship. In patients with type 1 diabetes with ED, DHEA showed the most pronounced improvement of all hormonal parameters analyzed. All these effects were observed after 16 weeks of treatment but not after 8 weeks. The role of DHEA in sexual function in women has been explored in several studies. It has been shown replacement therapy with DHEA in women with androgen insufficiency, and sexual dysfunction showed a significant decrease in sexual distress and a significant increase in desire, arousal, lubrication, satisfaction, and orgasm. It has also been shown that DHEA significantly improved desire, arousal, activity, interest, fantasy and drive, and relationship. Low DHEA-S levels but not testosterone levels correlated with sexual dysfunction symptoms. Although DHEA has improved sexual function in men and women to varying degrees, perhaps in certain subpopulations, this improvement may be related to the levels of baseline DHEA-S in the individuals recruited for the studies. In addition, most domains of sexual function are impacted by the levels of circulating testosterone. Thus, testosterone deficiency, albeit derived from reduced testosterone biosynthesis or via reduced DHEA conversion to testosterone, may contribute to sexual dysfunction. Thus, baseline testosterone should be considered together with baseline DHEA when investigating decline of DHEA and sexual function. The discrepancies between the aforementioned studies deserve further investigation. The effects of DHEA in the CNS are at least mediated by its direct modulation of central receptors like N-methyl D-aspartate receptors, sigma1, and GABA_A as well as by stimulation of substances like allopregnanolone and beta-endorphin, which are crucial for behavior and mood. The action of DHEA may be responsible for the beneficial clinical results seen in patients with different forms of depression, stress, and psychiatric disorders. In addition, the observed DHEA effects on neurogenesis and neuronal survival have important implications for future therapy. DHEA levels in men and women between 18 and 40 years of age ranges from 1.330 to 7.780 ng/mL with median concentrations of 2-3 ng/mL, respectively. Thus, the levels of free DHEA are approximately 100-fold higher than free testosterone. Because DHEA is rapidly converted to DHEA-S, the levels of the latter are thought to be more physiologically relevant. DHEA-S levels are approximately three orders of magnitude higher than total testosterone (3-5 µg/mL vs 3-5 ng/mL) and approximately five orders of magnitude higher than free T (3-5 µg/mL vs 20 pg/mL), which strongly argue against the notion that DHEA is a biologically irrelevant steroid hormone. More specifically, age-related decline of DHEA-S by 80-90 percent is more pronounced than the decline in testosterone. There is a lack of large, prospective, long-term trials with DHEA to establish evidence-based medicine. In the current environment where DHEA is regarded as a nutritional supplement, there is no financial incentive to invest in long-term large clinical studies. The latter explains why the majority of the clinical studies on DHEA were carried out by academic institutions with smaller numbers of patients and short-time durations. Such studies may be viewed as pilot studies. Conflicting results or discrepancies in data obtained in the various reported studies may be attributed to poor study designs and confounding factors. These include
- dose of DHEA and duration of the study
- clinical trials versus pharmacological intervention
- healthy adults versus patients with chronic illnesses
- young versus older patients
- normal versus patients with adrenal insufficiency
- use of other medications

Epidemiological, observational, or intervention studies differ significantly in design and end points. In addition, differences in dosages, duration of treatment, formulations, route of administration, study population, sample size, statistical power, and clinical end points as well as adjustments (e.g. for smoking, alcohol consumption, and body weight) have an important influence on the outcome. DHEA administration in men is not yet an established medical treatment. In addition, in most countries, there are no preparations available that have been approved by the regulatory agencies. In the United States, DHEA is categorized as nutritional supplement with little quality control, with varying preparations having different DHEA concentrations and are available over the counter. For these reasons, caution should be exercised in treating with DHEA and physicians should educate their patients before recommending DHEA as a supplement. Progress in this area is likely to be minimal until a pharmaceutical-grade oral preparation is established. Pharmacokinetics of absorption and its variability among people has to be determined. Large, long-term prospective studies will have to be commenced, which will have to identify beneficial clinical effects due to DHEA itself and those due to its conversion products, estradiol, testosterone, and 7 hydroxy derivatives. There are enough preliminary clinical and preclinical data to warrant pursuing further clinical studies [11451].

Normal values related to age

Dehydroepiandrosterone (DHEA) is a “pro-hormone” or precursor of testosterone that is available as an over-the-counter supplement in many countries. It is promoted as a wonder supplement capable of promoting youthfulness, virility and enhanced strength or body composition. In men aged 20-70 years, ingesting a single dose of 50-100 mg DHEA increases serum DHEA concentrations up to sevenfold and increases serum androstenedione concentrations approximately fourfold, but serum testosterone and dihydrotestosterone (DHT) concentrations are not changed. In older men (50-70 years), ingesting a single dose of 50-100 mg DHEA raises serum estradiol concentrations by 24-39 percent to the upper normal range. Prolonged ingestion of DHEA in doses ranging from 50 to 1600 mg/day in 20-65-year-old men produces dose-dependent increases in serum DHEA, DHEA sulphate and androstenedione, but has no effect on serum testosterone or DHT. Thus, ingestion of DHEA in men increases only weak precursor hormones, with little or no increase in more potent androgens or estrogens. More chronic intake of DHEA in doses of 50-1600 mg/day in men does not alter energy or protein metabolism, body mass or lean body mass. Intake of DHEA during resistance training does not augment the gains in lean mass or muscle strength in male college students or middle-aged men. It appears that DHEA does not promote fat loss or muscle gain or augment adaptations to resistance training in healthy men. Surprisingly, DHEA is classified as an androgenic/anabolic steroid and banned by the World Anti-Doping Agency and other anti-doping codes [10347].

Influence of menstrual cycle

Mood changes occur often in the luteal phase of menstrual cycle. Steroids modulating GABAA and NAMD receptors in the brain, namely allopregnanolone, were suggested as a
factor of premenstrual syndrome. Another neurosteroid influencing the well-being is dehydroepiandrosterone. In the past decade it was shown by several authors that some dehydroepiandrosterone derivatives, especially those with 7-hydroxy- or 7-oxo group, exert a higher activity than dehydroepiandrosterone itself. It was also reasonable to see whether the levels of circulating 7-hydroxy-derivatives of dehydroepiandrosterone differ in the follicular and luteal phase of the menstrual cycle. Steroids known to exert neuroprotective effects, namely 7alpha- and 7beta-hydroxy-dehydroepiandrosterone, 5-androstene-3beta,7alpha, 17beta-triol and 5-androstene-3beta,7beta,17beta-triol, were determined in midfollicular and midluteal phase of the menstrual cycle of 22 healthy women with a regular menstruation cycle. Whereas the maternal steroids, dehydroepiandrosterone and androstene-3beta,17beta-diol showed no significant difference between the phases of menstrual cycle, the levels of their 7-hydroxylated metabolites were significantly lower in the luteal phase. It is suggested that the observed decrease of 7-hydroxylated metabolites during the luteal phase may be a factor related to the etiopathogenesis of mood change and neurocognitive disturbances, which are known to be more accented in that particular phase of the menstrual cycle [11332].

In women

One study was intended to investigate various hormones during competition among female athletes. Subjects were elite female soccer players (n=9). Blood samples and profiles of mood states (POMS) were obtained before (Pre), during 3 days of competition (Competition), and after competition (Post-3 days). Serum concentrations were determined for dehydroepiandrosterone sulfate (DHEAS), cortisol (C), prolactin (PRL), testosterone (T), adrenocorticotropic hormone (ACTH), luteinising hormone (LH), and follicle stimulating hormone (FSH). Levels of C and PRL increased significantly during competition compared with Pre. Levels of LH increased significantly during competition (3rd day) compared with Pre. Levels of DHEAS changed significantly during competition, whereas levels of T, ACTH, and FSH were unchanged during competition. The fatigue score of POMS increased significantly during competition (2nd day) compared with Pre. It was thus demonstrated that competitive stress affected hormonal status in female athletes. These findings suggest that hormonal changes reflect physical and mental stress during competition [06118].

Elderly women

Studies disputed the widely promoted anti-aging effect of dehydroepiandrosterone (DHEA) supplementation; however, conflicting data exist on whether physiological DHEA supplementation enhances exercise training effects on body composition, physical performance, and cardiometabolic risk in healthy postmenopausal women. The aim of one study was to determine whether 12 weeks of DHEA supplementation (50 mg/d) in postmenopausal women enhances exercise-related changes in body composition, physical performance, and cardiometabolic risk. Thirty-one sedentary, postmenopausal, Caucasian women (mean 64 years) completed the study. Participants were randomized to one of two 12-week interventions: exercise training plus 50 mg/d of DHEA (n=17), or exercise training plus placebo (n=14). The exercise intervention consisted of both endurance (4 d/wk) and resistance (3 d/wk) exercise components. The main outcomes were measures of body composition, physical performance, and measures of cardiometabolic risk. DHEA treatment with exercise resulted in increases in circulating sulfated DHEA (650 %), total testosterone (100 %), estradiol (165 %), estrone (85 %), and IGF-I (30 %). Although exercise training alone significantly improved physical performance, body composition, and insulin sensitivity, administration of DHEA provided no additional benefits. It was concluded that 12 weeks of combined endurance and resistance training significantly improved body composition,
physical performance, insulin sensitivity, and low-density lipoprotein cholesterol particle number and size, whereas DHEA had no additional benefits [07093].

**Efficacy in performance enhancement**

Athletes use testosterone precursors with the belief that they will boost testosterone levels and, thereby, achieve the same anabolic effects of AASs. They are promoted to enhance performance by reducing fat, building muscle mass, increasing strength, and improving sexual performance. Studies of the efficacy of these testosterone precursors have not demonstrated any significant improvements in enhancing performance. The “Andro Project” in 2000 studied the effects of healthy men, 35 to 65 years of age, taking androstenedione or androstenediol, 200 mg daily, in the setting of a 12-week high-intensity resistance-training program. Total testosterone levels increased by 16 percent after 1 month, but had returned to pretreatment levels by the end of the 12 weeks. The study also found that estrone and estradiol levels remained elevated significantly (up to 97%). Neither androstenedione nor androstenediol significantly improved lean body mass or increased muscle strength compared with placebo. They also found that there was an adverse effect on high-density lipoprotein (HDL) cholesterol and an increase in the coronary heart disease risk. It has also been studied the effects of daily oral supplementation with androstenedione, 300 mg, or DHEA, 150 mg, in healthy men 19 to 29 years of age with 8 weeks of resistance training. They found that serum androstenedione levels were elevated, but no significant increases in testosterone levels occurred, and it did not significantly enhance the adaptations to resistance training with changes in body composition or strength. They also examined the effects of a single dose of DHEA, 50 mg, and found a 150 percent increase in androstenedione levels within 60 minutes but no increases in testosterone levels. A 2002 review of studies found that most studies showed

- acute oral ingestion of androstenedione or androstenediol, at least 200 mg, modestly and transiently increased serum testosterone levels in men
- elevation of circulating estrogen levels
- dosages of androstenedione or androstenediol, less than 300 mg/day for as long as 12 weeks, had no effects on body composition, muscle mass, or performance
- significantly decreased serum HDL cholesterol levels and increases in the cardiovascular disease risk

A summary of research from 1999 in 2002, showed a consensus in that no significant increases in muscle mass or strength was found with a single dose or daily ingestion. High dosages of androstenedione, 200 to 300 mg/day, significantly increased serum testosterone levels in young healthy men by 34 percent and increased estradiol levels by 128 percent. The increases in testosterone levels were temporary, and daily doses up to 12 weeks showed significant increases in estradiol and estrone levels but did not show any significant increases in baseline testosterone levels. Other studies also showed no increase in muscle protein synthesis with supplementation with DHEA or androstenedione and no increase in muscle fiber cross-sectional area with a combination of androstenedione ingestion and resistance training compared with placebo. Studies also showed that androstenedione supplementation in men increased serum androstenedione levels; a low daily dose (100 mg) did not increase serum testosterone levels significantly, but a high daily dose (300 mg) increased serum testosterone levels and serum estrogen levels. Investigations on the efficacy of DHEA, with dosages up to 150 mg/d, revealed neither change in testosterone levels, nor significant changes in lean muscle mass or strength compared with placebo. Several reasons have been suggested for the lack of efficacy of androstenedione on improving muscle mass and strength. First, oral supplementation must undergo first-pass
hepatic metabolism before it reaches the circulation. Second, only 2 percent of an oral dose is converted to testosterone; a large percentage is converted to testosterone glucuronide, which is biologically inactive. Third, the peripheral aromatization of testosterone to estradiol may limit the available level of testosterone to bind and activate androgen receptors. In summary, the testosterone precursors DHEA, androstenedione, and androstenediol have little inherent androgen action. The ergogenic claims regarding prohormone supplementation have not been supported by controlled studies. Studies have demonstrated repeatedly that acute and long-term administration of oral androstenedione, androstenediol, or DHEA does not effectively increase serum testosterone levels and fails to produce any significant changes in lean body mass, muscle strength, or performance improvement compared with placebo. High doses of androstenedione seem to increase serum levels of estrogens, primarily through peripheral aromatization [07009].

The purpose of one study was to examine salivary cortisol, dehydroepiandrosterone (DHEA), and testosterone responses to the bench press in an international powerlifting competition and to determine whether these salivary hormone concentrations could be used to predict performance. Twenty-six elite athletes (13 females and 13 males) provided saliva samples during the official weighing-in and after the last attempt at the bench press, as well as at baseline on a non-competition day. Performance index was determined with the Wilks formula, which adjusts powerlifting scores according to body mass. Salivary cortisol concentrations were significantly increased in all subjects after the bench press, whereas DHEA concentrations were significantly increased in women but not in men after the bench press. No significant change in testosterone concentrations was observed during the experiment in either men or women, which resulted in a marked decrease in the testosterone/cortisol ratio. The performance index showed no significant correlation with any of the hormone responses to competition. In conclusion, despite the increase in stress adrenocortical hormone responses to an international powerlifting competition, these hormone concentrations alone are not predictors of bench press performance in elite powerlifting athletes [10462].

Effects of walking training

It was hypothesized that physical exercise in postmenopausal women could interfere with the molecular interrelationship of the immune-endocrine system and be effective even in women in whom training determined a reduction of spontaneous physical activity (SPA). For this reason, we investigated the effects of an aerobic program on plasma dehydroepiandrosterone sulfate (DHEA-S) and cytokine levels in relationship to SPA modification. Thirty-two postmenopausal women (mean age, 56 years) were enrolled in the study. Inclusion criteria were as follows: age younger than 65 years, body mass index higher than 18.5 and lower than 35 kg/m, no pharmacological treatments, and no history of chronic, cardiovascular, or orthopedic diseases. Before and after 3 months of walking training at moderate intensity (40-50 min, 4 d/wk), they were evaluated for SPA, body composition, energy intake, and levels of plasma cytokines (tumor necrosis factor alpha [TNF-alpha], interleukin [IL]-1alpha, IL-1beta, IL-2, IL-8, and IL-10), C-reactive protein, DHEA-S, cortisol, and estrogen. At baseline, SPA did not correlate with either DHEA-S level or cytokine levels. There was negative correlation between DHEA-S and both TNF-alpha and IL-2. After the intervention program, 16 women showed increased SPA, and 16 women showed decreased SPA. Independent of these changes in SPA, both TNF-alpha levels and cortisol-to-DHEA-S ratio decreased, whereas DHEA-S levels increased. In postmenopausal women, walking training, rather than SPA, influences DHEA-S and cytokine concentrations and their correlations, thus interfering with adrenal steroids and the inflammatory markers network. Physical exercise acts in parallel on menopausal neuroendocrine alterations and on the systemic inflammatory profile independent of SPA changes [12175].
Effects on asthma

Dehydroepiandrosterone (DHEA) is considered as an important immunomodulating and anti-inflammatory hormone. Despite the continuing interest in DHEA replacement therapy, our knowledge of its effects upon asthma is very limited. DHEA is able to reverse cytokine imbalances associated with asthma, may prevent and attenuate allergic inflammation in airways, and does not possess the undesirable side effects of glucocorticoids; therefore, it may be potentially applied in the treatment of asthma. The steroid-sparing effect observed with DHEA clinically could appear especially favorable in asthmatic patients receiving oral treatment and those inhaling high doses of glucocorticoids. In addition, DHEA and its analogs might prove useful in reversing relative glucocorticoids insensitivity in patients with corticosteroid-resistant asthma [10460].

Adverse effects

The adverse effects of oral supplementation with testosterone precursors are not well known. The lack of long-term studies makes it difficult to identify the dangers and risks of using these nutritional supplements. Adverse effects are most likely underreported because it is difficult to determine the prevalence of prohormone use and the disparity in product labeling of nutritional supplements. DHEA was reported to cause irreversible virilization in women (hair loss, hirsutism, deepened voice) and gynecomastia in men. Androstenedione use was found to have an adverse effect on coronary heart disease risk and to cause a significant reduction in serum HDL cholesterol. Long-term increased levels of unopposed circulating estrogens potentially could induce hormone-sensitive malignancies, such as uterine, breast, and prostate cancer. Theoretically, high doses of prohormones could increase androgen levels and have the same adverse as AAS use, such as liver dysfunction, glucose intolerance and diabetes, malignancies, menstrual irregularities, infertility, testicular atrophy, impotence, male pattern baldness, acne, or aggressive behavior [07009].

DHEA metabolites activate estrogen receptors

Dehydroepiandrosterone (DHEA) levels were reported to associate with increased breast cancer risk in postmenopausal women, but some carcinogen-induced rat mammary tumor studies question this claim. The purpose of this study was to determine how DHEA and its metabolites affect estrogen receptors alpha or beta (ERalpha or ERbeta)-regulated gene transcription and cell proliferation. In transiently transfected HEK-293 cells, androstenediol, DHEA, and DHEA-S activated ERalpha. In ERbeta transfected HepG2 cells, androstenedione, DHEA, androstenediol, and 7-oxo DHEA stimulated reporter activity. ER antagonists ICI 182,780 (fulvestrant) and 4-hydroxytamoxifen, general P450 inhibitor miconazole, and aromatase inhibitor exemestane inhibited activation by DHEA or metabolites in transfected cells. ERbeta-selective antagonist R,R-THC (R,R-cis-diethyl tetrahydrochrysene) inhibited DHEA and DHEA metabolite transcriptional activity in ERβ-transfected cells. Expression of endogenous estrogen-regulated genes: pS2, progesterone receptor, cathepsin D1, and nuclear respiratory factor-1 was increased by DHEA and its metabolites in an ER-subtype, gene, and cell-specific manner. DHEA metabolites, but not DHEA, competed with 17beta-estradiol for ERα and ERβ binding and stimulated MCF-7 cell proliferation, demonstrating that DHEA metabolites interact directly with ERalpha and ERbeta in vitro, modulating estrogen target genes in vivo [12176].
DHEA in vascular disease

Dehydroepiandrosterone (DHEA) is a steroid hormone derived from cholesterol synthesized by the adrenal glands. DHEA and its 3beta-sulphate ester (DHEA-S) are the most abundant circulating steroid hormones. In human, there is a clear age-related decline in serum DHEA and DHEA-S and this has suggested that a relative deficiency in these steroids may be causally related to the development of a series of diseases associated with aging including cardiovascular diseases (CVD). This commentary aims to highlight the action of DHEA in CVD and its beneficial effect in therapy. We thus discuss the possible impact of serum DHEA decline and DHEA supplementation in diseases such as hypertension, coronary artery disease and atherosclerosis. More specifically, we provide evidence for a beneficial action of DHEA in the main disease of the pulmonary circulation: pulmonary hypertension. We also examine the potential cellular mechanism of action of DHEA in terms of receptors (membrane/nuclear) and associated signaling pathways (ion channels, calcium signaling, PI3K/AKT/eNos pathway, cGMP, RhoA/RhoK pathway). It was shown that DHEA acts as an anti-remodeling and vasorelaxant drug. Since it is a well-tolerated and inexpensive drug, DHEA may prove to be a valuable molecule in CVD but it deserves further studies both at the molecular level and in large clinical trials [12177].

Androst-5-ene-3beta,7 beta,17beta-triol (betaAET) is an anti-inflammatory metabolite of DHEA that is found naturally in humans, but in rodents only after exogenous DHEA administration. Unlike DHEA, C-7-oxidized DHEA metabolites cannot be metabolized into potent androgens or estrogens, and are not peroxisome proliferators in rodents. The objective of our current studies was to characterize the pharmacology of betaAET to enable clinical trials in humans. The pharmacology of betaAET was characterized by pharmacokinetics, drug metabolism, nuclear hormone receptor interactions, androgenicity, estrogenicity, and systemic toxicity studies. betaAET’s acute anti-inflammatory activity and immune modulating characteristics were measured in vitro in RAW264.7 cells and in vivo in murine models with parenteral administration. betaAET was rapidly metabolized and cleared from circulation in mice and monkeys. betaAET was weakly androgenic and estrogenic in immature rodents, but not bound by androgen, estrogen, progesterone, or glucocorticoid nuclear hormone receptors. betaAET did not induce peroxisome proliferation, nor was it systemically toxic or trophic for sex hormone responsive tissues in mature rats and monkeys. betaAET significantly attenuated acute inflammation both in vitro and in vivo, augmented immune responses in adult mice, and reversed immune senescence in aged mice. betaAET may contribute to the anti-inflammatory activity in rodents attributed to DHEA. Unlike DHEA, betaAET’s anti-inflammatory activity cannot be ascribed to activation of PPARs, androgen, or estrogen nuclear hormone receptors. Exogenous betaAET is unlikely to produce untoward toxicity or hormonal perturbations in humans [11333].

Laboratory techniques

It was recently taken advantage of the unique power of DNA microarrays to compare the genomic expression profile of tetrahydrogestrinone (THG) with that of dihydrotestosterone (DHT), the most potent natural androgen, thus clearly demonstrating that THG is an anabolic steroid. In 2004, the U.S. Controlled Substances Act has been modified to include androstenedione (4-dione) as an anabolic steroid. However, despite the common knowledge that dehydroepiandrosterone (DHEA) is the precursor of testosterone, DHEA has been
excluded from the list of anabolic steroids. It was thus used the same DNA microarray technology to analyze the expression profile of practically all the 30,000 genes of the mouse genome modulated by DHEA and DHT in classical androgen-sensitive tissues. Daily subcutaneous injections of DHT (0.1 mg) or DHEA (3 mg) for 1 month in gonadectomized C57BL6/129 SV mice increased ventral prostate, dorsal prostate, seminal vesicle and preputial gland weight (p<0.01 for all tissues). As early as 24h after single injection of the two steroids, 878, 2681 and 14 probe sets were commonly stimulated or inhibited in the prostate (ventral+dorsal), seminal vesicles and preputial glands, respectively, compared to tissues from gonadectomized control animals. After 7 days of daily treatment with DHEA and DHT, 629, 919 and 562 probe sets were commonly modulated in the same tissues while after 27 days of treatment, 1195, 5127 and 2883 probe sets were modulated, respectively. In analogy with the data obtained with THG, the present microarray data provide an extremely precise and unquestionable genomic signature and proof of the androgenic/anabolic activity of DHEA. Such data add to the literature showing that DHEA is transformed into androgens in the human peripheral tissues as well as in laboratory animal species, including the monkey, thus exerting potent androgenic/anabolic activity. The present microarray approach to identify anabolic compounds is applicable to all potential androgenic/anabolic compounds [06119].

According to the WADA rules urine samples containing dehydroepiandrosterone (DHEA) concentrations greater than 100 ng/mL shall be submitted to isotope ratio mass spectrometry (IRMS) analysis. The threshold concentration is based on the equivalent to the glucuronide, and the DHEA concentrations have to be adjusted for a specific gravity value of 1.020. In 2006, 11,012 doping control urine samples from national and international federations were analyzed in the Cologne doping control laboratory, 100 (0.9 %) of them yielding concentrations of DHEA greater than 100 ng/mL. Sixty-eight percent of the specimens showed specific gravity values higher than 1.020, 52 percent originated from soccer players, 95 percent were taken in competition, 85 percent were male urines, 99 percent of the IRMS results did not indicate an application of testosterone or related prohormones. Statistical evaluation showed significantly different DHEA concentrations between specimens taken in- and out-of-competition, whereas females showed smaller DHEA values than males for both types of control. Also a strong influence of the DHEA excretion on different sport disciplines was detectable. The highest DHEA values were detected for game sports (soccer, basketball, handball, ice hockey), followed by boxing and wrestling. In 2007, 6622 doping control urine samples were analyzed for 3alpha,5-cyclo-5alpha-androstan-6beta-ol-17-one (3alpha,5-cyclo), a DHEA metabolite which was described as a useful gas chromatography-mass spectrometry (GC-MS) screening marker for DHEA abuse. Nineteen urine specimens showed concentrations higher than the suggested threshold of 140 ng/mL, six urine samples yielded additionally DHEA concentrations higher than 100 ng/mL, none of them showing positive IRMS findings. These results should be taken into consideration in future discussions about threshold values for endogenous steroids in doping control [08156].

According to World Anti-Doping Agency (WADA) rules (WADA Technical Document-TD2004EAAS) urine samples containing dehydroepiandrosterone (DHEA) concentrations greater than 100 ng/mL shall be submitted to isotope ratio mass spectrometry (IRMS) analysis. The threshold concentration is based on the equivalent to the glucuronide, and the DHEA concentrations have to be adjusted for a specific gravity value of 1.020. In 2006, 11,012 doping control urine samples from national and international federations were analyzed in the Cologne doping control laboratory, 100 (0.9 %) of them yielding concentrations of DHEA greater than 100 ng/mL. Sixty-eight percent of the specimens showed specific gravity values higher than 1.020, 52 percent originated from soccer players, 95 percent were taken in competition, 85 percent were male urines, 99 percent of the IRMS results did not indicate an application of testosterone or related prohormones. Only one urine
sample was reported as an adverse analytical finding having 319 ng/mL DHEA (screening result), more than 10,000 ng/mL androsterone and depleted carbon isotope ratio values for the testosterone metabolites androsterone and etiocholanolone. Statistical evaluation showed significantly different DHEA concentrations between specimens taken in- and out-of-competition, whereas females showed smaller DHEA values than males for both types of control. Also a strong influence of the DHEA excretion on different sport disciplines was detectable. The highest DHEA values were detected for game sports (soccer, basketball, handball, ice hockey), followed by boxing and wrestling. In 2007, 6622 doping control urine samples were analyzed for 3alpha,5-cyclo-5alpha-androstan-6beta-ol-17-one (3alpha,5-cyclo), a DHEA metabolite which was described as a useful gas chromatography-mass spectrometry (GC-MS) screening marker for DHEA abuse. Nineteen urine specimens showed concentrations higher than the suggested threshold of 140 ng/mL, six urine samples yielded additionally DHEA concentrations higher than 100 ng/mL, none of them showing positive IRMS findings. These results should be taken into consideration in future discussions about threshold values for endogenous steroids in doping control [07094].

**Current medical recommendations**

Current evidence shows that the testosterone precursors DHEA, androstenedione, and androstenediol do not offer any substantial performance-enhancing benefits. The potential adverse effects of these substances produce a significant health risk, especially to the pediatric and adolescent population. At higher doses, they were shown to minimally and temporarily elevate testosterone levels and to increase estrogen levels. No significant health or medical benefits exist, except for the possible antiaging properties of DHEA. As a result of the increasing evidence of the potential harmful health effects of these prohormones, the FDA has placed androstenedione on the schedule III controlled substance list and has not approved androstenedione or DHEA for any indication [07009].
BOLDENONE, BOLDIONE AND BOLANDIOL

Overviews

Boldenone, which was synthesized with the aim of producing a long-acting injectable methandrostenolone, is meanwhile one of the most frequently detected AAS, generally exposed via GC/MS, that allows identification of the active drug and its main metabolite, 5beta-androst-1-en-17beta-ol-3-one. Boldenone and epiboldenone sulphates, which are markers for the exogenous origin of boldenone, may be employed to decrease the number of samples to be analyzed by isotope ratio mass spectrometry. Boldenone and epiboldenone sulphates, which are markers for the exogenous origin of boldenone, may be employed to decrease the number of samples to be analyzed by isotope ratio mass spectrometry [12011].

Boldenone is an androgenic anabolic steroid intensively used for growth promoting purposes in animals destined for meat production and as a performance enhancer in athletics. Therefore its use is officially banned either in animals intended for consumption or in humans. Because most anabolic steroids are completely metabolized and usually no parent steroid is excreted, metabolite identification is crucial to detect the illegal use of anabolic steroids either in humans or in livestock. 17alpha- and 17beta-boldenone 17-glucuronides were synthesized, purified and characterized in order to provide suitable standards for the identification and quantification of these metabolites [08163].

Boldenone (BOL) is an androgenic steroid that improves the growth and food conversion in food-producing animals. In most countries worldwide, this anabolic steroid is forbidden for human uses and meat production as it was developed for veterinary use. Recently, BOL is used by bodybuilders in both off season and pre-contest, where it is well known for increasing vascularity while preparing for a bodybuilding contest. One study was designed to investigate the physiological and biochemical changes in rabbits after injection with the growth promoter BOL. A total of 32 adult New Zealand rabbits were divided into four groups, where the control group includes animals that were injected intramuscularly with olive oil and dissected after 3 weeks. The remaining three experimental groups included animals that received one, two and three intramuscular injections of 5 mg/kg body weight BOL, respectively, and were dissected after 3, 6 and 9 weeks, respectively. The animals from practice appeared healthy and did not show clinical signs of disease and none of the rabbits died during the experimental period. Serum total protein, globulin, alanine aminotransferase, asparate aminotransferase, urea, creatinine, testosterone, luteinizing hormone and follicle-stimulating hormone levels were significantly increased while serum direct bilirubin, albumin and albumin/globulin ratio were significantly decreased after one, two and three intramuscular injections of BOL as compared to their relative values in the control group. These findings explain the common phenomena in athletes and bodybuilders who suffer from infertility, renal and hepatic alterations following injection with some drugs as steroids (BOL) to build muscles [13245].

Aiming at the facilitated differentiation of endogenous boldenone production from exogenous (and thus illicit) administration, potential markers were desirable and subject of a recent application and metabolism study. In urine specimens of a volunteer who ingested 20 mg of boldenone, the sulfates of boldenone and epiboldenone were detected and their structures confirmed by isolation, solvolysis, and subsequent comparison to reference material of the unconjugated compounds. In contrast to the presence of these analytes in post-administration urine samples, three out of four routine doping control specimens containing boldenone and its phase-I-metabolite 5beta-androst-1-en-17beta-ol-3-one of natural
(endogenous) origin (as demonstrated by isotope-ratio mass spectrometry) did not contain the sulfoconjugates of boldenone and its epimer. These two analytes might therefore support the distinction of boldenone application and its endogenous production [13012].

Since administration studies, such-like those with boldenone are not possible with non-approved/designer steroids, metabolism studies with promagnon, methylclostebol, and methasterone were conducted with a chimeric mouse model transplanted with human hepatocytes. As demonstrated earlier, the humanized liver enables a proxy human metabolism to a certain extent, permitting the investigation of the metabolic fate and renal elimination of these compounds by means of GC-MS. While the elimination study with promagnon yielded mainly one equivocal metabolic product (methylclostebol), methylclostebol as the administered compound was found to generate a variety of singly and doubly hydroxylated and/or reduced products with promagnon (4-chloro-17alpha-methyl-androst-4-ene-3beta,17beta-diol) as metabolite of methylclostebol being an adequate target for doping controls. In addition, the degradation and elimination of methasterone in the chimeric mouse model was studied. The comparison of human and mouse post-administration urine samples however revealed rather limited similarities and it was suggested that the chimeric mice utilized different metabolic pathways. Nevertheless, two compounds assigned to 2alpha,17alpha-dimethyl-5alpha-androstane-2beta,3alpha,16,17beta-tetrol and x,16-dihydroxy-methasterone (with x being a non-identified position) were observed and proposed as additional putative human urinary metabolites. As in most of the aforementioned studies, the authors stressed that all structural assignments were not supported yet by chemical synthesis and that further work was necessary to confirm the tentatively postulated compositions [13012].

**Boldenone potency compared with other anabolic steroids**

Bolandiol is a synthetic anabolic steroid that increases lean body mass and bone mineral density without significant stimulation of sex accessory glands in castrate adult male rats. Since bolandiol suppresses gonadotropins and endogenous testosterone production, it was investigated its mechanism of action. It was compared the potency of bolandiol in vitro and in vivo with T, 5alpha-dihydrotestosterone (DHT), 19-nortestosterone (19-NT) and estradiol (E$_2$). Bolandiol bound with lower affinity to the recombinant rat androgen receptor (AR) than the other androgens and had low, but measurable, affinity for recombinant human progesterin receptors (PR-A, PR-B), and estrogen receptors (ERAlpha and beta-1). Functional agonist activity was assessed in transcription assays mediated by AR, PR, or ER. Bolandiol was stimulatory in all these assays, but only 4-9 percent as potent as T, DHT, and 19-NT via AR, 1 percent as potent as progesterone via PR, and 3 percent and 1 percent as potent as E$_2$ acting through ERAlpha or ERbeta, respectively. In immature castrate rats, bolandiol was equipotent to testosterone in stimulating growth of the levator ani muscle but less potent than T in stimulating growth of the sex accessory glands. Bolandiol also stimulated uterine weight increases in immature female rats, which were partly blocked by ICI 182,780, but it was not aromatized in vitro by recombinant human aromatase. In contrast to testosterone, stimulation of sex accessory gland weights by bolandiol was not inhibited by concomitant treatment with the dual 5alpha-reductase inhibitor dutasteride. As bolandiol exhibits tissue selectivity in vivo, it may act via AR, PR, and/or ER, utilize alternative signaling pathway(s) or transcriptional coregulators, and/or be metabolized to a more potent selective steroid [09130].

**Effect on reproductive functions**
One study was conducted to evaluate the adverse effects of the anabolic steroid, boldenone undecylenate (BOL) on reproductive functions of male rabbits. Thirty white New Zealand mature male rabbits were divided into three groups (10 rabbits each). Group A rabbits served as a control group. Group B rabbits received 4.4 mg/kg body weight (bwt) BOL 5 percent oily solution. Group C rabbits received 8.8 mg/kg bwt BOL. Rabbits were injected intramuscularly twice weekly for two months. BOL had no significant effect on the bwt and bwt gain. Testes and epididymis weights were decreased significantly in the BOL-treated groups. BOL caused significant reduction in serum testosterone level, seminal volume, sperm motility, and sperm count. No abnormalities were detected in the sperm morphology of the BOL-treated groups. Histopathological alterations in the testes and epididymis were marked in the group C rabbits. These results indicate that administration of BOL exerts a significant harmful effect on the reproductive functions of male rabbits [12165].

Boldenone (androgenic steroid) is used in improvement of the growth and food conversion in food-producing animals. In addition, it is used by bodybuilders during both off-season and precontest, where it is well known for increasing vascularity while preparing for a bodybuilding contest. One study was designed to investigate the possible effect of growth promoter boldenone undecylenate on the structure and functions of rabbit testes. A total of 32 adult New Zealand rabbits were divided into 4 groups. The first group in the control group includes animals that were intramuscularly injected with olive oil and dissected after 3 weeks. Three experimental groups include animals that receive 1, 2, and 3 intramuscular injections of 5 mg/kg body weight boldenone, and dissected after 3, 6, and 9 weeks, respectively. Treating rabbits with boldenone increased the testosterone levels compared to the control group. Seminiferous tubules of the rabbit testis treated with boldenone showed reduced development and degeneration of the germinal epithelium, leading to debris and syncytial cell formation in the lumina of seminiferous tubules. The immunohistochemical results indicated severe reduction in proliferating cell nuclear antigen-positive spermatogonia in boldenone-treated animals as compared to the control group. These findings explain the common phenomena among athletics and bodybuilders who suffer from infertility as they were injected with some drugs such as steroids (boldenone) to build muscles [12166].

Possible endogenous origin

Boldenone (17-hydroxy-androsta-1,4-diene-3-one) and boldione (androst-1,4-diene-3,17-dione), are currently listed as exogenous anabolic steroids by the World Anti-Doping Agency. However, it has been reported that these analytes can be produced endogenously. Interestingly, only for boldenone a comment is included in the list on its potential endogenous origin. In this study, the endogenous origin of boldione in human urine was investigated, and the potential influence of phytosterol consumption was evaluated. It was carried out a 5-week in vivo trial with both men (n=6) and women (n=6) and measured alpha-boldenone, beta-boldenone, boldione, androstenedione, beta-testosterone and alpha-testosterone in their urine using gas chromatography coupled to multiple mass spectrometry (GC-MS-MS). The results demonstrate that endogenous boldione is sporadically produced at concentrations ranging from 0.75 ngm/L to 1.73 ngm/L, whereas endogenous boldenone could not be proven. It was also tested the effect of the daily consumption of a commercially available phytosterol-enriched yogurt drink on the presence of these analytes in human urine. Results from this study could not indicate a relation of boldione-excretion with the consumption of phytosterols at the recommended dose. The correlations between boldione and other steroids were consistently stronger for volunteers consuming phytosterols than for those refraining from phytosterol consumption. This preliminary in vivo trial indicates the endogenous origin of boldione or boldione in human urine, independent on the presence of
any structural related analytes such as phytosterols [09129].

Boldenone (androsta-1,4-dien-17beta-ol-3-one, Bo) is an anabolic steroid known to have been used in cattle breeding or equine sport as a doping agent for many years. Although not clinically approved for human application, boldenone or its main metabolite 5beta-androst-1-en-17beta-ol-3-one (BM1) were detected in several doping control samples. For more than 15 years the possibility of endogenous boldenone production in human beings has been discussed. This is a challenging issue for doping control laboratories as boldenone belongs to the list of prohibited substances of the World Anti-Doping Agency and therefore the chance for false positive testing is significant. By GC/C/IRMS (gas chromatography/combustion/isotope ratio mass spectrometry) it should be possible to analyze the $^{13}$C/$^{12}$C ratio of either boldenone or BM1 and to distinguish whether their source is endogenous or exogenous. Therefore a method was developed to determine the $^{13}$C/$^{12}$C ratios of boldenone, BM1, pregnanediol, androsterone, etiocholanolone, and testosterone from a single urine specimen. The validity of the method was ensured by repeated processing of urine fortified with 2-50 ng/mL boldenone and BM1. The specificity of the method was ensured by gas chromatography/mass spectrometry determinations. Out of 23 samples investigated throughout the last four years, 11 showed $^{13}$C/$^{12}$C ratios of boldenone or BM1 inconsistent with an exogenous origin. Two of these samples were collected from the same athlete within a one-month interval, strongly indicating the chance of endogenous Bo production by this athlete [10087].

For over a decade there has been an intensive debate on the possible natural origin of boldenone (androst-1,4-diene-17beta-ol-3-one, 17beta-boldenone) in calf urine and several alternative markers to discriminate between endogenously formed boldenone and exogenously administered boldenone have been suggested. The currently approved method for proving illegal administration of beta-boldenone(ester) is the detection of beta-boldenone conjugates. Because of the fractionation approach used in this method there is no need for conjugated reference standards which often are not available. The disadvantage of needing three analytical runs to determine the conjugated status of each of the metabolites was overcome by using fast chromatography [08188].

Boldenone (17-hydroxy-androsta-1,4-diene-3-one, Bol) and boldione (androsta-1,4-diene-3,17-dione, ADD), are currently listed as exogenous anabolic steroids by the World Anti-Doping Agency. However, it has been reported that these analytes can be produced endogenously. Interestingly, only for Bol a comment is included in the list on its potential endogenous origin. In one study, the endogenous origin of ADD in human urine was investigated, and the potential influence of phytosterol consumption was evaluated. It was carried out a 5-week in vivo trial with both men (n=6) and women (n=6) and measured alpha-boldenone, beta-boldenone, boldione, androstenedione, beta-testosterone and alpha-testosterone in their urine using gas chromatography coupled to multiple mass spectrometry (GC-MS-MS). The results demonstrate that endogenous ADD is sporadically produced at concentrations ranging from 0.751 ng/mL to 1.73 ng/mL, whereas endogenous Bol could not be proven. We also tested the effect of the daily consumption of a commercially available phytosterol-enriched yogurt drink on the presence of these analytes in human urine. Results from this study could not indicate a relation of ADD-excretion with the consumption of phytosterols at the recommended dose. The correlations between ADD and other steroids were consistently stronger for volunteers consuming phytosterols (test) than for those refraining from phytosterol consumption (control). Excretion of AED, bT and aT did not appear to be dependent on the consumption of phytosterols. This preliminary in vivo trial indicates the endogenous origin of boldione or ADD in human urine, independent on the presence of any structural related analytes such as phytosterols [09128].
Urinary samples contaminated with faecal boldenone

One potential explanation for the presence of beta-boldenone in calf urine is contamination of the sample with feces containing beta-boldenone. It has been demonstrated that after oral and intramuscular administration of beta-boldenone esters, several metabolites are formed and excreted in urine. One of the (minor) metabolites is 6beta-hydroxy-17alpha-boldenone. This paper describes an analytical method that can discriminate between unconjugated boldenone, its glucuronide- and sulphate-conjugates, 6beta-hydroxy-17alpha/beta-boldenone and coprostanol, a marker for fecal contamination. The method was applied to all samples suspected to contain boldenone within the Dutch National Residue Control Plan. Approximately 10,000 samples of urine were screened (LC-MS) in 2004-2005 by VWA-East, one of the official Dutch control laboratories, from which 261 samples were suspected to contain boldenone. These samples were all analyzed for their conjugation state, 6beta-hydroxy-17alpha/beta-boldenone and for the presence of coprostanol. Alfa-boldenone, the major metabolite in bovine urine after boldenone-ester administration, was found in a large number of these samples. The presence of alpha-boldenone was proven also to be a result of fecal contamination. None of the samples tested contained residues of the metabolite 6beta-hydroxy-17alpha/beta-boldenone. Not finding this metabolite indicates that the origin of alpha-boldenone conjugates is endogenous. The results confirm that the presence of unconjugated beta-boldenone and alpha-boldenone conjugates next to alpha-boldenone are no indicators for illegal administration of boldenone-esters. No indications were obtained that conjugated beta-boldenone can be of endogenous origin [06111].

Influence of renal function

The widespread use of reporting estimated glomerular filtration rate (eGFR) alongside serum creatinine has led to a heightened appreciation of renal disease. However, creatinine is recognized as an insensitive marker of true GFR and therefore can lead to misdiagnosis of renal dysfunction in the absence of true pathology. It was reported a case of a 37-year-old male referred due to abnormal eGFR and creatinine in the absence of clinical signs, symptoms or other biochemical abnormalities of renal disease. Subsequent investigations based on a high index of suspicion for exogenous substance abuse led to a novel observation of significantly raised creatinine due to the presence of boldenone, an equine anabolic steroid commonly abused in body building [11101].

Metabolism of 1-ene-steroids

5alpha-Androst-1-ene derivatives are widely known as potent anabolic steroids. Although the metabolism of boldenone was elucidated and an adequate detection method was developed boldenone remained a popular steroid for misuse. Hence, the boldenone analogues were next on the list to be introduced on the prohormone market after testosterone precursors and their 19-nor-analogues [06004].

Stenbolone

Stenbolone acetate (17beta-acetoxy-2-methyl-5alpha-androst-1- en-3-one) is a registered anabolic steroid. After oral administration of stenbolone acetate glucuronidated stenbolone and several metabolites were detected. Besides the 16-hydroxy and 17-keto metabolites a minor metabolite hydroxylated at C-18, 2-methyl-5alpha-androst-1-ene-18-ol-3,17-dione, was
also found [06004].

**Quinbolone**

Quinbolone (17beta-(1-cyclopenten-1-yloxy)-androsta-1,4-dien-3-one) is the 17beta cyclopentene ether of boldenone and is reportedly metabolised to boldenone [06004].

**Boldione**

Boldione (1,4-androstadiene-3,17-dione) is marketed as an orally active precursor of boldenone and is available as a nutritional supplement in the United States. In cattle it was shown to be a metabolite of boldenone and endogenous. In man, 1,4-androstadiene-3,17-dione was identified as a metabolite and precursor of boldenone. Two excretion studies with orally administered 1,4-androstadiene-3,17-dione have been performed so far and have shown that this steroid is partially excreted unchanged. Besides the major metabolite boldenone, two other unidentified metabolites has been detected, maybe 5beta-androst-1-ene-3alpha-ol,17-one as the major metabolite and 5beta-androst-1-ene-17beta-ol-3-one, 5beta-androst-1-ene-3alpha,17-one as the major metabolite and 5beta-androst-1-ene-6beta-ol-3,17-dione as other metabolites. These metabolites were previously identified as metabolites of boldenone [06004].

The occurrence of boldione metabolites conjugated with cysteine and N-acetylcysteine in human urine was evaluated. Methods based on precursor ion scan of the protonated aminoacid (m/z 122 and m/z 164 for cysteine and N-acetylcysteine respectively) and neutral losses of the aminoacids (121 Da and 163 Da for cysteine and N-acetylcysteine respectively) were applied for the open detection of conjugates. Results for urine samples collected before and after boldione administration were compared. Using this approach, 24 metabolites (eleven conjugates with cysteine and thirteen conjugated with N-acetylcysteine) were detected. The metabolites were characterized by mass spectrometry and their potential structures were proposed based on this information. The structures of nine of these metabolites were confirmed by the synthesis of the conjugates. According to these results, a metabolic pathway for boldione involving this type of conjugation was presented. This is the first time that cysteine conjugates are presented for exogenous anabolic androgenic steroids and the first report of N-acetylcysteine conjugates for steroids [12168].

Boldione (1,4-androstadien-3,17-dione) is included in the list of prohibited substances, issued by the World Anti-Doping Agency (WADA). Endogenous production of low concentrations of boldione has also been reported. The objective of this study was to assess boldione metabolism in humans. Detection of boldione metabolites was accomplished by analysis by liquid chromatography coupled to tandem mass spectrometry of urine samples obtained after administration of the drug and subjected to different sample preparation procedures to analyze the different metabolic fractions (free, glucuronides, sulphates and released in basic media). In addition to boldione, eight metabolites were detected in the free fraction. Four of them were identified by comparison with standards: 6β-hydroxy-boldenone (M3), androsta-1,4,6-triene-3,17-dione (M5), (5alpha)-1-androstenedione (M6) and (5alpha)-1-testosterone (M8). Metabolite M7 was identified as the 5β-isomer of 1-androstenedione, and metabolites M1, M2 and M4 were hydroxylated metabolites and tentative structures were proposed based on mass spectrometric data. After beta-glucuronidase hydrolysis, five additional metabolites excreted only as conjugates with glucuronic acid were detected: boldenone, (5beta)-1-testosterone (M9), and three metabolites resulting from reduction of the 3-keto group. Boldenone, epiboldenone, and hydroxylated metabolites of boldione, boldenone and 1-testosterone were detected as conjugates with sulfate. In addition, boldione and seven
metabolites (boldenone, M2, M3, M4, M5, M7 and M9) increased their concentration in urine after treatment of the urine in alkaline conditions. In summary, 15 boldione metabolites were detected in all fractions. The longer detection time was observed for metabolite M4 after alkaline treatment of the urine, which was detected up to 5 days after boldione administration [12169].

Experimentally

Boldenone (BOL) is a derivative of the testosterone that has dual effects on humans, both directly and indirectly; directly as injection to build muscles and indirectly as through consuming meat of animals that were treated with BOL. However, the action of these steroids on different body organs structures is still unclear; therefore, the aim of the present study was to investigate the effect of the intramuscular injection of BOL undecylenate on the different organ structures. A total of 10 adult New Zealand rabbits were divided into two main groups, the first group was the control group, which includes animals that were injected intramuscularly with olive oil and the second group included animals that received two intramuscular injections of 5 mg/kg body weight BOL dissected after 6 weeks. Our results showed that intramuscular injection of rabbits with BOL showed hypertrophy in both skeletal and cardiac muscles, disturbances of the hepatocytes radially arranged cords with multifocal hepatocellular vacuolations in the liver, glomerulus mass reduction with multifocal glomerular injury in the kidney, disturbances of the cycle of spermatogenesis in the testes. In conclusion, using BOL, while preparing for a young bodybuilding contest, may cause an alteration in the histological structure of most of the body organs; these findings suggested that especially young people who misuse anabolic androgenic steroids should be careful if they want to use such steroids to enhance their strength and endurance [013246].

Laboratory techniques

Boldenone is one of the most frequently detected anabolic androgenic steroids in doping control analysis. Boldenone misuse is commonly detected by the identification of the active drug and its main metabolite, 5beta-androst-1-en-17beta-ol-3-one (BM1), by gas chromatography-mass spectrometry (GC-MS), after previous hydrolysis with β-glucuronidase enzymes, extraction and derivatization steps. However, some cases of endogenous boldenone and BM1 have been reported. Nowadays, when these compounds are detected in urine at low concentrations, isotope ratio mass spectrometry (IRMS) analysis is needed to confirm their exogenous origin. The aim of the present study was to identify boldenone metabolites conjugated with sulphate and to evaluate their potential to improve the detection of boldenone misuse in sports. Boldenone was administered to a healthy volunteer and urine samples were collected up to 56 h after administration. After a liquid-liquid extraction with ethyl acetate, urine extracts were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using electrospray ionisation in negative mode by monitoring the transition of m/z 365-350, specific for boldenone sulphate. Boldenone sulphate was identified in the excretion study urine samples and, moreover, another peak with the same transition was observed. Based on the MS/MS behaviour the metabolite was identified as epiboldenone sulphate. The identity was confirmed by isolation of the LC peak, solvolysis and comparison of the retention time and MS/MS spectra with an epiboldenone standard. These sulphated metabolites have not been previously reported in humans and although they account for less than 1% of the administered dose, they were still present in urine when the concentrations of the major metabolites, boldenone and BM1, were at the level of endogenous origin. The sulphated metabolites were also detected in 10 urine samples tested
positive to boldenone and BM1 by GC-MS. In order to verify the usefulness of these new metabolites to discriminate between endogenous and exogenous origin of boldenone, four samples containing endogenous boldenone and BM1, confirmed by IRMS, were analysed. In 3 of the 4 samples, neither boldenone sulphate nor epiboldenone sulphate were detected, confirming that these metabolites were mainly detected after exogenous administration of boldenone. In contrast, boldenone sulphate and, in some cases, epiboldenone sulphate were present in samples with low concentrations of exogenous boldenone and BM1. Thus, boldenone and epiboldenone sulphates are additional markers for the exogenous origin of boldenone and they can be used to reduce the number of samples to be analysed by IRMS. In samples with boldenone and BM1 at the concentrations suspicion for endogenous origin, only if boldenone and epiboldenone sulphates are present, further analysis by IRMS will be needed to confirm exogenous origin [12167].

Boldione (1,4-androstanediene-3,17-dione) is a direct precursor (prohormone) to the anabolic steroid boldenone (1,4-androstanediene-17beta-ol-3-one). It is advertised as a highly anabolic/androgenic compound promoting muscularity, enhancing strength and overall physical performance, and is available on the Internet and in health stores. One work was undertaken to determine and characterize boldione and its metabolites in human urine, using both liquid chromatography with electrospray ionization mass spectrometry and gas chromatography with mass spectrometry and derivatization. Boldione and its three metabolites were detected in dosed human urine after dosing a healthy volunteer with 100 mg boldione. The excretion studies showed that boldione and its metabolites were detectable in urine for 48 h after oral administration, with maximum excretion rates after 1.8 and 3.6 h (boldenone case). The amounts of boldione and boldenone excreted in urine from this 100 mg dose were 34.45 and 15.95 mg, respectively [06109].

Sulfo-conjugates of boldenone and its phase-I metabolites were studied concerning their utility as markers for boldenone administration and as a means to differentiate endogenous boldenone (metabolite) production from the illicit use of the AAS. In an elimination study with 20 mg of boldenone, urine samples were collected up to 56 h post-administration and subjected to LLE and LC-MS/MS analysis comprising specific ion transitions for boldenone (and epiboldenone) sulfate. The sulfo-conjugates were indeed found up to 56 h, and the applicability of these marker compounds to routine doping control samples was tested by analyzing specimens of earlier AAFs with boldenone and samples containing evidently endogenously produced boldenone metabolites as verified by IRMS. In 3 out of 4 cases of naturally/endogenously occurring boldenone, sulfates of boldenone and epiboldenone were not detected, suggesting that these analytes can serve as indicators (though not as proof) for exogenous sources of boldenone in athletes’ doping control samples. Analogously, metandienone was subjected to elimination studies with particular emphasis on sulfo-conjugated metabolites. In agreement with an earlier report, the utility of sulfated 18-nor-17beta-hydroxymethyl,17alpha-methyl-androst-1,4,13-trien-3-one as long-term metabolite was outlined, which was detected in post-administration studies up to 26 days by means of LC-MS/MS [13009].

Boldione is an anabolic androgenic steroid (AAS) related to boldenone, androstenedione, and testosterone bearing two double bonds in C1 and C4 positions. Boldione is rapidly transformed to the well-known AAS boldenone, being both compounds included in the list of prohibited substances and methods published yearly by the World Anti-Doping Agency (WADA). After the administration of boldione to a male volunteer, the already described urinary metabolites of boldenone produced after reduction in C4, oxydoreduction in C3 and C17, and hydroxylation have been detected. In addition, minor new metabolites have been detected and their structure postulated after mass spectrometric analyses. Finally, the reduction of the double bound in C1 produces metabolites identical to the endogenously
produced ones. A method based on gas chromatography coupled to isotope ratio mass spectrometry (GC/IRMS) after a urine sample purification by high performance liquid chromatography (HPLC) permitted to confirm the main synthetic like boldione/boldenone metabolite (17beta-hydroxy-5beta-androst-1-en-3-one) and boldenone at trace levels (<5 ng/mL) and then to establish its synthetic or endogenous origin, and to determine the exogenous origin of metabolites with the same chemical structure of the endogenous ones. The detection of pseudoendogenous androgens of synthetic origin partially overlapped boldenone and its main metabolite detection, being an additional proof of synthetic steroids misuse. By the use of IRMS, the correct evaluation of the modifications of the steroid profile after the administration of synthetic AAS that could be converted into endogenous like ones is possible [13247].

**Conjugated and unconjugated**

Natural occurrence or illegal treatment of boldenone (BOLD) presence in cattle urine is under debate within the European Union. Separation of conjugated and unconjugated forms of 17alpha-boldenone (alpha-BOLD) and 17beta-boldenone (beta-BOLD) and presence of related molecules as androsta-1,4-diene-3,17-dione (ADD) appear critical points for the decision of an illegal use. The aim of one study was a new analytical approach of BOLD and ADD confirmation in cattle urine. The separation between conjugated and unconjugated forms of BOLD was obtained by a preliminary urine liquid-liquid extraction step with ethyl acetate. In this step the organic phase extracts only unconjugated BOLD and ADD, while BOLD in conjugated form remain in urine phase. Afterwards the urine phase, contains conjugated BOLD, was subjected to an enzymatic deconjugation. Solid-phase extraction (OASIS-HLB Waters) was used for the purification and concentration of analytes in organic and urine phases and liquid chromatography ion electrospray tandem mass spectrometry (LC-MS-MS) was applied for the confirmation of BOLD and ADD, using deuterium-labelled 17beta-boldenone (BOLD-d3) as internal standard. The method was validated as a quantitative confirmatory method according to the Commission Decision 2002/657/CE. The results obtained demonstrate that the developed method show very high specificity, precision, trueness and ruggedness. Decision limits (CCalpha) smaller than 0.5 ng/mL were obtained for each analyte [06110].

**In cattle**

17Beta-boldenone (17beta-BOLD) and Boldione (ADD) are steroid compounds with androgenic activity, likely to be used as growth promoters in cattle. Different studies still ongoing aiming to distinguish between "natural" occurrence or illegal BOLD source had already indicated that their metabolism in cattle is of relevant significance. To identify metabolites as in vivo markers to support the thesis of exogenous administration, a further approach to the in vitro biotransformation of 17beta-BOLD and ADD was performed using different subcellular fractions obtained from both liver and kidney of untreated cattle. Polar and non-polar metabolites obtained from incubated parent compounds were formerly separated by high performance liquid chromatography (HPLC) elution and successively identified by liquid chromatography tandem mass spectrometry (LC-MS/MS) detection. The bovine liver was the target tissue of the main metabolic reaction transforming 17beta-BOLD to ADD and vice versa. The presence of 6beta-hydroxy-17beta-BOLD, produced from both compounds when NADPH was added as cofactors to liver post mitochondrial and microsomal fractions suggests that cytochrome P450-dependent enzymes could be involved in the biotransformation, as it occurs for 6beta-hydroxylation of 17beta-testosterone. The results indicated that the urinary excretion profile in vivo of 6beta-hydroxy-17beta-BOLD and 16alpha-hydroxy-17beta-BOLD could be studied together with 17alpha- and 17beta-BOLD
Boldenone is an androgenic steroid that improves the growth and food conversion in food producing animals. In most countries worldwide, this anabolic steroid is forbidden for meat production. Until recently, the control of its illegal use was based either on 17beta-boldenone or 17alpha-boldenone (its main metabolite in cattle) identification in edible tissues, hair, faeces or urine. Recent observations and data tend to demonstrate the natural occurrence (but not ubiquitous) in cattle of these steroids, making the analytical strategy of the control more complicated. It was investigated the metabolism of boldenone in cattle after intramuscular and oral treatment of boldenone, boldenone esters and boldione. The central objective was to elucidate the structures of the main metabolites (phase I and phase II) in urine, with main objective to be further in position to compare boldenone urinary profiles of treated and non-treated animals. Nine metabolites have been identified, only four were present whatever the treatment and the administered boldenone source. Nevertheless, all of them have been detected at least once in non-treated animals which did not permit us to use them as biomarkers of an illegal treatment. At last, but not at least, all metabolites were found mainly glucuro-conjugated, and rarely sulfo-conjugated, with the only exception of 17beta-boldenone. Current investigations are showing the absence of 17beta-boldenone sulfoconjugate in non-treated animals; that would permit to distinguish non-treated from treated animals with boldione, boldenone and boldenone esters [06113].
OTHER SPECIFIED ANABOLIC ANDROGENIC STEROIDS

Oxandrolone

The discovery and implementation of the long-term metabolite of metandienone, namely 17beta-hydroxymethyl-17alpha-methyl-18-norandrost-1,4,13-trien-3-one, to doping control resulted in hundreds of positive metandienone findings worldwide and impressively demonstrated that prolonged detection periods significantly increase the effectiveness of sports drug testing. For oxandrolone and other 17-methyl steroids, analogs of this metabolite have already been described, but comprehensive characterization and pharmacokinetic data are still missing. In this report, the synthesis of the two epimeric oxandrolone metabolites-17beta-hydroxymethyl-17alpha-methyl-18-nor-2-oxa-5alpha-androsta-13-en-3-one and 17alpha-hydroxymethyl-17beta-methyl-18-nor-2-oxa-5alpha-androsta-13-en-3-one-using a fungus (Cunninghamella elegans) based protocol is presented. The reference material was fully characterized by liquid chromatography nuclear magnetic resonance spectroscopy and high resolution/high accuracy mass spectrometry. To ensure a specific and sensitive detection in athlete's urine, different analytical approaches were followed, such as liquid chromatography-tandem mass spectrometry (QqQ and Q-Orbitrap) and gas chromatography-tandem mass spectrometry, in order to detect and identify the new target analytes. The applied methods have demonstrated good specificity and no significant matrix interferences. Linearity (R(2) > 0.99) was tested, and precise results were obtained for the detection of the analytes (coefficient of variation <20%). Limits of detection (S/N) for confirmatory and screening analysis were estimated at 1 and 2 ng/mL of urine, respectively. The assay was applied to oxandrolone post-administration samples to obtain data on the excretion of the different oxandrolone metabolites. The studied specimens demonstrated significantly longer detection periods (up to 18 days) for the new oxandrolone metabolites compared to commonly targeted metabolites such as epioxandrolone or 18-nor-oxandrolone, presenting a promising approach to improve the fight against doping [13237].
metabolites such as epioxandrolone or 18-nor-oxandrolone, presenting a promising approach to improve the fight against doping [13238].

**Stanozolol**

Anabolic-androgenic steroids (AAS) represent one of the most frequently detected classes of prohibited substances in doping controls. Due to their long-lasting beneficial effects on athletic performance, utmost retrospection via urine analysis is desirable and accomplished by targeting long-term metabolites of the respective drugs. In case of stanozolol, a substantial variety of metabolites has enabled the identification of numerous adverse analytical findings in the past, and recent studies concerning complementary phase-I and phase-II metabolites has further expanded the windows of opportunity for detecting the abuse of stanozolol. In this study, the utility of liquid chromatography-high resolution/high accuracy (tandem) mass spectrometry (LC-MS/MS) for the detection of 3'-OH-stanozolol glucuronide in sports drug testing is presented and the identification of two additional and so far unreported metabolites is shown. The structures of the complementary glucuronic acid conjugates were attributed to stanozolol-N-glucuronide and 17-epistanozolol-N-glucuronide. By means of chemical synthesis, stanozolol-N-glucuronide was prepared and used to corroborate the suggested structures. The 3'-OH-stanozolol glucuronide and the newly identified target compounds were implemented into routine sports drug test assays consisting of direct injection LC-MS/MS or solid-phase extraction (SPE) followed by LC-MS/MS. A considerably expanded detection window for stanozolol abuse was demonstrated compared to the use of conventional phase-I metabolites and methodologies based on, for example, low resolution LC-MS/MS or gas chromatography-tandem mass spectrometry (GC-MS/MS). The commercial availability of 3'-OH-stanozolol glucuronide has been of great value for confirmatory purposes, and 17-epistanozolol-N-glucuronide was found to be a favourable long-term metabolite for doping controls as it was observed up to 28 days post-administration of the drug. Applying the established methodology over a period of six months to 659 routine sports drug testing samples, a total of 85 adverse analytical findings was uncovered, 72 of which would have remained undetected using earlier employed GC-MS/MS approaches [13239].

Stanozolol is one of the most frequently detected anabolic steroids in doping control samples. This compound is metabolized to a large extent and its metabolites can be detected in urine much longer than the parent compound. The main stanozolol metabolites are excreted in urine as glucuronide conjugates and 3'-hydroxy-stanozolol glucuronide is one of the most important in human urine. Therefore enzymatic hydrolysis is usually applied prior to extraction. In one article a method for the sensitive detection of intact 3'-hydroxy-stanozolol glucuronide, by liquid chromatography tandem mass spectrometry, is described. The method takes advantage of an easy and fast sample preparation based on a single solid-phase extraction avoiding enzymatic hydrolysis or derivatization. It allows to detect stanozolol abuse in human urine at 25 pg/mL. The method was validated according to Eurachem guidelines. The matrix effect, expressed as ion enhancement, was +14 percent. The extraction recovery of the method was 93%. The limit of detection (LOD), whereby all WADA-criteria in chromatography and mass spectrometry are fulfilled, was determined at 50 pg/mL. Application of the method to an excretion study revealed that the 3'-hydroxy-stanozolol glucuronide could be confirmed for 10 days after oral administration of 2 mg of stanozolol, prolonging detection times compared to other metabolites and methodologies by almost 50 percent [13799].

Aiming at the improved detection of stanozolol misuse in sport, an LC-MS/MS-based
methodology targeting the long-term metabolite 3'-hydroxystanozolol glucuronide was presented. Following SPE with cation-exchange mixed-mode adsorber resin, urine extracts were analyzed by conventional triple quadrupole detection in SRM mode; the selectivity and intensity of ion transitions allowed for LODs as low as 25 pg/mL, thus providing a viable tool for extended retrospection in sports drug test samples [13009].

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Androgenic steroids (AAS) represent one of the most frequently detected classes of prohibited substances in doping controls. Due to their long-lasting beneficial effects on athletic performance, utmost retrospection via urine analysis is desirable and accomplished by targeting long-term metabolites of the respective drugs. In case of stanozolol, a substantial variety of metabolites has enabled the identification of numerous adverse analytical findings in the past, and recent studies concerning complementary phase-I and phase-II metabolites has further expanded the windows of opportunity for detecting the abuse of stanozolol. In this study, the utility of liquid chromatography-high resolution/high accuracy (tandem) mass spectrometry (LC-MS/MS) for the detection of 3'-OH-stanozolol glucuronide in sports drug testing is presented and the identification of two additional and so far unreported metabolites is shown. The structures of the complementary glucuronic acid conjugates were attributed to stanozolol-N-glucuronide and 17-epistanozolol-N-glucuronide. By means of chemical synthesis, stanozolol-N-glucuronide was prepared and used to corroborate the suggested structures. The 3'-OH-stanozolol glucuronide and the newly identified target compounds were implemented into routine sports drug test assays consisting of direct injection LC-MS/MS or solid-phase extraction (SPE) followed by LC-MS/MS. A considerably expanded detection window for stanozolol abuse was demonstrated compared to the use of conventional phase-I metabolites and methodologies based on, for example, low resolution LC-MS/MS or gas chromatography-tandem mass spectrometry (GC-MS/MS). The commercial availability of 3'-OH-stanozolol glucuronide has been of great value
for confirmatory purposes, and 17-epistanozolol-N-glucuronide was found to be a favourable long-term metabolite for doping controls as it was observed up to 28 days post-administration of the drug. Applying the established methodology over a period of six months to 659 routine sports drug testing samples, a total of 85 adverse analytical findings was uncovered, 72 of which would have remained undetected using earlier employed GC-MS/MS approaches [13241].

Anabolic androgenic steroids are used in the sport context to enhance muscle mass and strength and to increase muscle fatigue resistance. Since muscle fatigue has been related to oxidative stress caused by an exercise-linked reactive oxygen species (ROS) production, we investigated the potential effects of a treatment with the anabolic androgenic steroid stanozolol against oxidative damage induced on rat skeletal muscle mitochondria by an acute bout of exhaustive exercise. Mitochondrial ROS generation with complex I- and complex II-linked substrates was increased in exercised control rats, whereas it remained unchanged in the steroid-treated animals. Stanozolol treatment markedly reduced the extent of exercise-induced oxidative damage to mitochondrial proteins, as indicated by the lower levels of the specific markers of protein oxidation, glycoxidation, and lipoxidation, and the preservation of the activity of the superoxide-sensitive enzyme aconitase. This effect was not due to an enhancement of antioxidant enzyme activities. Acute exercise provoked changes in mitochondrial membrane fatty acid composition characterized by an increased content in docosahexaenoic acid. In contrast, the postexercise mitochondrial fatty acid composition was not altered in stanozolol-treated rats. The results suggest that stanozolol protects against acute exercise-induced oxidative stress by reducing mitochondrial ROS production, in association with a preservation of mitochondrial membrane properties [11104].

The canine phase I and phase II metabolism of the synthetic anabolic-androgenic steroid stanozolol was investigated following intramuscular injection into a male greyhound. The major phase I biotransformation was hydroxylation to give 6alpha-hydroxystanozolol which was excreted as a glucuronide conjugate and was identified by comparison with synthetically derived reference materials. An analytical procedure was developed for the detection of this stanozolol metabolite in canine urine using solid phase extraction, enzyme hydrolysis of glucuronide conjugates and analysis by positive ion electrospray ionisation ion trap LC-MS [09124].

The abuse of stanozolol is quite widespread in sports. Its analysis is challenging and this has led to the development of new methods to improve its detection. A method was developed and validated for the detection of the three main monohydroxylated metabolites of stanozolol. The excretion profile of these metabolites was studied in four healthy male volunteers. The excretion study samples, after a single oral dose of drug, showed that 3'-OH-stanozolol was excreted at the highest concentration, followed by 16beta-OH stanozolol, with 4beta-OH stanozolol as the least excreted. Ninety-eight old doping samples with adverse analytical findings for 3'-OH-stanozolol were reanalysed using this method. This showed 3'-OH-stanozolol and 16beta-OH stanozolol in all the 98 samples whereas 4beta-OH-stanozolol was identified in 90 samples. The percentage of positive identifications of stanozolol in Indian sportspeople has increased markedly in the last five years, from 32 percent in 2004 to 82 percent in 2009; however, this may be due to the more effective detection of stanozolol metabolites. It can thus be concluded that the marked increase in percent positive of stanozolol in Indian sportspersons in 2009 may be due to the improved detection by a more effective LCMS/MS method [09125].

The applicability of LC-MS/MS in precursor ion scan mode for the detection of urinary stanozolol metabolites has been studied. The product ion at m/z 81 has been selected as specific for stanozolol metabolites without a modification in A- or N-rings and the product ions
at m/z 97 and 145 for the metabolites hydroxylated in the N-ring and 4-hydroxy-stanozolol metabolites, respectively. Under these conditions, the parent drug and up to 15 metabolites were found in a positive doping test sample. The study of a sample from a chimeric uPA-SCID mouse collected after the administration of stanozolol revealed the presence of 4 additional metabolites. The information obtained from the product ion spectra was used to develop a SRM method for the detection of 19 compounds. This SRM method was applied to several doping positive samples. All the metabolites were detected in both the uPA-SCID mouse sample and positive human samples and were not detected in none of the blank samples tested; confirming the metabolic nature of all the detected compounds. In addition, the application of the SRM method to a single human excretion study revealed that one of the metabolites (4xi,16xi-dihydroxy-stanozolol) could be detected in negative ionization mode for a longer period than those commonly used in the screening for stanozolol misuse (3'-hydroxy-stanozolol, 16beta-hydroxy-stanozolol and 4beta-hydroxy-stanozolol) in doping analysis. The application of the developed approach to several positive doping samples confirmed the usefulness of this metabolite for the screening of stanozolol misuse. Finally, a tentative structure for each detected metabolite has been proposed based on the product ion spectra measured with accurate masses using UPLC-QTOF MS [09126].

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Anabolic androgenic steroids, such as stanozolol, are typically misused by athletes during preparation for competition. Out-of-competition testing presents a unique challenge in the current anti-doping detection system owing to logistic reasons. Analysing hair for the presence of a prohibited drug offers a feasible solution for covering the wider window in out-of-competition testing. To assist in vivo studies aiming to establish a relationship between drug levels detected in hair, urine and blood, sensitive methods for the determination of stanozolol and its major metabolite 3'-hydroxystanozolol were developed in pigmented hair, urine and serum, using brown Norway rats as a model system and liquid chromatography tandem mass spectrometry (LC-MS/MS). For method development, spiked drug free rat hair, blood and urine samples were used. The newly developed method was then applied to hair, urine and serum samples from five brown Norway rats after treatment (intraperitoneal) with stanozolol for six consecutive days at 5.0 mg/kg/day. The assay for each matrix was linear within the quantification range with determination coefficient (r²) values above 0.995. The respective assay was capable of detecting 0.125 pg/mg stanozolol and 0.25 pg/mg 3'-hydroxystanozolol with 50 mg hair; 0.063 ng/mL stanozolol and 0.125 ng/mL 3'-hydroxystanozolol with 100 microL of urine or serum. The accuracy, precision and extraction
recoveries of the assays were satisfactory for the detection of both compounds in all three matrices. The average concentrations of stanozolol and 3'-hydroxystanozolol, were as follows: hair = 70 ± 22 pg/mg and 13 ± 3 pg/mg; urine = 4 ± 6 ng/mL and 9 ± 7 ng/mL; serum = 8 ± 4 ng/mL and 7 ± 2 ng/mL, respectively. The developed methods are sensitive, specific and reproducible for the determination of stanozolol and 3'-hydroxystanozolol in rat hair, urine and serum. These methods can be used for in vivo studies further investigating stanozolol metabolism, but also could be extended for doping testing. Owing to the complementary nature of these tests, with urine and serum giving information on recent drug use and hair providing retrospective information on habitual use, it is suggested that blood or urine tests could accompany hair analysis and thus avoid false doping results [12116].

Since stanozolol and 3'-hydroxystanozolol are structurally different from most AAS, they can be more difficult to detect in urine than other AAS, and thus require bespoke methods. Depending on the dose administered, once in the body, stanozolol gets rapidly metabolised and the metabolites are generally detected in urine until ca. 6 days. Thus, urinalysis generally fails to determine the long term history of an individual’s drug use, which is a major hindrance in cases of performance-enhancing drugs used in preparation for competition. Stanozolol, along with other AAS, is a so called “training drug” which is taken for a prolonged period, typically in cycles, during preparation, in order to obtain the desired performance-enhancing effects. Furthermore, urinalysis also fails to distinguish between chronic use and single, accidental exposure of drugs [12116].

An extensive literature has documented adverse effects on mental health in anabolic androgenic steroids (AAS) abusers. Depression seems a common adverse reaction in AAS abusers. Recently it has been reported that in a rat model of AAS abuse stanozolol induces behavioural and biochemical changes related to the pathophysiology of major depressive disorder. In the present study, we used the model of AAS abuse to examine possible changes in the monoaminergic system, a neurobiological substrate of depression, in different brain areas of stanozolol-treated animals. Wistar rats received repeated injections of stanozolol (5mg/kg, s.c.), or vehicle (propylene glycol, 1 ml/kg) once daily for 4 weeks. Twenty-four hours after last injection, changes of dopamine (DA) and relative metabolite levels, homovanillic acid (HVA) and 3,4-dihydroxy phenylacetic acid (DOPAC), serotonin (5-HT) and its metabolite levels, 5-hydroxy indolacetic acid (5-HIAA), and noradrenaline (NA) amount were investigated in prefrontal cortex (PFC), nucleus accumbens (NAC), striatum (STR) and hippocampus (HIPP). The analysis of data showed that after chronic stanozolol, DA levels were increased in the HIPP and decreased in the PFC. No significant changes were observed in the STR or in the NAC. 5-HT and 5-HIAA levels were decreased in all brain areas investigated after stanozolol exposure; however, the 5-HIAA/5-HT ratio was not altered. Taken together, the data indicate that chronic use of stanozolol significantly affects brain monoamines leading to neurochemical modifications possibly involved in depression and stress-related states [12170].

Methyltestosterone

Since a few years more and more products have appeared on the market for dietary supplements containing steroids that had never been marketed as approved drugs, mostly without proper labeling of the contents. Syntheses and few data on pharmacological effects are available dated back mainly to the 1950s or 1960s. Only little knowledge exists about effects and side effects of these steroids in humans. The present study reports the identification of Δ6-methyltestosterone in a product named “Jungle Warfare”, which was obtained from a web-based supplement store. The main urinary metabolites, 17alpha-hydroxy-17beta-
The androgen 17alpha-methyltestosterone (17alpha-meT) is one of the most commonly abused anabolic androgenic steroids (AAS). We assessed the impact of 17alpha-meT after bilateral infusion into the dorsomedial hypothalamus (DMH) in female anxiety. A paradoxical effect in Vogel conflict test (VCT) behavior was noted: while AAS infusion induced an increase in the latency to display the appetitive reaction of the task, it also increased the number of punished responses. No changes in elevated plus maze (EPM) behavior were noted. However, AAS infusion induced an increase in social interactions. Changes in social interactions were mimicked by muscimol infusion and counteracted by co-infusion of AAS plus the GABAA receptor (GABA-A-R) antagonist GABAzine. A reduction of systolic blood pressure was registered after AAS infusion in the DMH. No changes in fluid intake or locomotor behaviors were noted. It was concluded that the AAS 17alpha-meT modulates distinct anxiety domains in females through a fast-acting mechanism [06120].

Methyltestosterone (MT) is one of the most frequently detected anabolic androgenic steroids in doping control analysis. MT misuse is commonly detected by the identification of its two main metabolites excreted as glucuronide conjugates, 17alpha-methyl-5alpha-androstane-3alpha,17beta-diol and 17alpha-methyl-5beta-androstane-3alpha,17alpha-diol. The detection of these metabolites is normally performed by gas chromatography-mass spectrometry, after previous hydrolysis with beta-glucuronidase enzymes, extraction and derivatization steps. The aim of the present work was to study the sulphate fraction of MT and to evaluate their potential to improve the detection of the misuse of the drug in sports. MT was administered to healthy volunteers and urine samples were collected up to 30 days after administration. After an extraction with ethyl acetate, urine extracts were analysed by liquid chromatography tandem mass spectrometry using electrospray ionisation in negative mode by monitoring the transition m/z 385 to m/z 97. Three diol sulphate metabolites (S1, S2 and S3) were detected. Potential structures for these metabolites were proposed after solvolysis and mass spectrometric experiments: S1, 17alpha-methyl-5beta-androstane-3alpha,17beta-diol 3alpha-sulphate; S2, 17beta-methyl-5alpha-androstan-3alpha,17alpha-diol 3alpha-sulphate; and S3, 17beta-methyl-5beta-androstane-3alpha,17alpha-diol 3alpha-sulphate. Synthesis of reference compounds will be required in order to confirm the structures. The retrospectivity of these sulphate metabolites in the detection of MT misuse was compared with the obtained with previously described metabolites. Metabolite S2 was detected up to 21 days after MT administration, improving between 2 and 3 times the retrospectivity of the detection compared to the last long-term metabolite of MT previously described, 17alpha-hydroxy-17beta-methylandrostane-4,6-dien-3-one [12178].

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**Biomarkers**

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**Side effects**

*Hyperthyreoidimus*

In one paper it was reported different effects of methyltestosterone administration on thyroid function in two twin brothers, one of whom suffered from hypothyroidism, while the other was apparently healthy. Methyltestosterone, which is a non-aromatisable androgen, resulted in a marked reduction of thyroxine-binding globulin (TBG), irrespectively of the patient’s hormonal status, while the impact on free thyroid hormones depended on baseline thyroid function. The research shows that a possibility of the use of non-aromatisable androgens or other
drugs affecting TBG levels should be taken into consideration in all hypothyroid patients receiving levothyroxine, in whom thyroid hormone status suddenly changes without any apparent reason [13243].

**Neurological damage**

Anabolic-androgenic steroids (AAS) are lipophilic hormones often taken in excessive quantities by athletes and bodybuilders to enhance performance and increase muscle mass. AAS exert well known toxic effects on specific cell and tissue types and organ systems. The attention that androgen abuse has received lately should be used as an opportunity to educate both athletes and the general population regarding their adverse effects. Among numerous commercially available steroid hormones, very few have been specifically tested for direct neurotoxicity. It was evaluated the effects of supraphysiological doses of methandienone and 17alpha-methyltestosterone on sympathetic-like neuron cells. Vitality and apoptotic effects were analyzed, and immunofluorescence staining and western blot performed. In this study, we demonstrate that exposure of supraphysiological doses of methandienone and 17alpha-methyltestosterone are toxic to the neuron-like differentiated pheochromocytoma cell line PC12, as confirmed by toxicity on neurite networks responding to nerve growth factor and the modulation of the survival and apoptosis-related proteins ERK, caspase-3, poly (ADP-ribose) polymerase and heat-shock protein 90. It was observed, in contrast to some previous reports but in accordance with others, expression of the androgen receptor (AR) in neuron-like cells, which when inhibited mitigated the toxic effects of AAS tested, suggesting that the AR could be binding these steroid hormones to induce genomic effects. It was also noted elevated transcription of neuritin in treated cells, a neurotropic factor likely expressed in an attempt to resist neurotoxicity. Taken together, these results demonstrate that supraphysiological exposure to the AAS methandienone and 17alpha-methyltestosterone exert neurotoxic effects by an increase in the activity of the intrinsic apoptotic pathway and alterations in neurite networks [13244].

The combined analytical capabilities of GC-MS and LC-MS/MS were employed in a study concerning alternative long-term metabolites of methyltestosterone. In an elimination study with orally administered methyltestosterone, urine samples were collected over a period of 30 days, and sulfate-conjugated phase-II metabolites in particular were screened using a dedicated selected reaction monitoring (SRM) approach. Three analytes were observed and suggested to consist of 17alpha-methyl-5beta-androstane-3alpha,17beta-diol 3-sulfate, 17beta-methyl-5alpha-androstane-3alpha,17alpha-diol 3-sulfate, and 17beta-methyl-5beta-androstane-3alpha,17alpha-diol 3-sulfate as supported by GC-MS analyses (providing Kovats indices and EI mass spectra) of the deconjugated substances. Targeting of 17beta-methyl-5alpha-androstane-3alpha,17alpha-diol 3-sulfate by LC-MS/MS enabled the expanded retrospectivity (up to 21 days) and considering that, the synthesis of reference substance to verify the postulated structure of the metabolite(s) was suggested [13009].

**Laboratory techniques**

Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was used to investigate the effect of different substitutions introduced during metabolism on fragmentation patterns of four anabolic steroids including methyltestosterone, methandrostenolone, cis-androstosterone and adrenosterone, along with their metabolites. Collision-induced dissociation (CID) analysis was performed to correlate the major product ions of 19 steroids with structural features. The analysis is done to portray metabolic alteration, such as incorporation or reduction of double bonds, hydroxylations, and/or oxidation of hydroxyl moieties to keto functional group on steroidal skeleton which leads to drastically changed product ion spectra from the respective classes of steroids, therefore, making them difficult to identify. The comparative ESI-MS/MS study also revealed some characteristic peaks to differentiate
different steroidal metabolites and can be useful for the unambiguous identification of anabolic steroids in biological fluid. Moreover, LC-ESI-MS/MS analysis of fermented extract of methyltestosterone, obtained by Macrophomina phaseolina was also investigated [12180].

**Desoxymethyltestosterone (DMT)**

Desoxymethyltestosterone (DMT) is a steroid recently identified to be misused as a doping agent. Since, the knowledge of functions of this substance is rather limited, it was our aim to characterise the pharmacological profile of DMT and to identify potential adverse side effects. DMT was synthesised, its purity was confirmed and its biological activity was tested. The potency of Madol (DMT) to transactivate androgen receptor (AR) dependent reporter gene expression was two times lower as compared to dihydrotestosterone (DHT). Receptor binding tests demonstrate that DMT binds with high selectivity to the AR, binding to the progesterone receptor (PR) was low. In vivo experiments in orchietomised rats demonstrated that treatment with DMT resulted only in a stimulation of the weight of the levator ani muscle; the prostate and seminal vesicle weights remained unaffected. Like testosterone, administration of DMT resulted in a stimulation of IGF-1 and myostatin mRNA expression in the gastrocnemius muscle. In the prostate proliferation was stimulated by TP (testosteronepropionate), but remained unaffected by DMT. Remarkably, treatment with DMT, in contrast to TP, resulted in a significant increase of the heart weight. In the liver, DMT slightly stimulates the expression of the tyrosine aminotransferase gene (TAT). Our results demonstrate that DMT is a potent AR agonist with an anabolic activity. Besides the levator ani weight, DMT also modulates the gene expression in the musculus gastrocnemius. The observed stimulation of TAT expression in the liver and the significant increase of the heart weight after DMT treatment can be taken as an indication for side effects. Summarizing these data it is obvious that DMT is a powerful anabolic steroid with selective androgen receptor modulators (SARM) like properties and some indications for toxic side effects. Therefore, there is a need for a strict control of a possible misuse [06121].

**Metabolism**

Madol (17alpha-methyl-5alpha-androst-2-en-17beta-ol), also called desoxymethyltestosterone (DMT) was independently detected by two research groups in 2004. Madol was patented in 1961 but has never been approved by the FDA. In vitro and excretion studies with a baboon indicated that madol was only partially metabolised and that it is largely excreted unchanged in urine. Indications of the presence of a metabolite containing two more oxygen atoms and another less unsaturated metabolite were tentatively given, without further structural characterization. Madol was included as a target substance in the methods used to detect anabolic steroids at the 2004 Olympic Games in Athens. These analyses did not result in an adverse analytical finding. In 2005, madol evolved from the clandestine underground to the commercial supplement market and became directly available via the internet [06004].

**Methylnortestosterone**

The contribution of lymphatic transport to the oral bioavailability of methylnortestosterone (M) after oral administration of the lipophilic prodrug methylnortestosterone undecanoate (MU) has been evaluated, and the sensitivity of lymphatic MU transport to lymphatic lipid transport has been investigated. M and MU were administered intravenously and orally to greyhound dogs to determine absolute bioavailability after oral dosing of MU. MU was also administered orally with differing quantities of food (lipid) to lymph duct-cannulated greyhound dogs to
investigate the relative roles of lymph versus blood transport on M bioavailability and the effect of lipid load on systemic exposure. The relationship between lymphatic lipid and MU transport was further investigated in anesthetized rats. The oral bioavailability of M after administration of MU was found to be highly dependent on coadministration of food, and the bioavailability of M increased approximately 700 percent in fed versus fasted animals. In both cases, lymph diversion resulted in negligible systemic exposure of M, indicating almost complete dependence on lymphatic transport of MU for systemic exposure of M. Lymphatic transport of MU was even more highly dependent on the quantity of coadministered lipid and increased more than 50-fold with increasing lipid load. Therefore, increasing the quantity of food or lipid coadministered with MU stimulated a significant increase in the lymphatic transport of MU and systemic exposure of M. The lipid sensitivity of lymphatic transport of MU is significantly higher than previously observed for more metabolically stable compounds, suggesting a role for coadministered lipid in promoting avoidance of enterocyte-based cleavage of MU [09131].

Testing

The misuse of the anabolic steroid methyltestosterone is currently routinely monitored in doping control laboratories by gas chromatography-mass spectrometry (GC-MS) of two of its metabolites: 17alpha-methyl-5beta-androstane-3beta,17beta-diol and 17alpha-methyl-5beta-androstane-3beta,17beta-diol. Due to the absence of any easy ionizable moiety these metabolites are poorly detectable using liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI). In one study, the metabolism of methyltestosterone has been reinvestigated by the use of a precursor ion scan method in LC-ESI-MS/MS. Two metabolites have been detected using this method. Both compounds have been confirmed in post-administration urine samples of an uPA-SCID mouse with humanized liver and were characterized by LC-MS/MS and GC-MS using both quadrupole and time of flight (TOF) analysers. From the detailed study of the fragmentation, these metabolites were proposed to be epimethyltestosterone and a dehydrogenated compound. Epimethyltestosterone has previously been described as a minor metabolite while the occurrence of the oxidized metabolite has not been reported. Comparison with the synthesis reference revealed that the structure of the dehydrogenated metabolite is 6-ene-epimethyltestosterone. A selected reaction monitoring method including 3 transitions for each metabolite has been developed and applied to samples from an excretion study and to samples declared positive after GC-MS analysis. 6-Ene-epimethyltestosterone was found in all samples showing its applicability in the detection of methyltestosterone misuse [09132].

Fluoxymesterone

Anabolic androgenic steroids (AAS) are testosterone derivatives used either clinically, in elite sports, or for body shaping with the goal to increase muscle size and strength. Clinically developed compounds and nonclinically tested designer steroids often marketed as food supplements are widely used. Despite the considerable evidence for various adverse effects of AAS use, the underlying molecular mechanisms are insufficiently understood. Here, we investigated whether some AAS, as a result of a lack of target selectivity, might inhibit 11beta-hydroxysteroid dehydrogenase 2 (11beta-HSD2)-dependent inactivation of glucocorticoids. Using recombinant human 11beta-HSD2, we observed inhibitory effects for several AAS. Whereas oxymetholone, oxymesterone, danazol, and testosterone showed medium inhibitory potential, fluoxymesterone was a potent inhibitor of human 11beta-HSD2 (half-maximal inhibitory concentration [IC50] of 60-100 nM in cell lysates; IC50 of 160 nM in intact SW-620, and 530nM in MCF-7 cells). Measurements with rat kidney microsomes and
lysates of cells expressing recombinant mouse 11beta-HSD2 revealed much weaker inhibition by the AAS tested, indicating that the adverse effects of AAS-dependent 11beta-HSD2 inhibition cannot be investigated in rats and mice. Furthermore, we provide evidence that fluoxymesterone is metabolized to 11-oxofluoxymesterone by human 11beta-HSD2. Structural modeling revealed similar binding modes for fluoxymesterone and cortisol, supporting a competitive mode of inhibition of 11beta-HSD2-dependent cortisol oxidation by this AAS. No direct modulation of mineralocorticoid receptor (MR) function was observed. Thus, 11beta-HSD2 inhibition by fluoxymesterone may cause cortisol-induced MR activation, thereby leading to electrolyte disturbances and contributing to the development of hypertension and cardiovascular disease [12172].

**Dihydrotestosterone (DHT, androstanolone)**

Following a transdermal application of 250 mg of dihydrotestosterone (DHT) or an oral administration of 50 mg of dehydroepiandrosterone (DHEA), the ratios of DHT/EpiT, DHT/5beta-androstan-3alpha,17beta-diol, and 5alpha-androstan-3alpha,17beta-diol/5beta-androstan-3alpha,17beta-diol or DHEA/EpiT, 16alpha-OH-dehydroepiandrosterone/EpiT, 7beta-OH-dehydroepiandrosterone/EpiT, and 5beta-androstan-3alpha,17beta-diol/5alpha-androstan-3alpha,17beta-diol were found suitable to support the detection of a DHT or DHEA abuse respectively [12016].

Steroid 5alpha-reductase inhibitors are used to treat benign prostatic hyperplasia and androgenic alopecia, but the role of 5alpha-dihydrotestosterone (DHT) in mediating testosterone's effects on muscle, sexual function, erythropoiesis, and other androgen-dependent processes remains poorly understood. To determine whether testosterone's effects on muscle mass, strength, sexual function, hematocrit level, prostate volume, sebum production, and lipid levels are attenuated when its conversion to DHT is blocked by dutasteride (an inhibitor of 5alpha-reductase type 1 and 2) the 5alpha-Reductase Trial was a randomized controlled trial of healthy men aged 18 to 50 years comparing placebo plus testosterone enanthate with dutasteride plus testosterone enanthate from 2005 through 2010. Eight treatment groups received 50, 125, 300, or 600 mg/week of testosterone enanthate for 20 weeks plus placebo (4 groups) or 2.5 mg/d of dutasteride (4 groups). The primary outcome was change in fat-free mass; secondary outcomes: changes in fat mass, muscle strength, sexual function, prostate volume, sebum production, and hematocrit and lipid levels. A total of 139 men were randomized; 102 completed the 20-week intervention. Men assigned to dutasteride were similar at baseline to those assigned to placebo. The mean fat-free mass gained by the dutasteride groups was 0.6 kg when receiving 50 mg/week of testosterone enanthate, 2.6 kg for 125 mg/week, 5.8 kg for 300 mg/week, and 7.1 kg for 600 mg/week. The mean fat-free mass gained by the placebo groups was 0.8 kg when receiving 50 mg/wk of testosterone enanthate, 3.5 kg for 125 mg/week, 5.7 kg for 300 mg/week, and 8.1 kg for 600 mg/week. The dose-adjusted differences between the dutasteride and placebo groups for fat-free mass were not significant. Changes in fat mass, muscle strength, sexual function, prostate volume, sebum production, and hematocrit and lipid levels did not differ between groups. It was concluded that changes in fat-free mass in response to graded testosterone doses did not differ in men in whom DHT was suppressed by dutasteride from those treated with placebo, indicating that conversion of testosterone to DHT is not essential for mediating its anabolic effects on muscle [12173].

It was performed a pilot study using human peripheral blood lymphocytes (PBL) as a novel system to identify new biomarkers of dihydrotestosterone (DHT) and insulin-like growth factor-1 (IGF-1) abuse in sport. First, to obtain a gene signature, we treated cultures of lymphocytes from sedentary males with three doses of 0.237 microg/ml DHT, each of which is 80-fold the physiological concentration in young adult male serum, at days 0, 2 and 4, or
with a single dose of 1.25 microg/ml IGF-1, which is 5-fold the physiological concentration in young adult male serum. It was then used the Human Genome U133 Plus 2.0 microarray to identify a gene signature related to DHT or IGF-1 administration. Gene expression was evaluated after 7 and 21 days of DHT treatment, and after 24 h, 72 h and 7 days of IGF-1 treatment. Microarray analysis yielded a list of genes whose expression was altered after DHT or IGF-1 treatment. Among these it was selected the genes that are most representative of the pathways associated with skeletal and muscular disorders using the IPA bioinformatics tool. It was identified six (IDO1, CXCL13, CCL1, GZMB, VDR and IL2RA) and two (FN1 and RAB31) genes that were up-regulated in lymphocytes from sedentary subjects after 7 days of DHT and IGF-1 treatment, respectively. The expression of these genes in lymphocytes from differently trained athletes was either down-regulated or similar to that in lymphocytes from sedentary subjects. This finding suggests that up-regulation was due to the drug and not to physical exercise. In conclusion, it was demonstrate that PBL can be useful in anti-doping checks, and we describe new biomarkers of DHT and IGF-1 abuse which can be included in the Athlete’s Biological Passport [13258].

Dihydrotestosterone (DHT) exerts both functional and signaling effects extending beyond the effects of testosterone in rodent skeletal muscle. As a primer for investigating the role of DHT in human skeletal muscle function, this study aimed to determine whether circulating DHT is acutely elevated in men following a bout of repeat sprint exercise and to establish the importance of training status and sprint performance to this response. Fourteen healthy active young men performed a bout of repeat sprint cycle exercise at a target workload based on an incremental work-rate maximum (10 × 30 s at 150 % $W_{\text{max}}$ with 90-s recovery). Venous blood samples were collected preexercise and 5 and 60 min after exercise. Five minutes after exercise, there were significant elevations in total testosterone, free testosterone, and DHT, which returned to baseline after 1 h. Changes in DHT with exercise (5 min postexercise - preexercise) correlated significantly with changes in TT and FT. Sprinting cadence correlated with changes in FT, DHT and TT, and habitual training volume correlated with the change in TT. In conclusion, the data demonstrate that DHT is acutely elevated following sprint cycle exercise and that this response is influenced by cycling cadence. The importance of DHT in the context of exercise training and sports performance remains to be determined [13259].

Enzyme immunoassays (EIA) are commonly utilized for the evaluation of androgens in biological fluids; however, careful consideration must be given to cross-reactivity with other endogenous sex-steroid hormones. Our purpose was to determine the validity of a commonly-utilized commercially-available dihydrotestosterone (DHT) EIA. Serum samples obtained from older hypogonadal men who participated in a 12-month randomized controlled trial evaluating the effects of testosterone-enanthate (125 mg/week) or vehicle in combination with finasteride (5 mg/day) or placebo were assayed for DHT via EIA and using a validated gold-standard LC-MS/MS approach. Additionally, commercially-available (DHT-free) buffer containing graded testosterone doses was evaluated by DHT immunoassay. DHT concentrations measured via EIA were 79 percent to >1000 percent higher than values obtained by LC-MS/MS, with the largest differences (415-1128 %) occurring in groups receiving finasteride. Both LC-MS/MS and EIA indicated that testosterone-enanthate increased serum DHT to a similar magnitude. In contrast, finasteride-induced reductions in DHT were detected by LC-MS/MS, but not EIA. No significant associations were present for DHT concentrations between measurement techniques. Cross-reactivity of testosterone with the immunoassay ranged from 18 to 99 percent and DHT concentrations measured by EIA were highly associated with the spiked testosterone concentrations in DHT-free buffer. In conclusion, we provide evidence invalidating a commonly-utilized commercially-available DHT immunoassay because significant cross-reactivity exists between testosterone and the EIA and because the changes in DHT observed via EIA were not associated with a validated
gold-standard measurement technique. The cross-reactivity of testosterone is particularly concerning because testosterone is present in 100-fold greater concentrations than is DHT within the circulation [13260].

**Dehydroepiandrosterone**

One study aimed to determine the role of DHEA-S (dehydroepiandrosterone-sulphate) in coping against the exercise training mixing aerobic and resistance components. During 5-day successive exercise training, 16 young male participants (19 ± 2 years) received either a placebo (flour capsule) or DHEA (100 mg/day) in a double-blinded and placebo-controlled design. Oral DHEA supplementation significantly increased circulating DHEA-S by 2.5-fold, but a protracted drop (35 %) was observed from Day 3 during training. In the placebo group, only a minimal DHEA-S reduction (17 %) was observed. Changes in testosterone followed a similar pattern as DHEA-S. Muscle soreness was elevated significantly on Day 2 for both groups to a similar extent. Lower muscle soreness was observed in the DHEA-supplemented group on Day 3 and Day 6. In the placebo group, training increased circulating creatine kinase (CK) levels by approximately ninefold, while only a threefold increase was observed in the DHEA-supplemented group. This mix-type exercise training improved glucose tolerance in both groups, while lowering the insulin response to the glucose challenge, but no difference between treatments was observed. The results suggest that DHEA-S may play a role in protecting skeletal muscle from exercise training-induced muscle damage [13261].

Evidence links dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) to crucial military health issues, including operational stress, resilience, and traumatic brain injury. This study evaluated the anabolic, neuroprotective, and neuroexcitatory properties of DHEA(S) in healthy military men. A salivary sample was obtained from 42 men and assayed for DHEA(S), testosterone, nerve growth factor (NGF; which supports nerve cell proliferation), and salivary alpha amylase (sAA; a proxy of sympathetic nervous system function). Separate regression analyses were conducted with DHEA and DHEAS as independent variables, and testosterone, NGF, and sAA as dependent variables, respectively. The models explained 23 percent of variance in testosterone, 17 percent of variance in NGF, and 7.4 percent of variance in sAA. Standardized beta coefficients revealed that DHEA independently influenced testosterone, whereas DHEAS independently influenced NGF and sAA. DHEA demonstrated anabolic properties, whereas DHEAS demonstrated neuroprotective and neuroexcitatory properties in military men. This area of study has broad implications for stress inoculation, traumatic brain injury rehabilitation, and regenerative medicine in military personnel [13262].

**Diurnal secretion**

Diurnal patterns of cortisol and dehydroepiandrosterone (DHEA) secretion, the two main peripheral secretory products of the hypothalamic-pituitary-adrenal neuroendocrine stress axis, have been well characterized in rest conditions but not in relation to physical exercise. The purpose of one investigation was therefore to determine the effects of an intense 90-min aerobic exercise on the waking diurnal cortisol and DHEA cycles on three separate days [without exercise, with morning exercise (10:00-11:30 h), and with afternoon exercise (14:00-15:30 h)] in nine recreationally trained soccer players. Saliva samples were collected at awakening, 30 min after awakening, and then every 2 h from 08:00 to 22:00 h. A burst of secretory activity was found for cortisol but not for DHEA after awakening. Overall, diurnal decline for both adrenal steroids was observed on resting and exercise days under all conditions. However, there was a significant increase in salivary cortisol concentrations on
the morning-exercise and afternoon-exercise days at, respectively, 12:00 h and 16:00 h, versus the other trials. This acute response to exercise was not evident for DHEA. The results of this investigation indicate that 90 min of intense aerobic exercise does not affect the circadian pattern of salivary adrenal steroids in recreationally trained athletes over a 16-h waking period, despite a transitory increase in post-exercise cortisol concentration. Further studies are necessary to determine whether these results are applicable to elite athletes or patients with cortisol or DHEA deficiency [13263].

**Deposterone**

The indiscriminate use of anabolic androgenic steroids (AAS) has motivated researchers to investigate the mutagenic action of these substances. One study, using the mouse bone marrow micronucleus test, evaluates the genotoxic potential of testosterone cypionate (deposteron). Male Swiss mice received intramuscular injections of deposteron at three doses. The animals were sacrificed 24, 48, or 72h after treatment and bone marrow was removed immediately, followed by scoring to count the micronuclei in 2000 polychromatic erythrocytes (PCE). Two-hundred erythrocytes/animal were analyzed to determine the PCE-NCE (normochromatic erythrocyte) relationship and to determine the cytotoxic effects. The animals treated with deposteron at the highest dose presented greater numbers of micronuclei. The highest dose caused a decrease in the PCE/NCE relationship, indicating a cytotoxic effect. It was conclude that Deposteron is genotoxic and cytotoxic in mice [13264].

**Dromastanolone**

A 26-year-old male presented with three weeks of jaundice after the self-initiation of the injectable anabolic steroid, Mastabol (Dromastanolone Di-Propionate; 17 beta-Hydroxy-2alpha-methyl-5alpha-androstan-3-one propionate). He reported dark urine, light stools, and pruritus. He denied abdominal pain, intravenous drug use, intranasal cocaine, blood transfusions, newly placed tattoos, or sexually transmitted diseases. He used alcohol sparingly. Physical exam revealed jaundice with deep scleral icterus. The liver was palpable 2 cm below the right costal margin with no ascites. The peak bilirubin was 23.6 mg/dL, alkaline phosphatase was 441 units/L, aspartate aminotransferase/alanine aminotransferase were 70 units/L and 117 units/L respectively. A working diagnosis of acute intrahepatic cholestasis was made. Liver biopsy revealed a centrilobular insult with neutrophilic infiltrates and Ito cell hyperplasia consistent with acute drug induced cholestasis. The patient's clinical symptoms resolved and his liver enzymes, bilirubin, and alkaline phosphatase normalized. Anabolic steroids with 17 alpha carbon substitutions have been associated with a bland variety of cholestatic injury with little hepatocellular injury. Cholestasis, under these circumstances, may be secondary to the binding of drugs to canalicular membrane transporters, accumulation of toxic bile acids from canalicular pump failure, or genetic defects in canalicular transport proteins. Mastabol is an injectable, 17 beta hydroxyl compound with no alpha alkyl groups at the 17 carbon position. As such, it has been reported to have little potential toxic effects on the liver. This is the first known reported case of Mastabol-induced cholestatic liver injury. It highlights the need for physicians to consider such widely available substances when faced with hepatic injury of unclear etiology [13265].

**Adrenosterone**

767
Adrenosterone (androst-4-ene-3,11,17-trione, 11-oxoandrostenedione) is an endogenous steroid hormone that has been promoted as a dietary supplement capable of reducing body fat and increasing muscle mass. It is proposed that adrenosterone may function as an inhibitor of the 11beta-hydroxysteroid dehydrogenase type 1 enzyme (11beta-HSD1), which is primarily responsible for reactivation of cortisol from cortisone. The urinary metabolism of adrenosterone was investigated, after a single oral administration in two male subjects, by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Substantially increased excretion of 11beta-hydroxyandrosterone, 11beta-hydroxyetiocholanolone, 11-oxoandrosterone and 11-oxoetiocholanolone was observed. Minor metabolites such as 3alpha,17beta-dihydroxy-5beta-androstan-11-one, 3alpha-hydroxyandrost-4-ene-11,17-dione and 3alpha,11beta-dihydroxyandrost-4-en-17-one were also identified. The exogenous origin of the most abundant adrenosterone metabolites was confirmed by GC-C-IRMS according to World Anti-Doping Agency criteria. Through analysis of a reference population data set obtained from urine samples provided by elite athlete volunteers (n=85), GC-MS doping control screening criteria are proposed: 11beta-hydroxyandrosterone concentration greater than 10 000 ng/mL (specific gravity adjusted to 1.020) or 11beta-hydroxyandrosterone/11beta-hydroxyetiocholanolone ratio greater than 20. Urine samples fulfilling these screening criteria may be subjected to GC-C-IRMS analysis for confirmation of adrenosterone administration [09133].

It was examined the glucuronidation of androsterone (5alpha-androstane-3alpha-ol-17-one), etiocholanolone (5beta-androstane-3alpha-ol-17-one), 5alpha-androstan-3alpha-ol-17-one, 5beta-androstan-3alpha-ol-17-one, androsterone and 5beta-diol (5alpha-diol) and 5beta-androstane-3alpha,17beta-diol (5beta-diol), by 19 recombinant human UDP-glucuronosyltransferases (UGTs). The results reveal large differences in stereo- and regio-selectivity between UGT2B7, UGT2B15 and UGT2B17. UGT2B7 conjugated all 4 androgens at the 3-OH, but not at the 17-OH that is available in both diols. UGT2B7 exhibited a higher glucuronidation rate towards the steroids with a flat backbone, androsterone and 5alpha-diol, in comparison to etiocholanolone and 5beta-diol that have a bent backbone. UGT2B17 readily glucuronidated androsterone and, particularly, etiocholanolone at the 3-OH, but in the two diols it exhibited high preference for the 17-OH and low glucuronidation rate at the 3-OH. UGT2B15 did not glucuronidate any of the studied 4 androgens at the 3-OH, but it did conjugate both diols at the 17-OH, with a clear preference for 5alpha-diol. Of subfamily 1A UGTs, only UGT1A4 catalyzed the glucuronidation of androsterone and 5alpha-diol at measurable rates, even if low. UGT2A1 and UGT2A2 glucuronidated most compounds in this study, but mostly at rather low rates. An exception was the glucuronidation of etiocholanolone by UGT2A1 that revealed a very low substrate affinity in combination with very high Vmax value. The results shed new light on the substrate selectivity of individual UGTs in steroids glucuronidation. In addition they bear implications for doping analyses and its dependence of genetic polymorphism since testosterone is a precursor in the biosynthesis of all these 4 androgens, while the contribution of UGT2B17 to their glucuronidation vary greatly [09134].

Evidence suggests that in manipulating prostaglandin E2 (PGE2), it is unknowingly implicated 3alpha-hydroxysteroid dehydrogenase [E.C. 1.1.1.50], 3(or 17)alpha-hydroxysteroid dehydrogenase [E.C. 1.1.1.209] and their respective products, androsterone (ADT) and epitestosterone (EpiT), in the developmental masculinization of sex behavior. EpiT is generally regarded as a hormonally inactive 17alpha-epimer of testosterone (T). In rats, the kidney is the primary site of EpiT formation, whereas in humans it originates from the gonads, with only a small contribution secreted by the adrenals. Because the ratio of T to EpiT is nearly constant, it is presently used for assessing steroid abuse in competitive sports, where the World Anti-Doping Agency (WADA) considers a T/EpiT ratio >4 evidence of T doping. Despite its central role in the detection of illicit anabolic steroid use, our knowledge of
factors effecting EpiT production is poor. Clues in the literature, however, reveal that prostaglandin-mediated processes, such as LHRH release, may influence its production. Antimycotics, NSAIDs, and opioid analgesics used in sports medicine are all known to effect prostaglandin E2 synthesis. Primary PGs are potent inhibitors of ADT oxidation, while indomethacin, a prostaglandin blocker, powerfully inhibits 3alpha-HSD reduction and ADT oxidation. This is significant because ADT inhibits the oxidation of EpiT, and may modulate its antiandrogenic and neuroprotective effects. It is hypothesized that the T/EpiT ratio is increased by COX-2 inhibitors and opioid analgesics, and decreased by antimycotics that do not impair testosterone biosynthesis. Given the devastating personal and career consequences that may result from false positive drug tests, substantive research on the effects of PGE2 manipulations on EpiT is warranted [07095].

Androstenediol

Doping control laboratories accredited by the World Anti-Doping Agency (WADA) require criteria that allow endogenous steroids to be distinguished from their synthetic analogues in urine. Methodology based on "looking outside the metabolic box" was used in this study to identify diagnostic urinary markers of 4-androstenediol (4-ADIOL) administration. Androst-2,4-diene-17-one and androstan-3,5-diene-17-one are proposed to be formed in urine from acid-catalyzed hydrolysis of 4-ADIOL sulfoconjugate, a major phase II metabolic product of 4-ADIOL. The presence of these markers in the routine gas chromatography-mass spectrometry (GC-MS) steroid screen was suitable to identify samples requiring confirmation by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), to measure the carbon isotope ratio (delta^{13}C) of the androstadiene markers and confirm their likely synthetic origin based on depleted^{13}C content [11100].

Sublingual intake of 21.4 mg androstenediol increases serum testosterone concentrations whereas swallowing 200 mg androstenediol does not. The duration of increase in serum testosterone following sublingual androstenediol (SL-DIOL) is unknown. Resistance exercise (EX) following SL-DIOL may cause larger increases in serum estradiol concentrations than while at rest. This project evaluated the duration of change in, and the effects of acute EX on, the hormonal response to SL-DIOL. Six young resistance trained males consumed either placebo (PL) or SL-DIOL before a single session of EX or no exercise (Rest) in a random, double blind, crossover manner (for a total of four trials). Blood samples were collected before supplementation, and at 60, 120, 180, 240, 480, and 720 min post-supplementation, with the exercise occurring between 60 and 120 min. The serum concentration of total testosterone increased significantly at 60 min similarly in SL-DIOL-EX and SL-DIOL-Rest by approximately 115 percent, and at 120 min by approximately 107 percent with no differences due to exercise. The serum concentration of estradiol increased also significantly similarly in SL-DIOL-EX and SL-DIOL-Rest by approximately 33 percent at 60 min and approximately 45 percent at 120 min, with no differences due to exercise. Serum testosterone returned to baseline by 240 min and serum estradiol returned to baseline by 720 min post-intake. These findings indicate that SL-DIOL acutely elevates serum testosterone and estradiol concentrations, that EX does not alter the endocrine response to SL-DIOL, and that the increases in serum estradiol last between 480 and 720 min while the increases in serum testosterone last <240 min following acute SL-DIOL intake [06114].

Metabolism of 1-androstenediol and 1-androstenedione

1-Androstenediol (5alpha-androst-1-ene-3beta,17beta-diol) and 1-androstenedione (5beta-androst-1-ene-3,17-dione) were introduced on the prohormone market early this millenium.
Androstenedione was previously reported as the only metabolite of boldenone with a 5alpha-configuration. So far, only the in vivo metabolism of 1-androstenediol has been investigated and 1-androstenedione was detected as its major metabolite. An in vitro study using rat liver homogenates showed an extensive mutual metabolism of 1-androstenediol, 1-androstenedione and 1-testosterone, as well as various other hydroxylated metabolites [06004].

3alpha-Androstanediol

The abuse of anabolic-androgenic steroids (AS) is a growing problem; however, the effects and mechanisms underlying their addictive effects are not well understood. Research findings regarding androgen abuse in people and hedonic effects of androgens in laboratory rats are reviewed. Androgens, like other steroids, can have traditional actions via cognate intracellular steroid receptors, as well as other substrates. There are results that indicate that testosterone (T) metabolites may have actions in part via gamma-aminobutyric acid (GABA)(A)/benzodiazepine receptor complexes (GBRs) and/or dopaminergic neurons in the nucleus accumbens, to mediate T's positive hedonic states. This may provide the basis for positive reinforcing effects of androgen seeking and use behavior. Following a comprehensive review of the background literature, findings are presented that have explored the extent to which metabolites of T mediate euphorogenic effects of androgens by acting in the nucleus accumbens. Then results regarding whether GBRs are necessary substrates for androgens' positive hedonic effects are discussed. Lastly, research that addresses if dopaminergic neurons in the nucleus accumbens are necessary for these effects of androgens are discussed. The review provides a comprehensive examination of the hedonic properties and abuse/addiction potential of androgens and the putative mechanisms underlying these effects [07096].

Androstenedione

Androstenedione, the natural precursor of testosterone, is marketed to produce increases in free testosterone. It was widely used by the East German athletes in the 1970s and 1980s and received extensive media attention in 1998 when it was photographed in the locker of Mark McGwire during his bid to break Roger Maris’ single season home run record. Neither androstenedione nor DHEA has ever been definitively shown to increase protein synthesis, body composition, or exercise performance [07002].

Androstenedione (C_{19}H_{26}O_{2}), a precursor to testosterone, was marketed as a natural alternative to anabolic steroids, and was purported to raise blood testosterone levels and promote muscle size and strength. Current research does not support the efficacy of this supplement. In young men, 100-200 mg androstenedione taken once does not increase blood testosterone levels or stimulate muscle protein synthesis, and 100 mg androstenedione three times a day for 8 weeks or twice a day for 12 weeks does not augment gains in muscle size and strength during resistance training. Although a single dose of 300 mg androstenedione may raise blood testosterone levels slightly in young men, it is unlikely that this increase in testosterone would increase muscle size or strength. In women and middle-aged men, acute androstenedione intake raises blood testosterone levels to those typically seen in young men. The effect of a chronic intake of androstenedione on muscle strength and size has not been studied in women. In middle-aged men, 200 mg androstenedione per day does not produce gains in strength more than resistance training alone, and appears to suppress the endogenous production of testosterone. Chronic androstenedione intake may pose significant health risks. High-density lipoprotein cholesterol
is reduced with chronic androstenedione intake, corresponding to a 10-15 percent increase in cardiovascular disease risk. Androstenedione intake raises blood dihydrotestosterone and oestrogen levels, which have been linked to benign prostate hypertrophy, baldness, an increased risk of cardiovascular disease, various forms of cancer and gynecomastia in men. High blood levels of androstenedione may increase the risk of prostate cancer and pancreatic cancer as well as causing neural/behavioural changes such as increased hostility. Elevated blood levels of testosterone in both men and women may pose health risks. In summary, androstenedione does not produce either anabolic or ergogenic effects and may raise the risk of negative health consequences [09135].

Studies have shown that the administration of androstenedione significantly increases the urinary ratio of testosterone glucuronide to epitestosterone glucuronide (T/E) - measured by gas chromatography/mass spectrometry (GC/MS) - in subjects with a normal (approximately 1) or naturally high (>1) initial values. However, the urinary T/E ratio has been shown not to increase in subjects with naturally low (<1) initial values. Such cases then rely on the detection of C(6)-hydroxylated metabolites shown to be indicative of androstenedione administration. While these markers may be measured in the routine GC/MS steroid profile, their relatively low urinary excretion limits the use of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) to specifically confirm androstenedione administration based on depleted (13)C content. A mass spectrometry strategy was used in this study to identify metabolites of androstenedione with the potential to provide compound-specific detection. C(4)-hydroxylation was subsequently shown to be a major metabolic pathway following androstenedione administration, thereby resulting in urinary excretion of 4-hydroxyandrostenedione [09136].

**Hydroxyandrostenedione**

4-Hydroxyandrost-4-ene-3,17-dione is a second generation, irreversible aromatase inhibitor and commonly used as anti breast cancer medication for postmenopausal women. 4-Hydroxytestosterone is advertised as anabolic steroid and does not have any therapeutic indication. Both substances are prohibited in sports by the World Anti-Doping Agency, and, due to a considerable increase of structurally related steroids with anabolic effects offered via the internet, the metabolism of two representative candidates was investigated. Excretion studies were conducted with oral applications of 100mg of 4-hydroxyandrostenedione or 200mg of 4-hydroxytestosterone to healthy male volunteers. Urine samples were analyzed for metabolic products using conventional gas chromatography-mass spectrometry approaches, and the identification of urinary metabolites was based on reference substances, which were synthesized and structurally characterized by nuclear magnetic resonance spectroscopy and high resolution/high accuracy mass spectrometry. Identified phase-I as well as phase-II metabolites were identical for both substances. Regarding phase-I metabolism 4-hydroxyandrostenedione (1) and its reduction products 3beta-hydroxy-5alpha-androstane-4,17-dione (2) and 3alpha-hydroxy-5beta-androstane-4,17-dione (3) were detected. Further reductive conversion led to all possible isomers of 3xi,4xi-dihydroxy-5xi-androstan-17-one (4, 6-11) except 3alpha,4alpha-dihydroxy-5beta-androstane-4,17-dione (5). Out of the 17beta-hydroxylated analogs 4-hydroxytestosterone (18), 3beta,17beta-dihydroxy-5alpha-androstane-4-one (19), 3alpha,17beta-dihydroxy-5beta-androstane-4-one (20), 5alpha-androstane-3beta,4beta,17beta-triol (21), 5alpha-androstane-3alpha,4beta,17beta-triol (26) and 5alpha-androstane-3alpha,4alpha,17beta-triol (28) were identified in the post administration urine specimens. Furthermore 4-hydroxyandrost-4,6-diene-3,17-dione (29) and 4-hydroxyandrost-3,17-dien-3,17-dione (30) were determined as oxidation products. Conjugation was diverse and included glucuronidation and sulfatation [07098].
Androst-4-en-3-one-based steroids

Structure elucidation of steroids by mass spectrometry has been of great importance to various analytical arenas and numerous studies were conducted to provide evidence for the composition and origin of (tandem) mass spectrometry-derived product ions used to characterize and identify steroidal substances. The common product ion at m/z 97 generated from androst-4-en-3-one analogs has been subject of various studies, including stable isotope-labeling and (high resolution/high accuracy) tandem mass spectrometry, but its gas-phase structure has never been confirmed. Using high resolution/high accuracy mass spectrometry and low resolution tandem mass spectrometry, density functional theory (DFT) calculation, and infrared multiple photon dissociation (IRMPD) spectroscopy employing a free electron laser, the structure of m/z 97 derived from testosterone was assigned to protonated 3-methyl-2-cyclopenten-1-one. Product ions of m/z 97 obtained from MS(2) and MS(3) experiments of protonated 3-methyl-2-cyclopenten-1-one, 2-methyl-2-cyclopenten-1-one, 2-cyclohexen-1-one, and testosterone corroborated the suggested gas-phase ion structure, which was eventually substantiated by IRMPD spectroscopy yielding a spectrum that convincingly matched the predicted counterpart. Finally, the dissociation pathway of the protonated molecule of testosterone to m/z 97 was revisited and an alternative pathway was suggested that considers the exclusion of C-10 along with the inclusion of C-5, which was experimentally demonstrated with stable isotope labeling [12181].

Dehydrochloromethyltestosterone

The biotransformation of dehydrochloromethyltestosterone (DHCMT, 4-chloro-17β-hydroxy,17α-methylandrosta-1,4-dien-3-one) in man was studied with the aim to discover long-term metabolites valuable for the antidoping analysis. Having applied a high performance liquid chromatography for the fractionation of urinary extract obtained from the pool of several DHCMT positive urines, about 50 metabolites were found. Most of these metabolites were included in the GC-MS/MS screening method, which was subsequently applied to analyze the post-administration and routine doping control samples. As a result of this study, 6 new long-term metabolites were identified tentatively characterized using GC-MS and GC-MS/MS. The most long-term metabolite M3 was shown to be superior in the majority of cases to the other known DHCMT metabolites, such as 4-chloro-18-nor-17β-hydroxymethyl,17alpha-methylandrosta-1,4,13-trien-3-one and 4-chloro-3alpha,6beta,17beta-trihydroxy-17alpha-methyl-5beta-androst-1-en-16-one [12182].

Six formerly unrecognized urinary metabolites of dehydrochloromethyltestosterone (DHCMT, Oral-Turinabol) were characterized in post-administration study urine samples by means of GC-MS and GC-MS/MS. These additional target analytes were tentatively identified and the candidate referred to as 4-chloro-18-nor-17beta-hydroxymethyl-17alpha-methyl-5beta-androst-13-en-3alpha-ol was found to be traceable for a longer period of time than those commonly used to uncover doping with DHCMT. Proof of the attributed composition of the metabolite by means of chemical synthesis (or nuclear magnetic resonance spectroscopy, NMR) however remains to be presented [13012].

Fluoxymesterone

Employing LC-HRMS, the metabolism of fluoxymesterone was revisited and three metabolic products were described, potentially complementing routine doping controls. The structures
of these analytes were reported as 9-fluoro-17beta-ol-17alpha-methyl-11-en-5alpha-<br>androstan-3-one, its isomer 9-fluoro-17beta-ol-17alpha-methyl-11-en-5beta-androstan-3-one,<br>and 9-fluoro-17beta-ol-17alpha-methyl-5-androstan-3,6,11-trione as attributed on the basis of<br>HRMS and MS/MS data. Unfortunately, neither isotope-labeling nor comprehensive MS° or<br>H/D exchange experiments were conducted to corroborate the comparably speculative<br>dissociation pathways presented in the article, which represented the sole basis of structure<br>assignments. Also here, substantiated evidence (e.g. by chemical synthesis or NMR from<br>metabolites isolated from urine) remains to be provided [13012].

**Desoxymethyltestosterone**

In a commendable manner, the in vitro and chemical synthesis of urinary metabolites of<br>desoxymethyltestosterone (DMT, madol) followed by NMR characterization and comparison<br>to authentic administration study urine samples with GC-MS was presented. Although<br>employed as a target analyte in routine doping controls for several years, proof for the<br>assumed structure of the main metabolite was not available; hence, the proposed<br>composition of the metabolite was to be substantiated, which was accomplished by means of<br>human hepatocytes as well as chemical synthesis that eventually enabled the confirmation of<br>the analyte as 17alpha-methyl-2beta,3alpha,17beta-trihydroxy-5alpha-androstane [13012].

**Keto-androgens**

Prostate cancer is the most frequently diagnosed form of cancer in males in the United<br>States. The disease is androgen driven and the use of orchiectomy or chemical castration,<br>known as androgen deprivation therapy (ADT) has been employed for the treatment of<br>advanced prostate cancer for over 70 years. Agents such as GnRH agonists and non-<br>steroidal androgen receptor antagonists are routinely used in the clinic, but eventually<br>relapse occurs due to the emergence of castration-resistant prostate cancer. With the<br>appreciation that androgen signaling still persists in these patients and the development of<br>new therapies such as abiraterone and enzalutamide that further suppress<br>es androgen synthesis or signaling, there is a renewed need for sensitive and specific methods to quantify<br>androgen precursor and metabolite levels to assess drug efficacy. It was described the<br>development, validation and application of a stable isotope dilution liquid chromatography<br>electrospray ionization selected reaction monitoring mass spectrometry (SID-LC/ESI/SRM/MS) method for quantification of serum keto-androgens and their sulfate and<br>glucuronide conjugates using Girard-T oxime derivatives. The method is robust down to 0.2-<br>4pg on column, depending on the androgen metabolite quantified, and can also quantify<br>dehydroepiandrosterone sulfate (DHEA-S) in only 1 microL of serum. The clinical utility of<br>this method was demonstrated by analyzing serum androgens from patients enrolled in a<br>clinical trial assessing pharmacological agents to maximally suppress gonadal and adrenal androgens (Targeted Androgen Pathway Suppression, TAPS clinical<br>trial). The method was validated by correlating the results obtained with a hydroxylamine<br>derivatization procedure coupled with tandem mass spectrometry using selected reaction<br>monitoring that was conducted in an independent laboratory [13266].

**Trenbolone**

Trenbolone (17beta-hydroxy-estra-4,9,11-trien-3-one) and its derivatives such as 17alpha-<br>methyltrenbolone represent a class of highly potent anabolic-androgenic steroids, which are
prohibited in sports according to the regulation of WADA. Due to marginal gas chromatographic properties of these compounds but excellent proton affinities resulting from a large and conjugated pi-electron system, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been the method of choice for the detection of these analytes in sports drug testing. Recent findings of trenbolone and methyltrenbolone in doping control urine samples of elite athletes demonstrated the importance of a sensitive and robust analytical method, which was based on an enzymatic hydrolysis of target compounds, liquid-liquid extraction and subsequent LC-MS/MS measurement. Diagnostic product ions obtained after collision-induced dissociation of protonated molecules were found at m/z 227, 211, 199 and 198, which enabled targeted screening using multiple reaction monitoring. Using 7 model compounds (trenbolone, epitrenbolone, methyltrenbolone, ethyltrenbolone, propyltrenbolone, 17-ketotrenbolone and altrenogest), the established method was validated for specificity, lower limits of detection (0.3-3 ng/mL), recovery (72-105 %), intraday and interday precision < 20 percent [08162].

Selective androgen receptor modulators (SARMs) now under development can protect against muscle and bone loss without causing prostate growth or polycythemia. 17β-Hydroxyestra-4,9,11-trien-3-one (trenbolone), a potent testosterone analog, may have SARM-like actions because, unlike testosterone, trenbolone does not undergo tissue-specific 5α-reduction to form more potent androgens. It was tested the hypothesis that trenbolone-enanthate (TREN) might prevent orchietomy-induced losses in muscle and bone and visceral fat accumulation without increasing prostate mass or resulting in adverse hemoglobin elevations. Male F344 rats aged 3 mo underwent orchietomy or remained intact and were administered graded doses of TREN, supraphysiological testosterone-enanthate, or vehicle for 29 days. In both intact and orchietomized animals, all TREN doses and supraphysiological testosterone-enanthate augmented androgen-sensitive levator ani/bulbocavernosus muscle mass by 35-40 percent above shams and produced a dose-dependent partial protection against orchietomy-induced total and trabecular bone mineral density losses and visceral fat accumulation. The lowest doses of TREN successfully maintained prostate mass and hemoglobin concentrations at sham levels in both intact and orchietomized animals, whereas supraphysiological testosterone-enanthate and high-dose TREN elevated prostate mass by 84 and 68 percent, respectively. In summary, low-dose administration of the non-5α-reducible androgen TREN maintains prostate mass and hemoglobin concentrations near the level of shams while producing potent myotrophic actions in skeletal muscle and partial protection against orchietomy-induced bone loss and visceral fat accumulation. The findings indicate that TREN has advantages over supraphysiological testosterone and supports the need for future preclinical studies examining the viability of TREN as an option for androgen replacement therapy [11102].

Trenbolone (TRE) is a steroid used by veterinarians on livestock to increase appetite and body weight. The use of TRE has been restricted because of its harmful side effect for consumers. To effectively control TRE residue in food and food product, a rapid and convenient immunoassay was developed by preparing an anti-TRE monoclonal antibody. The immunogen and coating antigen were prepared by coupling TRE hapten with carrier proteins via 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) method. The optimized method gave an average IC₅₀ value of 0.323 ng/mL towards TRE and an average detection limit (LOD) of 0.06 ng/mL, which is much lower than the maximum residue levels (2.0 ng/g) accepted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The specificity of the antibody was evaluated by measuring cross-reactivity of six structurally related compounds, including 19-nortestosterone (9.7 %), testosterone (0.13 %), methyltestosterone (<0.01 %), methandrosteneolone (<0.01 %), (+)-dehydroisoandrosterone (<0.001 %) and beta-estradiol (<0.001 %). The recovery rates of the test in detection of TRE-fortified animal tissue, urine and animal feed samples were in the range of 81-89 percent,
while the intra- and inter-assay coefficients of variation were less than 12 percent [11103].

Despite the widespread use of the anabolic androgen trenbolone acetate (TBA) in animal agriculture, evidence demonstrating the occurrence of TBA metabolites such as 17beta-trenbolone (17beta-TBOH), 17alpha-trenbolone (17alpha-TBOH), and trenbolone (TBO) is relatively scarce, potentially due to rapid transformation processes such as direct photolysis. Therefore, we investigated the phototransformation of TBA metabolites and associated ecological implications by characterizing the photoproducts arising from the direct photolysis of 17beta-TBOH, 17alpha-TBOH, and TBO and their associated ecotoxicity. LC-HRMS/MS analysis identified a range of hydroxylated products that were no longer photoactive, with primary photoproducts consisting of monohydroxy species and presumptive diastereomers. Also observed were higher-order hydroxylated products probably formed via subsequent reaction of primary photoproducts. NMR analysis confirmed the formation of 12,17-dihydroxy-estra-5(10),9(11),dien-3-one (12-hydroxy-TBOH; 2.2 mg), 10,12,17-trihydroxy-estra-4,9(11),dien-3-one (10,12-dihydroxy-TBOH; 0.7 mg), and a ring-opened 11,12-dialdehyde oxidation product (TBOH-11,12-dialdehyde; 1.0 mg) after irradiation of about 14 mg of 17beta-trenbolone. Though unconfirmed by NMR, our data suggest that the formation of additional isomeric products may occur, likely due to the reactivity of the unique 4,9,11 conjugated triene structure of trenbolone. In vivo exposure studies employing Japanese medaka (Oryzias latipes) indicate that low concentrations of 17alpha-TBOH photoproduct mixtures can alter ovarian follicular development, and photoproducts alter whole-body 17beta-estradiol levels. Therefore, direct photolysis yields photoproducts with strong structural similarity to parent steroids, and these photoproducts still retain enough biological activity to elicit observable changes to endocrine function at trace concentrations. These data indicate that environmental transformation processes do not necessarily reduce steroid hormone ecotoxicity [13248].

17beta-Hydroxyestra-4,9,11-trien-3-one or trenbolone is an anabolic steroid used in some meat producing countries where its use is licenced. In cattle it is metabolised into 17alpha-trenbolone. It was required to make 17alpha-[4-14C]trenbolone for use in environmental fate studies. At the same time we also had a request to make 17alpha-[4-14C]estradiol so it was combined the two syntheses and made use of the synergy to allow us to make a batch of 17alpha-[4-14C]estradiol by known methodology and then elaborate a portion of this into 17alpha-[4-14C]trenbolone. The synthesis of 17alpha-[4-14C]trenbolone from 17alpha-[4-14C]estradiol was achieved in 8 steps and 3.1 percent overall yield to give material with a radiochemical purity of 99.5 percent and specific activity of 59 mCi/mmol [13249].

Both androgenic and estrogenic steroids are widely used as growth promoters in feedlot steers because they significantly enhance feed efficiency, rate of gain, and muscle growth. However, despite their widespread use relatively little is known about the biological mechanism by which androgenic and estrogenic steroids enhance rate and efficiency of muscle growth in cattle. Treatment of feedlot steers with a combined estradiol (E2) and trenbolone acetate (TBA) implant results in an increased number of muscle satellite cells, increased expression of IGF-1 mRNA in muscle tissue, and increased levels of circulating IGF-1. Similarly, treatment of bovine satellite cell (BSC) cultures with either TBA or E2 results in increased expression of IGF-1 mRNA, increased rates of proliferation and protein synthesis, and decreased rates of protein degradation. Effects of E2 on BSC are mediated at least in part through the classical E2 receptor, estrogen receptor-alpha (ESR1), the IGF-1 receptor (IGFR1), and the G protein-coupled estrogen receptor-1 (GPER-1), formerly known as G protein-coupled receptor-30 (GPR30). The effects of TBA appear to be primarily mediated through the androgen receptor. Based on current research results, it is becoming clear that anabolic steroid-enhanced bovine muscle growth involves a complex interaction of numerous pathways and receptors. Consequently, additional in vivo and in vitro studies are
necessary to understand the mechanisms involved in this complex process. The fundamental information generated by this research will help in developing future, safe, and effective strategies to increase rate and efficiency of muscle growth in beef cattle [13250].

Trenbolone acetate (TBA) is a high-value steroidal growth promoter often administered to beef cattle, whose metabolites are potent endocrine-disrupting compounds. We performed laboratory and field phototransformation experiments to assess the fate of TBA metabolites and their photoproducts. Unexpectedly, we observed that the rapid photohydration of TBA metabolites is reversible under conditions representative of those in surface waters (pH 7, 25°C). This product-to-parent reversion mechanism results in diurnal cycling and substantial regeneration of TBA metabolites at rates that are strongly temperature- and pH-dependent. Photoproducts can also react to produce structural analogs of TBA metabolites. These reactions also occur in structurally similar steroids, including human pharmaceuticals, which suggests that predictive fate models and regulatory risk assessment paradigms must account for transformation products of high-risk environmental contaminants such as endocrine-disrupting steroids [13251].

Experimentally

The toxicokinetics of trenbolone was characterized during 500 ng/l water exposures in female rainbow trout (Oncorhynchus mykiss) and fathead minnows (Pimephales promelas). Related experiments measured various toxicodynamic effects of exposure. In both species, trenbolone was rapidly absorbed from the water and reached peak plasma levels within 8 h of exposure. Afterwards, trenbolone concentrations in trout (66-95 ng/ml) were 2-6 times higher compared with minnows (15-29 ng/ml), which was attributable to greater plasma binding in trout. During water exposures, circulating levels of estradiol (E2) rapidly decreased in both species to a concentration that was 25-40 percent of control values by 8-24 h of exposure and then remained relatively unchanged for the subsequent 6 days of exposure. In trout, changes in circulating levels of follicle-stimulating hormone were also significantly greater after trenbolone exposure, relative to controls. In both species, the pharmacokinetics of injected E2-d3 was altered by trenbolone exposure with an increase in total body clearance and a corresponding decrease in elimination half-life. The unbound percentage of E2 in trout plasma was 0.25 percent, which was similar in pre- or postvitellogenic female trout. Subsequent incubation with trenbolone caused the unbound percentage to significantly increase to 2.4 percent in the previtellogenic trout plasma. iTRAQ-based toxicoproteomic studies in minnows exposed to 5, 50, and 500 ng/l trenbolone identified a total of 148 proteins with 19 downregulated including vitellogenin and 18 upregulated. Other downregulated proteins were fibrinogens, α-2-macroglobulin, and transferrin. Upregulated proteins included amine oxidase, apolipoproteins, parvalbumin, complement system proteins, and several uncharacterized proteins. The results indicate trenbolone exposure is a highly dynamic process in female fish with uptake and tissue equilibrium quickly established, leading to both rapid and delayed toxicodynamic effects [13252].

1-Testosterone

It is assumed that 1-testosterone (5alpha-androst-1-ene-17beta-ol-3-one) cannot be produced through metabolism of 1,4-androstadiene-type steroids due to inhibition of 5alpha-reductase by the 1,2 double bond. In addition to the parent drug, 5alpha-androst-1-ene-3alpha-ol-17-one and 5alpha-androst-1-ene-3,17-dione were identified as metabolites in the glucuronide fraction. 5alpha-androst-1-ene-3alpha-ol-17-one was identified as the major metabolite of 1-testosterone. Moreover, reduction of the delta1 double bond resulted in
increased dihydrotestosterone concentrations (DHT) and consequently the rise of androsterone and other 5alpha-steroids (5alpha-androstane-3alpha,17beta-diol). A notable increase of the testosterone to epitestosterone ratio was also noticed [06004].

**Metabolism**

Since the beginning of the year 2005, the use of steroid precursors (prohormones) is illegal in the United States; nevertheless, there is still an enormous abuse of such substances. One of the most frequently misused steroids, often declared to be a prohormone, is 1-testosterone (17beta-hydroxy-5alpha-androst-1-en-3-one, 1-Testo). In one study, it was characterised molecular mechanisms of its action, determined its tissue specific androgenic and anabolic potency and investigated potential adverse effects. 1-Testo binds highly selective to the androgen receptor (AR) and has a high potency to stimulate AR dependent transactivation. In vivo an equimolar dose of 1-Testo has the same potency to stimulate the growth of the prostate, the seminal vesicles and the androgen sensitive levator ani muscle as the reference compound testosterone propionate (TP). Administration of 1-Testo, in contrast to TP, results in a significant increase of liver weight. The results demonstrate that 1-Testo, even without being metabolised, is a very potent androgen. It binds selectively to the AR and transactivates AR dependent reporter genes. In vivo it has a high androgenic and anabolic potency and increases liver weight. In summary 1-Testo can be characterised as a typical anabolic steroid. It has to be assumed that consumption of this substance is associated with adverse side effects typical for this class of compounds [06115].

**Metenolone**

The aim of one study was the investigation of effects of the metenolone enanthate (ME) that is used among athletes as doping and muscle amplifier, on hearts of male and female rats that are in puberty using morphometrical methods. A total of 36 rats which were divided into three separate groups (experiment, ME; vehicle, PO; control, C) each consisting of 6 male and 6 female rats were used. 0.5 mg/kg metenolone enanthate was applied intraperitoneally into experiment subjects 5 times a week over a period of 4 weeks. At the end of experiment, rats were euthanized and their hearts were cut at the level of musculus papillaris after the fixation in formalin. Hearts were taken out and embedded in paraffin wax. Photos were taken at cut surfaces, and thickness, diameters and surface area levels were measured. Left ventriculus mass (LVM) and left ventriculus mass index (LVMI) were calculated. In the study LVM and LVMI were found to be significantly higher in the ME group in females whereas left ventricular lumen diameter (LVLD) were found to be significantly lower. Thus left ventricular hypertrophy development was observed. LVM and LVMI were found to be similar in ME and C groups among male rats and the highest level of these data were found in the group. LVM and LVMI were higher among females. In conclusion, it has been shown that the adverse effects of ME on heart were developing starting from puberty and resulting with the enlargement of the heart and left ventricular hypertrophy and especially among females this condition was more evident. It has also been discussed that the continuous use of drugs may further enhance this condition [13253].

**Metandienone**

The metabolism of a variety of anabolic steroids frequently misused for doping purposes has been investigated in the last years. This research mainly focused on main and long-term metabolites suitable for detection, but detailed clearance mechanisms have rarely been
elucidated. Recent studies on metandienone focused on the identification of 17beta-hydroxymethyl-18alpha-methyl-18-norandrosta-1,4,13-trien-3-one (20betaOH-NorMD) as long-term metabolite, however, the metabolic pathway of its generation remained unclear. Metandienone and its Wagner-Meerwein rearrangement product 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one (NorMD) were hydroxylated by different human cytochrome P450 enzymes (CYPs). Some of their hydroxylation products were chemically synthesized and characterized by mass spectrometry to allow for their trace detection in urine samples. Following oral administration of metandienone or NorMD in one human volunteer each the post administration urines were checked for the presence of those hydroxylated metabolites using GC-MS/MS analysis. The human mitochondrial steroid hydroxylating enzymes CYP11B1 and CYP11B2 were capable to metabolize metandienone leading to the formation of 11beta-hydroxymetandienone and 18-hydroxymetandienone. Following Wagner-Meerwein rearrangement, the resulting products could be assigned to 20betaOH-NorMD and 11betaOH-NorMD. The contribution of CYP11B1 and CYP11B2 in human metabolism of metandienone was confirmed by analysis of post-administration samples of metandienone and NorMD. Combined with the results from a previous study, enzymatic pathways were identified that involve CYP21 and CYP3A4 in the hydroxylation of NorMD, while CYP21, CYP3A4 and CYP11B2 take part in 20betaOH-NorMD generation from MD. The current study represents a valuable contribution to the elucidation of clearance mechanisms of anabolic steroids and also indicates that mainly non-liver CYPs seem to be involved in these processes [12512].

There is increasing evidence that the biological activity of myostatin (MSTN), a negative regulator of muscle growth, is affected by training but also anabolic steroids. In this study, we analyzed the effects of the frequently abused anabolic steroid methandienone (Md) on the hypothalamic-pituitary-testicular axis and androgen-sensitive tissues in intact rats performing a treadmill training to simulate the situation of abusing athletes. The anabolic effects were correlated with the expression of members of the MSTN signaling cascade. Md treatment resulted in a significant stimulation of anabolic activity of the levator ani muscle, which was further increased by training, while prostate and seminal vesicle weights decreased in conformance with hormone concentrations of LH and testosterone. In gastrocnemius muscle, mRNA expression of genes of the MSTN signaling cascade (MSTN, Smad7 and MyoD) was reduced by training but not after Md treatment, in soleus muscle MSTN and its inhibitors, follistatin (FLST) and Smad-7 were only affected after training in combination with Md treatment. In summary, our data demonstrate that Md treatment of intact rats results in anabolic effects which are enhanced in combination with physical activity. Interestingly, the anabolic activity on the levator ani was increased in combination with training, although the levator ani muscle was not specifically stimulated by our training protocol. In the m. gastrocnemius and soleus, the anabolic effects correlate with changes in the expression patterns of genes involved in MSTN signaling. The data provide evidence that the decrease in the weight of androgen-sensitive sexual glands, observed after Md treatment, is caused by a suppression of endogenous testosterone synthesis. These observations provide new insights into the molecular mechanisms of the interaction between anabolic steroids, training and MSTN signaling during skeletal muscle adaptation [12096].

**Laboratory technique**

Metandienone is one of the most frequently detected anabolic androgenic steroids in sports drug testing. Metandienone misuse is commonly detected by monitoring different metabolites excreted free or conjugated with glucuronic acid using gas chromatography mass spectrometry (GC-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) after hydrolysis with beta-glucuronidase and liquid-liquid extraction. It is known that several
metabolites are the result of the formation of sulphate conjugates in C17, which are converted to their 17-epimers in urine. Therefore, sulphation is an important phase II metabolic pathway of metandienone that has not been comprehensively studied. The aim of this work was to evaluate the sulphate fraction of metandienone metabolism by LC-MS/MS. Seven sulphate metabolites were detected after the analysis of excretion study samples by applying different neutral loss scan, precursor ion scan and SRM methods. One of the metabolites (M1) was identified and characterised by GC-MS/MS and LC-MS/MS as 18-nor-17beta-hydroxymethyl-17alpha-methylandrost-1,4,13-triene-3-one sulphate. M1 could be detected up to 26 days after the administration of a single dose of metandienone (5 mg), thus improving the period in which the misuse can be reported with respect to the last long-term metandienone metabolite described (18-nor-17beta-hydroxymethyl-17alpha-methylandrost-1,4,13-triene-3-one excreted in the glucuronide fraction) [13254].

In sports drug testing, comprehensive studies on the metabolism of therapeutic agents with misuse potential are necessary to identify metabolites that provide utmost retrospectivity and specificity. By commonly employed approaches minor and/or long-term metabolites in urine might remain undetected. Hence, an alternative strategy to unambiguously identify the majority of urinary metabolites including low-abundance representatives is desirable. Urine samples were collected for 20 days during an elimination study with an oral dose of 5 mg of 17alpha-C2H3-metandienone. The specimens were processed according to established sample preparation procedures (including fractionation and deconjugation) and subjected to gas chromatography/hydrogen isotope ratio mass spectrometry (GC/IRMS) analysis. Due to the deuteration of the administered drug, urinary metabolites bearing the deuterium label yield abundant and specific signals on the GC/IRMS instrument resulting from the substantially altered D/H ratio. The sample aliquots were measured by gas chromatography/time-of-flight (GC/Q-TOF) mass spectrometry using identical GC conditions, allowing high-resolution/high-accuracy mass data to be obtained on all urinary metabolites previously identified by IRMS. Within the IRMS chromatograms, labeled metabolites were identified up to 20 days after administration at urinary concentration down to 0.25 ng/mL. More than 50 metabolites were observed with the earlier described long-term metabolite of metandienone, 18-nor-17beta-hydroxymethyl,17alpha-methyl-androst-1,4,13-trien-3-one, being the most prominent glucuronidated metabolite in the studied time window. In the sulfocojugated steroids fraction, a yet unknown metabolite was observed at m/z 283.1997 comprising the experimentally determined elemental composition of C20H21O. In conclusion, combining IRMS with high-resolution mass spectrometry considerably facilitates and accelerates metabolite identification of deuterium-labeled compounds in urine. Of particular relevance in doping control, the principle is applicable also to other arenas of drug research, allowing the preparation and administration of e.g. radioactively labeled substances to be omitted [0255].

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improving the period in which the misuse can be reported with respect to the last long-term metandienone metabolite described (18-nor-17beta-hydroxymethyl-17alpha-methylandrost-1,4,13-triene-3-one excreted in the glucuronide fraction) [13256].

In urine
Anabolic-androgenic steroids are some of the most frequently detected drugs in amateur and professional sports. Doping control laboratories have developed numerous assays enabling the determination of administered drugs and/or their metabolic products that allow retrospectives with respect to pharmacokinetics and excretion profiles of steroids and their metabolites. A new metabolite generated from metandienone has been identified as 18-nor-17beta-hydroxymethyl,17alpha-methyl-androst-1,4,13-trien-3-one in excretion study urine samples providing a valuable tool for the long-term detection of metandienone abuse by athletes in sports drug testing. The metabolite was characterized using gas chromatography/(tandem) mass spectrometry, liquid chromatography/(tandem mass spectrometry and liquid chromatography/high-resolution/high-accuracy (tandem) mass spectrometry by characteristic fragmentation patterns representing the intact 3-keto-1,4-diene structure in combination with typical product ions substantiating the proposed C/D-ring structure of the steroid metabolite. In addition, structure confirmation was obtained by the analysis of excretion study urine specimens obtained after administration of 17-CD(3)-labeled metandienone providing the deuterated analogue to the newly identified metabolite. 18-Nor-17beta-hydroxymethyl,17alpha-methyl-androst-1,4,13-trien-3-one was determined in metandienone administration study urine specimens up to 19 days after application of a single dose of 5 mg, hence providing an extended detection period compared with commonly employed strategies [06116].

In hair
A sensitive, specific and reproducible method for the quantitative determination of the anabolic metandienone in human hair has been developed. The preparation involved a decontamination step with methylene chloride. The hair sample (about 50 mg) was solubilised in 1 ml 1 M NaOH, 10 min at 95 degrees C, in presence of 2 ng of nandrolone-d(3) used as internal standard. The homogenate was neutralized and extracted using consecutively a solid-phase extraction (Isolute C(18) eluted with methanol) and a liquid-liquid extraction with pentane. The residue was derivatized by adding 5 microl MSTFA/NH(4)I/2-mercaptoethanol (250 microl; 5 mg; 15 microl) and 45 microl MSTFA, then incubated for 20 min at 60 degrees C. A 1 microl aliquot of derivatized extract was injected into the column (HP5-MS capillary column, 5 % phenyl-95 % methylsiloxane, 30 m x 0.32 mm i.d., 0.25 microm film thickness) of a Hewlett Packard (Palo Alto, CA, USA) gas chromatograph (6890 Series). Metandienone was identified using three transitions (its daughter ions at m/z 339 and 206 for the parent 444 and 191 for 206) using a Waters Quattro Micro MS-MS system. The transition m/z 444 to 206 has been used as quantification transition and the others as identification transitions. The assay was capable of detecting 2 pg/mg of metandienone when approximately 50 mg of hair material was processed. Linearity was observed for metandienone concentrations ranging from 2 to 500 pg/mg with a correlation coefficient of 0.9997. Intra-day and between-day precisions at 50 pg/mg were 13.4-16.5 percent and 22 percent, respectively, with an extraction recovery of 48 percent. The analysis of hair, cut into four segments, obtained from an athlete, revealed the presence of metandienone at the concentrations of 78, 7, 10 and 108 pg/mg in each segment of hair (0-1, 1-2, 2-3 and 3 cm to the tip) [06117].

Recently, a previously unknown urinary metabolite of metandienone, 17beta-hydroxymethyl-17 alpha-methyl-18-norandrosa-1,4,13-trien-3-one (20OH-NorMD), was discovered via LC-MS/MS and GC-MS. This metabolite was reported to be detected in urine samples up to 19 days after administration of metandienone. However, so far it was not possible to obtain
purified reference material of this metabolite and to confirm its structure via NMR. Eleven recombinant strains of the fission yeast Schizosaccharomyces pombe that express different human hepatic or steroidogenic cytochrome P450 enzymes were screened for production of this metabolite in a whole-cell biotransformation reaction. 17,17-Dimethyl-18-norandrosta-1,4,13-trien-3-one, chemically derived from metandienone, was used as substrate for the bioconversion, because it could be converted to the final product in a single hydroxylation step. The obtained results demonstrate that CYP21 and to a lesser extent also CYP3A4 expressing strains can catalyze this steroid hydroxylation. Subsequent 5 l-scale fermentation resulted in the production and purification of 10 mg of metabolite and its unequivocal structure determination via NMR. The synthesis of this urinary metandienone metabolite via S. pombe-based whole-cell biotransformation now allows its use as a reference substance in doping control assays [10088].

Mestranolon

Skeletal muscle has the ability to respond to new endogenous and exogenous physiological demands by changing its phenotypic characteristics. The large diversity of myosin heavy chain (MHC) isoforms expressed in muscle helps to form the basis for this remarkable plasticity. It is the expression and co-expression of these various MHC isoforms within a given fiber that ultimately delineates the entire range of fiber types. The prevalence of certain types of these fibers accounts for the functional and structural characteristics of a given muscle, and hence its phenotype. In an attempt to shorten recovery time and improve performance, strength and endurance athletes occasionally turn to the illicit use of anabolic-androgenic steroids (AAS). The high-intensity, aerobic exercise caused a significant increase in muscle wet weights with an additional increase following mesterolone treatment. In all three muscles, the effect of mesterolone in sedentary mice was stronger than in exercised mice suggesting that the effect of exercise partially blunted the anabolic effect of mesterolone. Skeletal muscles express a variety of different types of proteins associated with metabolism, inflammation, and contractile activity in response to exercise. A large number of different systemic and muscular proteins could also result from anabolic-androgenic steroid treatment. Although the elucidation of the complex molecular mechanisms underlying the ergogenic effects of AAS treatment was not the aim of the present study, muscle function may be improved by increasing protein synthesis or membrane stabilization. This may be accomplished via a competitive occupancy of glucocorticoid receptors by AAS which would act antagonistically to the catabolic action of glucocorticoid hormones. In the present study, adaptive differences observed among the three muscles examined implies alternative binding of the steroid to a number of androgen receptors resulting in agonistic promotion of skeletal muscle protein synthesis. Androgens receptors (AR) are expressed in satellite cells, differentiated myofibers, intramuscular fibroblasts, and different types of motoneurons. In addition, the regulation of plasmatic levels of insulin-like growth factor-1 (IGF-1), growth hormone, and thyroid hormone (as well as, the antagonism of glucocorticoids) are roles linked to anabolic-androgenic signaling. One study was undertaken to examine the effects of mesterolone (an anabolic-androgenic steroid) on the fiber type composition and cross-sectional area of skeletal muscle fibers of sedentary and high-intensity, aerobically-exercised transgenic mice. Thus, the aim was to investigate the role of mesterolone in a supposed catabolic environment. Does the interaction of anabolic hormone treatment and high-intensity aerobic exercise produce an increase in muscle mass and redistribution of skeletal muscle fiber types? Are muscles with distinct metabolic and contractile properties (i.e., fast/glycolytic vs slow/oxidative) differently modulated by the anabolic-androgenic steroid treatment combined with a high-intensity, endurance-type training program? As such, three skeletal muscles were studied under these experimental conditions: soleus (SOL), tibialis anterior.
(TA) and gastrocnemius (GAS). The entire range of pure and hybrid fiber types were delineated using myofibrillar adenosine triphosphatase (mATPase) histochemical methods allowing the detection of subtle changes in type composition. The mice used in the current study were genetically engineered to exhibit a lipid profile closer to humans allowing for a more relevant comparison. The transgenic mice (CETP(+/-)LDLr(-/-)) were engineered to exhibit a lipid profile closer to humans. Animals were divided into groups of sedentary (Sed) and/or training (Ex) mice (each treated orally with AAS or gum arabic/vehicle: Sed-C, Sed-M, ex-C, ex-M). The effects of AAS (mesterolone: M) on specific phenotypic adaptations (muscle wet weight, cross-sectional area, and fiber type composition) in three hindlimb muscles (soleus:SOL, tibialis anterior:TA and gastrocnemius:GAS) were assessed. In order to detect subtle changes in fiber type profile, the entire range of fiber types (I, IC, IIAC, IIA, IIAD, IID, IIB) was delineated using mATPase histochemistry. Body weight gain occurred throughout the study for all groups. However, the body weight gain was significantly minimized with exercise. This effect was blunted with mesterolone treatment. Both AAS treatment (Sed-M) and high-intensity, aerobic training (ex-C) increased the wet weights of all three muscles and induced differential hypertrophy of pure and hybrid fibers. Combination of AAS and training (ex-M) resulted in enhanced hypertrophy. In the SOL, mesterolone treatment (Sed-M and ex-M) caused dramatic increases in the percentages of fiber types IC, IIAC, IIAD, IID, with concomitant decrease in IIA, but had minimal impact on fiber type percentages in the predominantly fast muscles. Overall, the AAS-induced differential adaptive changes amounted to significant fiber type transformations in the fast-to-slow direction in SOL. AAS treatment had a significant effect on muscle weights and fiber type composition in SOL, TA and GAS which was even maximized in animals subjected to metabolically high-intensity aerobic exercise [13257].

**Norbolethone**

More than 100 synthetic derivatives of testosterone have been developed. As an example, norbolethone is a steroid that was developed in the 1960s by Wyeth Pharmaceuticals in Philadelphia to treat children with growth problems; it was never marketed. However, It was popular as a performance-enhancing drug because of its high anabolic activity and low androgenic activity [07002].

Norbolethone (17beta-hydroxy-13beta,17alpha-diethylgon-4-en-3-one) is an anabolic steroid found to be 16.3 times more effective in retaining nitrogen in rats than methyltestosterone. Although its promising anabolic:androgenic dissociation, this steroid was never commercialized due to its toxicity and was not explicitly listed as a prohibited substance in sports until its detection. Norbolethone is excreted as the parent compound and is metabolised to 13beta,17alpha-diethyl-5beta-gonane-3alpha,17beta-diol diol (minor metabolite) [06004].

**Phytosterols**

Cholesterol is a well-known component in fats of animal origin and it also is the precursor of natural hormones. Phytosterols appear in plants and only differ slightly in structure from cholesterol. An important difference however is the low absorption in the gut of phytosterols and their saturated derivatives, the phytostanols. As a result, there is time for all kind of reactions in faecal material inside and outside of the gut. Determination of the abuse of natural hormones may be based on gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Abuse of natural hormones changes the $^{13}$C/$^{12}$C ratio of some
metabolites during a relatively long time. The formation of (natural) hormones in the gut may interfere with this method. Designer drugs are mainly known from sports doping. In animal fattening, designer drugs may be used as well. Small changes in the structure of (natural) hormones may lead to a new group of substances asking for new strategies for their detection and the constatation of their abuse [06126].

Plant sterols are also known as phytosterols (or phytostanols when saturated). Most commonly, phytosterols are produced from a cycloartenol precursor which makes their synthesis different from animal sterols (zoosterols) which are derived from a lanosterol precursor. Various phytosterols have been discussed previously including gamma-oryzanol, octosanol and policosanol, and supplements containing Cissus quadrangularis. The most abundant phytosterol is sitosterol. There are differing outcome data from studies using sitosterol supplementation in terms of immune function and plasma lipid regulation. No precise effect on the athletic performance of phytosterols in general, including sitosterol, has been observed. Several studies have demonstrated that sitosterol is the most abundant phytosterol in plants and consequently in Western diets. Ultramarathon runners supplemented with β-sitosterol capsules displayed fewer cellular markers of inflammation (particularly those related to neutrophils) after a race compared with placebo-treated controls, suggesting that phytosterols may mitigate the effects of acute, strenuous exercise on immunity. However, in a study of trained runners subjected to an exercise bout of increasing intensity until exhaustion, the heart rate and blood lactate were uninfluenced by phytosterols. In a study of older sedentary adults, 6 months of endurance training reduced plasma cholesterol levels while increasing the absorption of phytosterols contained in a normal diet, suggesting that phytosterols may have beneficially regulated plasma lipids. The current limited research suggests that beta-sitosterol may mitigate the exercise-associated dysregulation of immune function and improve blood lipid profiles, though it has no direct ergogenic effects [12467].

Phytoecdysteroids
Phytoecdysteroids are analogues of arthropod steroid hormones found in plants, where they deter predation by non-adapted predators. There is potential to exploit this to develop new strategies for pest control, either by using ecdysteroids as lead molecules for the design of novel pest control agents or by alteration of ecdysteroid levels/profiles in crop plants through plant breeding or genetic modification. However, it is other properties of phytoecdysteroids that have led to a rapid recent increase in scientific and commercial interest in these molecules. They are apparently non-toxic to mammals and a wide range of beneficial pharmacological (adaptogenic, anabolic, anti-diabetic, hepatoprotective, immunoprotective, wound-healing, and perhaps even anti-tumour) activities is claimed for them. In particular, this has led to a large (and unregulated) market for ecdysteroid-containing preparations for body-builders, sportsmen, and pets, among others. Ecdysteroids are also being considered as nutraceutical additives to food products. Further, ecdysteroids are good candidates as elicitors for gene-switch systems to be used in medical gene therapy and research applications [09137].

Evolution of steroids such as sex hormones and ecdysteroids occurred independently in animal and plant kingdoms. Plants use phytoecdysteroids (PEs) to control defence interactions with some predators; furthermore, PEs can exert beneficial influence on many aspects of mammalian metabolism. Endocrine disrupting compounds such as the estrogen agonist bisphenol A (BPA) are widespread in the environment, posing a potential hormonal risk to animals and plants. Adverse BPA effects on reproductive development and function are coupled with other toxic effects. BPA bioremediation techniques could be developed by exploiting some tolerant plant species [10352].

Phytoecdysteroids are structural analogs of the insect molting hormone ec dysone. Plants comprise rich sources of ecdysteroids in high concentration and with broad structural diversity. Ecdysteroids have a number of proven beneficial effects on mammals but the hormonal effects of ecdysteroids have been proven only in arthropods. Their structures are somewhat similar to those of the vertebrate steroid hormones but there are several structural differences between the two steroid groups. Despite of these essential structural differences, ecdysteroids exert numerous effects in vertebrates that are similar to those of vertebrate hormonal steroids, and they may serve as effective anabolic, hepatoprotective, immunoprotective, antioxidant and hypoglycemic agents. Ecdysteroids do not bind to the cytosolic steroid receptors, instead, they are likely to influence signal transduction pathways, like the anabolic steroids, possibly via membrane bound receptors. The application of phytoecdysteroids is a promising alternative to the use of anabolic-androgenic steroids because of the apparent lack of adverse effects. The prospective use of phytoecdysteroids may extend to treatments of pathological conditions where anabolic steroids are routinely applied. One of the most cited aspects of phytoecdysteroid application (on the Internet) is the increase of muscle size. However in this field too stringent research is needed as an adequate cytological explanation is not yet available for the anabolic [08164].

20-Hydroxyecdysone (20E) is an ecdysteroid hormone that regulates moulting in insects. Interestingly, 20E is also found most abundantly in plant species and has anabolic effects in vertebrates, i.e. increasing muscle size without androgen influence. The effect of 20E on slow and fast fiber types of skeletal muscle has not been reported yet. Here we present that 20E affects the size (cross-sectional area) of the different fiber types in a muscle-specific manner. The effect on fiber size was modified by the distance from the site of the treatment and the presence of a regenerating soleus muscle in the animal. Besides the fiber size, 20E also increased the myonuclear number in the fibers of normal and regenerating muscles, suggesting the activation of satellite cells. According to our results 20E may provide an alternative for substitution of anabolic-androgenic steroids in therapeutic treatments against muscle atrophy [08165].
17-hydroxyandrost-3,5-diene ("Syntrax Tetrabol")

Yeast transactivation system

Anabolic-androgenic steroids are frequently misused compounds in sports, and they belong to the controlled substances according to the requirements of the World Anti-Doping Agency. The classical techniques of steroid detection are mass spectrometry coupled to gas or liquid chromatography. Biological methods that base on the ability of substances to bind the steroid receptor are not applied in routine doping control procedures so far, but they appear to be useful for characterization of steroid androgenic potential. In this study we used the yeast androgen receptor reporter system (YAS), which in the past has already successfully been applied to both various androgenic substances and also urine samples. Giving attention to the androgenic potential of steroidal dietary supplements, we exemplified the analysis using both mass spectrometry techniques and the YAS-based assay on the product "Syntrax Tetrabol" which was a confiscated dietary supplement and marketed as a steroid precursor. Identification, structure and the kinetic behavior of its excreted metabolites were analyzed by NMR, GC-MS and LC-MS/MS. The androgenic potential of the parent compound as well as its metabolites in urine was evaluated with the help of the YAS. The application of urine samples with a previous deconjugation and the inclusion of urine density values were carried out and led to increased responses on the YAS. Further, the possibility of a complementary application of structure-based instrumental analysis and biological detection of androgenicity with the help of the YAS seems to be desirable and is discussed [12183].

Designer steroids

Despite progress in the development of GC/MS and ionmobility based methodology and though it plays a key role in ASS abuse detection, it has failed to detect the so-called designer drugs, the prototype being tetrahydrogestrinone. Because these emerging drugs are devised specifically to evade detection, they impose a considerable burden on antidoping methods. A recently launched paradigm is "1-androsterone", administration of which modifies the urinary steroid profile, and in particular the ratios of androsterone/etiocholanolone and 5alpha-/5beta-androstane-3alpha,17beta-diol and the concentration of 5alpha-dihydrotestosterone. Meanwhile, 3alpha-hydroxy-5alpha-androst-1-en-17-one, a characteristic metabolite, is likely to play an important role by permitting a wider time frame of detection of steroid abuse, since screening performed as much as 9 days after a single administration of one capsule enabled its detection [12011].

The issue of a growing number of so-called designer steroids requires proper monitoring of illicitly distributed compounds. Upon detection and characterization, the knowledge regarding metabolism and excretion are particularly important to provide adequate target analytes for routine doping controls [12016].

Historically, dope-testing methods have been developed to target specific and known threats to the integrity of sport. Traditionally, the source of new analytical targets for which testing was required were derived almost exclusively from the pharmaceutical industry. More recently, the emergence of designer drugs, such as tetrahydrogestrinone that are specifically intended to evade detection, or novel chemicals intended to circumvent laws controlling the sale and distribution of recreational drugs, such as anabolic steroids, stimulants and cannabinoids, have become a significant issue. In one review, it was considered the
emergence of designer drugs and the response of dope-testing laboratories to these new threats, in particular developments in analytical methods, instrumentation and research intended to detect their abuse, and the likely future impact of these approaches [12185].

Desoxymethyltestosterone (DMT; 17beta-hydroxy-17alpha-methyl-5alpha-androst-2-ene) is a designer steroid present in hormonal supplements distributed illegally as such or in combination with other steroids, for self-administration. It figures on the list of substances prohibited in sports and its detection in athlete's urine samples is based upon the presence of the parent compound or the main urinary metabolite, which has not been characterized yet. Following its isolation from cultures of human fresh hepatocytes and S9 fractions of liver homogenates, it was possible to identify this metabolite as being 17alpha-methyl-2beta, 3alpha,17beta-trihydroxy-5alpha-androstane. Other minor metabolites were also characterized. The production, isolation, NMR, mass spectral analyses and chemical synthesis were presented [12186].

Cholesterol is a well-known component in fats of animal origin and it also is the precursor of natural hormones. Phytosterols appear in plants and only differ slightly in structure from cholesterol. An important difference however is the low absorption in the gut of phytosterols and their saturated derivatives, the phytostanols. As a result, there is time for all kind of reactions in faecal material inside and outside of the gut. Determination of the abuse of natural hormones may be based on gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Abuse of natural hormones changes the $^{13}$C/$^{12}$C ratio of some metabolites during a relatively long time. The formation of (natural) hormones in the gut may interfere with this method. Designer drugs are mainly known from sports doping. In animal fattening, designer drugs may be used as well. Small changes in the structure of (natural) hormones may lead to a new group of substances asking for new strategies for their detection and the constatation of their abuse [07092].

Anabolic steroids have been studied for over 50 years and during that time numerous compounds with a variety of functional groups have been produced and many have been published. Of these only a small number have been introduced to the pharmaceutical market. WADA has continued the work begun by the IOC banning the use of these agents within sport as performance enhancing substances. Athletes, however, continue to use these anabolic steroids but tighter testing and the introduction of unannounced sample collection has made this form of cheating harder. In order to try to evade detection, athletes who continue to dope are having to resort to the use of a far more dangerous form of drug – the designer steroid. These steroids are manufactured to closely resemble existing known compounds, but with sufficient chemical diversity to ensure that their detection by the WADA accredited laboratories this is more difficult. A worrying feature of the use of these compounds is that no data is available to evaluate either the efficacy or the safety of these substances. Many such drugs are now being made in clandestine ways (as demonstrated by the recent BALCO case) and then passed on to athletes who become the guinea pigs determining the potential of the substances as doping agents. Methods for the detection of these new compounds are being developed using emerging techniques such as gas chromatography or liquid chromatography attached to a variety of mass spectrometry instruments. This technology as well as vigilance by laboratories and enforcement agencies can all help in early detection of designer steroids being used for doping [10089].

Effective detection of the abuse of androgenic-anabolic steroids in human and animal sports often requires knowledge of the drug's metabolism in order to target appropriate urinary metabolites. "Designer" steroids are problematic since it is difficult to obtain ethical approval for in vivo metabolism studies due to a lack of a toxicological profile. In one study, the in vitro metabolism of estra-4,9-diene-3,17-dione was reported for the first time. This is also the first
study comparing the metabolism of a designer steroid in the three major species subject to sport's doping control; namely the equine, canine and human. In order to allow the retrospective analysis of sample testing data, the use of a high-resolution accurate-mass Thermo LTQ-Orbitrap LC-MS instrument was employed for metabolite identification of underivatised sample extracts. The full scan HR-LC-MS Orbitrap data was complimented by several further experiments targeted at elucidating more detailed structural information for the most abundant metabolites. These included; HR-LC-MS/MS of the underivatised metabolites, functional group selective chemical derivatisation followed by full scan HR-LC-MS, enzyme inhibition experiments and full scan electron ionization GC-MS analysis of methoxyamine-trimethylsilyl derivatives. The major metabolite detected in all species, and therefore the most suitable candidate for screening of estra-4,9-diene-3,17-dione abuse, was proposed to be an isomer of 17-hydroxy-estra-4,9-dien-3-one. Less significant metabolic pathways in all species included hydroxylation and reduction followed by hydroxylation. Reductive metabolism in the canine was less significant than in the other two species, while the equine was unique in producing a di-reduced metabolite (proposed to be an isomer of estra-4,9-diene-3,17-diol) and also relatively large quantities of d-ring hydroxy and hydroxy-reduced metabolites [10459].

New analogues of androgens that had never been available as approved drugs are marketed as "dietary supplement" recently. They are mainly advertised to promote muscle mass and are considered by the governmental authorities in various countries, as well as by the World Anti-doping Agency for sport, as being pharmacologically and/or chemically related to anabolic steroids. In the present study, it was reported the detection of a steroid in a product seized by the State Bureau of Criminal Investigation Schleswig-Holstein, Germany. The product "1-Androsterone" of the brand name "Advanced Muscle Science" was labeled to contain 100 mg of "1-Androstene-3b-ol,17-one" per capsule. The product was analyzed underivatized and as bis-TMS derivative by GC-MS. The steroid was identified by comparison with chemically synthesized 3beta-hydroxy-5alpha-androst-1-en-17-one, prepared by reduction of 5alpha-androst-1-ene-3,17-dione with LS-Selectride (Lithium tris-isooamyloborohydride), and by nuclear magnetic resonance. Semi-quantification revealed an amount of 3beta-hydroxy-5alpha-androst-1-en-17-one in the capsules as labeled. Following oral administration to a male volunteer, the main urinary metabolites were monitored. 1-Testosterone (17beta-hydroxy-5alpha-androst-1-en-3-one), 1-androstenedione (5alpha-androst-1-ene-3,17-dione), 3alpha-hydroxy-5alpha-androst-1-en-17-one, 5alpha-androst-1-ene-3alpha,1betaβ-diol, and 5alpha-androst-1-ene-3beta,17beta-diol were detected besides the parent compound and two more metabolites (up to now not finally identified but most likely C-18 and C-19 hydroxylated 5alpha-androst-1-ene-3,17-diones). Additionally, common steroids of the urinary steroid profile were altered after the administration of "1-Androsterone". Especially the ratios of androsterone/etiocolanolone and 5alpha-/5beta-androstane-3alpha,17beta-diol and the concentration of 5alpha-dihydrotestosterone were influenced. 3alpha-Hydroxy-5alpha-androst-1-en-17-one appears to be suitable for the long-term detection of the steroid (ab-)use, as this characteristic metabolite was detectable in screening up to nine days after a single administration of one capsule [11106].

Detection of androgenic-anabolic steroid abuse in equine sports requires knowledge of the drug's metabolism in order to target appropriate metabolites, especially where urine is the matrix of choice. Studying "designer" steroid metabolism is problematic since it is difficult to obtain ethical approval for in vivo metabolism studies due to a lack of toxicological data. In one study, the equine in vitro metabolism of eight steroids available for purchase on the Internet is reported; including androsta-1,4,6-tirome-3,17-dione, 4-chloro,17alpha-methylandrosta-1,4-diene-3,17beta-diol, estra-4,9-diene-3,17-dione, 4-hydroxyandrostenedione, 20-hydroxyecdyson, 11-keto-androstenedione, 17alpha-methyltestosterone, and tetrahydrogestrinone. In order to allow for retrospective analysis of sample testing data, the use of
a high-resolution accurate-mass Thermo LTQ-Orbitrap liquid chromatography-mass spectrometry (LC-MS) instrument was employed for metabolite identification of underivatized sample extracts. The full scan LC-HRMS Orbitrap data were complimented by LC-HRMS/MS and gas-chromatography-mass spectrometry (GC-MS) experiments in order to provide fragmentation information and to ascertain whether GC-MS was capable of detecting any metabolite not detected by LC-HRMS. With the exception of 20-hydroxyecdysone, all compounds would be metabolized by equine liver S9 and/or microsomes. With the exception of 17alpha-methyltestosterone, which produced metabolites that could only be detected by GC-MS, the metabolites of all other compounds could be identified using LC-HRMS, thus allowing retrospective analysis of previously acquired full-scan data resulting from routine equine drug testing screens. In summary, while in vitro techniques do not serve as a replacement for more definitive in vivo studies in all situations, their use does offer an alternative in situations where it would not be ethical to administer untested drugs to animals [11107].

The metabolic fate of the emerging drug candidate S107, possessing the potential for misuse as performance-enhancing agent in sports, was investigated by in vitro phase I and II experiments with human microsomal and S9 liver enzymes. The metabolites were identified by liquid chromatography-mass spectrometry with electrospray ionisation in positive mode (LC-ESI-MS/MS). Their collision-induced dissociation behaviour was studied by high-resolution/high accuracy Orbitrap MS(n) analysis, supported by stable isotope labelling, H/D-exchange experiments and density functional theory calculations. Monoxygenation accounted for the main phase I metabolic transformation due to N- and S-oxidation of the 1,4-benzothiazepine core, as substantiated by chemical synthesis, selective reduction methods and characteristic APCI in source fragmentation behaviour of the metabolites. Another dominant metabolic pathway was demethylation, yielding the N- and O-demethylated metabolite, respectively. The latter was further conjugated by glucuronidation as well as sulfonation in subsequent phase II metabolic reactions, whereas the N-demethylated metabolite was not amenable to conjugation. The active drug molecule itself was converted to two glucuronic acid conjugates, which are proposed to consist of two quaternary S107-N(+)-glucuronide isomers. All glucuronides were susceptible to enzymatic hydrolysis with β-glucuronidase (Escherichia coli). A comprehensive LC-ESI-MS(/MS)-based detection method for urine was developed and its fitness for purpose was assessed. The assay can serve as a potential screening and/or confirmation method for S107 in clinical drug testing and doping control analysis in the future [11108].

What is an androgen (anabolic-androgenic steroid)? Most would state that it is a derivative of the major sex steroid in men and most mammals, testosterone (T). Others might answer in the more generic sense: any compound that is an agonist (or partial agonist) at the androgen receptor (AR). There are seemingly enough forms of testosterone activity available to satisfy the needs of the medical population with oral, buccal, cutaneous patches (really, drug delivery devices) and gels, injectables of various durations (days to months), and implantable forms with a duration of action of 6 months or more. In the 1940s and 1950s, many pharmaceutical companies evaluated a myriad of compounds with purported anabolic activity searching for those that had anabolic (at muscle) activity without the more androgenic adverse events. It was to little avail given that there is but one AR, albeit with a greater affinity for dihydrotestosterone than for testosterone itself. Arcane testosterone chemistry produced many compounds that had sat on shelves for years without any clinical trials for evaluation for human or veterinary use or commercial promotion. In the future, the concept of selective AR agonists may come to play a much more prominent role. Such compounds may selectively produce the beneficial effects of androgens on musculoskeletal mass and strength without the androgenic adverse events. It appeared quite straightforward to begin with the steroid structure of testosterone, add multiple decorations to the various carbon
atoms, test in vitro with various cell lines that contain the AR linked to an objective outcome, for example, AR nuclear translocation or myogenic activity. Then comes the nefarious part: all certified antidoping laboratories have libraries of chromatography/mass spectroscopy (MS)/MS fragments, and each anabolic/androgenic steroid has its own unique signature. The skill is to find a compound active at the AR, but that is not yet in the library. Is this just an academic exercise in steroid biochemistry? I think not, given the example of tetrahydrogestrinone (THG). THG had been invisible to the antidoping laboratories until a sample of pure THG was presented to an antidoping lab. It had been synthesized by an underground laboratory and likely underwent enough testing (in vitro and in vivo) to determine its anabolic/androgenic activity and that it could not be detected by the then extant WADA testing procedure. There are also multiple proofs that the designer steroid methyl-1-testosterone (M1T) is an active anabolic/androgenic steroid. They set out to prove this point from the perspective of a multifaceted strategy from the source, the supplement M1T. The compound was strongly anabolic (in a dose-dependent manner) to increase the weight of the prostate, seminal vesicles, and levator ani muscle in orchidectomized rats, according to standard androgen assays in use for more than 60 year. Multiple metabolites were found in the urine of rats and men administered M1T yielding a signature for doping detection in athletes as well as a notion of the kinetics of metabolism and excretion [11554].

Various products containing rarely characterized anabolic steroids are nowadays marketed as dietary supplements. Herein, the designer steroid methyl-1-testosterone (M1T) (17beta-hydroxy-17alpha-methyl-5alpha-androst-1-en-3-one) was identified, and its biological activity, potential adverse effects, and metabolism were investigated. The affinity of M1T toward the androgen receptor (AR) was tested in vitro using a yeast AR transactivation assay. Its tissue-specific and anabolic potency and potential adverse effects were studied in a Hershberger assay (sc or oral), and tissue weights and selected molecular markers were investigated. Determination of M1T and its metabolites was performed by gas chromatography mass spectrometry. In the yeast AR transactivation assay, M1T was characterized as potent androgen. In rats, M1T dose-dependently stimulated prostate and levator ani muscle weight after sc administration. Oral administration had no effect but stimulated proliferation in the prostate and modulated IGF-I and AR expression in the gastrocnemius muscle in a dose-dependent manner. Analysis of tyrosine aminotransferase expression provided evidence for a strong activity of M1T in the liver (much higher after oral administration). In rat urine, 17alpha-methyl-5alpha-androstane-3alpha,17beta-diol, M1T, and a hydroxylated metabolite were identified. In humans, M1T was confirmed in urine in addition to its main metabolites 17alpha-methyl-5alpha-androst-1-ene-3alpha,17beta-diol and 17alpha-methyl-5alpha-androstane-3alpha,17beta-diol. Additionally, the corresponding 17-epimers as well as 17beta-hydroxymethyl-17alpha-methyl-18-nor-5alpha-androsta-1,13-dien-3-one and its 17-epimer were detected, and their elimination kinetics was monitored. It was demonstrated that M1T is a potent anabolic and anabolic steroid after oral and sc administration. Obviously, this substance shows no selective AR modulator characteristics and might exhibit liver toxicity, especially after oral administration [11450].

The pharmaceutical industry has made substantial innovations in the area of targeted drug delivery and optimization of pharmacokinetics for existing drugs to try to maximize therapeutic effect while simultaneously minimizing adverse consequences of medication abuse. These innovations, however, have not come without a price. Designer drugs and high content modified release formulations have been exploited both in casual recreational drug abuse as well as, on a much larger scale, by the criminal diversion of these products for profit. In one paper it was considered the challenges before manufacturers and regulators as they approach the problem of abuse potential of these new drug products and some of the solutions specifically designed to counteract abuse [06123].
Recently several new steroids, specifically synthetised and distributed for misuse in sports, have been detected in urine samples from athletes. The difference between these other steroids is that they have never been marketed neither as a pharmaceutical preparation nor as a “nutritional” supplement. Examples are norbolethone in 2002, tetrahydrogestrinone (THG) in 2003, and desoxymethyltestosterone, madol (17alpha-methyl-5alpha-androst-2-ene-17beta-ol) in 2004 [06004].

By using in silico models of the complexes formed by analogues of a cancer drug and its receptor, it may be possible to strategically redesign existing drugs and win the race against mutations that lead to drug resistance in prostate cancer. In Lewis Carroll's Through the Looking-Glass, the Red Queen reveals to Alice that in her world 'it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!'. The predicament in prostate cancer is much the same: researchers and clinicians alike are forced to keep running merely to stay still, because the receptors targeted by prostate cancer drugs are continually undergoing mutations that prevent the drugs from working. Now, in eLife, Minna Balbas, Charles Sawyers and their colleagues at the Memorial Sloan-Kettering Cancer Center and the University of Chicago may have found a way to run twice as fast, by using a novel approach to redesign drugs to restore their clinical efficacy. Prostate cancer is driven largely by male hormones, otherwise known as androgens, acting through the androgen receptor. Consequently, the majority of cases can be treated by depriving the tumors of androgens: this is achieved by suppressing the production of androgens and by administering antiandrogen drugs, such as flutamide and bicalutamide. By binding to the same site on the receptor as endogenous androgens, these drugs – which are known as receptor antagonists – prevent the hormones from activating the receptor. With time, however – sometimes only a matter of months – the cancer returns in a form resistant to these therapies, termed “castration-resistant prostate cancer”. Resistance can arise through various means, but many cases result from mutation of the androgen receptor. This is because the gene encoding the androgen receptor is on the X chromosome, which means that men have only a single copy and therefore any change in the gene must be expressed in the protein. Over the past few years, Sawyers and collaborators have brought a number of more effective antiandrogens to the clinic, most notably enzalutamide (Enz). This drug binds to the androgen receptor with high affinity and effects a more complete blockade of receptor signaling than earlier drugs, providing significant survival benefit in advanced castration-resistant prostate cancer. Nevertheless, cases of resistance to Enz are being encountered in the clinic. Prostate cancer is poised to enter a new era of personalized health care. The next breakthroughs in therapeutics will occur with an in-depth mechanistic understanding of the events underlying disease progression and the development of resistance. The approach taken by Balbas et al. provides a compelling paradigm for improving the durability of therapies, by strategically redesigning drugs to compensate for resistance arising from mutations in their targets. The same approach could also used to extend the effectiveness of other targeted therapies before resistance arises [13267].

**Tetrahydrogestrinone (THG)**

Tetrahydrogestrinone (THG) is a steroid recently identified to be misused as doping agent. However, the knowledge on functions of this substance in humans or animal models is rather limited. Therefore, it was the aim to further characterize the pharmacological profile of THG and identify potential adverse side effects. THG was synthesized, the purity was confirmed and its biological activity was tested. The potency of THG to transactivate AR dependent reporter gene expression was two orders of magnitude lower compared to dihydrotestosterone. THG binds with high affinity but unselective to the androgen (AR),
progesterone (PR), glucocorticoid (GR) and mineralocorticoid (MR) receptor. Treatment of orchiectomised rats with THG resulted in a stimulation of prostate, seminal vesicle and levator ani muscle, indicating androgenic and anabolic properties. In the liver THG, in contrast to testosteronepropionate (TP), down regulates the expression of the GR dependent tyrosine aminotransferase gene (TAT). In summary, our results demonstrate that THG is not a specific AR agonist. THG exhibits a high binding affinity to all tested steroid hormone receptors and binds with highest affinity to the GR. The in vivo data are indicative of an anabolic and androgenic potency of THG, but the repression of TAT demonstrates that THG also interferes with the glucocorticoid hormone system. Therefore, it is conceivable that an intake will result in adverse side effects [06124].

Tetrahydrogestrinone (18a-homo-pregna-4,9,11-trien-17beta-ol-3-one) was first identified in a syringe that allegedly contained an anabolic steroid undetectable by routine doping control in sports and that was anonymously sent to USADA and the WADA-accredited doping control laboratory in Los Angeles. Although THG was never approved or marketed for any clinical indication it has been demonstrated that it was a potent androgen and progestin. Moreover, THG also binds to glucocorticoid and mineralocorticoid receptors. Interestingly, one of the side-products of the synthesis of THG starting from gestrinone is norbolethone. The detection of THG as a designer steroid caused international commotion and mass media attention, especially because it seemed to have been synthetised to remain undetectable by routine screening methods in WADA accredited doping control laboratories. THG is however detectable using liquid chromatography-tandem mass spectrometry. No in-vivo studies on the metabolism of THG have been performed. In vitro studies, however, indicate that THG is metabolised to 18a-homo-pregna-4,9,11-trien-17beta-ol-3-one and 18a-homo-pregna-4,9,11-trien-17beta,16epsilon-diol-3-one [06004].

The “designer” steroid tetrahydrogestrinone (THG) had been developed by Wyeth in the 1960s and taken to clinical trials as a potential anabolic agent, but never brought to market. It was impossible to locate any records from the clinical trials 40 years after the fact. THG had been shown to have anabolic activity. There were no publications about toxicological studies, but as a 17alpha-methyl steroid, comparison to similar structures strongly suggested the potential for some degree of hepatotoxicity. Based on this information, THG was added to the Prohibited List in 2006 [12006].

In the 1940s and 1950s, many pharmaceutical companies evaluated a myriad of compounds with purported anabolic activity searching for those that had anabolic (at muscle) activity without the more androgenic activities. It was to little avail given that there is but one androgen receptor (AR), albeit with a greater affinity for dihydrotestosterone than for testosterone itself. Arcane testosterone chemistry produced many compounds that had sat on shelves for years without any clinical trials for evaluation for human or veterinary use or commercial promotion. In the future, the concept of selective AR agonists may come to play a much more prominent role. Such compounds may selectively produce the beneficial effects of androgens on musculoskeletal mass and strength without the androgenic adverse events. Clandestine laboratories seeking to make a product to give an edge to athletes began to screen compounds for their anabolic/androgenic activities with the proviso that the compound and its metabolites be invisible to the WADA screening protocol. It appeared quite straightforward to begin with the steroid structure of testosterone, add multiple decorations to the various carbon atoms, test in vitro with various cell lines that contain the AR linked to an objective outcome, for example, AR nuclear translocation or myogenic activity. Then comes the nefarious part: all certified antidoping laboratories have libraries of chromatography/mass spectroscopy (MS)/MS fragments, and each anabolic/androgenic steroid has its own unique signature. The skill is to find a compound active at the AR, but that is not yet in the library. For example tetrahydrogestrinone (THG) had been invisible to the antidoping laboratories
until a sample of pure THG was presented to an antidoping lab. It had been synthesized by an underground laboratory and likely underwent enough testing (in vitro and in vivo) to determine its anabolic/androgenic activity and that it could not be detected by the then extant WADA testing procedure. However, within a week of obtaining that pure sample of THG, the University of California at Los Angeles (UCLA) Olympic Analytic Laboratory was able to devise a test for this compound and its metabolic products. This task was later verified by the synthesis of THG and characterized by MS and nuclear magnetic resonance spectroscopy. The UCLA laboratory developed and verified sensitive and specific methods for the rapid screening of urine samples by liquid chromatography/tandem MS, both in the natural state and with trimethylsilyl ether-oxime derivatives (as is done in the standard antidoping testing procedure). Further proof of the administration of THG was obtained from the urine of baboons injected with authentic THG. To close the loop concerning athletes, further proof of activity was determined in vitro in an AR-mediated myogenic cell line and in vivo by muscle accretion in orchidectomized male rats [12113].

**Digoxin-like effects**

For a long time, athletes have used androgenic anabolic steroids (AASs) in an inappropriate and veiled manner with the aim of improving exercise performance or for cosmetic purposes. Abuse of AASs triggers adverse effects such as hepatocarcinogenesis, heart attacks, and aggressive behavior. However, AAS-induced toxicity is not completely understood at the molecular level. In one study, it was shown, by performing a dioxin response element (DRE)-luciferase reporter gene assay, that tetrahydrogestrinone (THG), a popular and potent androgen receptor agonist, has dioxin-like effects. In addition, we showed that THG increased cytochrome P-450 1A1 (CYP1A1) mRNA and protein levels, and enzyme activity. The gene encoding CYP1A1 is involved in phase 1 xenobiotic metabolism and a target gene of the aryl hydrocarbon receptor (AhR). Using the AhR antagonist CH-223191, we also examined whether the effects of THG on DRE activation depended on AhR. The results suggest that synthetic anabolic steroids may have dioxin-like side effects that can disturb endocrine systems and may cause other side effects including cancer through AhR [12187].

**YK11**

A novel steroid compound, (17alpha,20E)-17,20-[(1-methoxyethylidene)bis(oxy)]-3-oxo-19-norpregna-4,20-diene-21-carboxylic acid methyl ester (YK11), was found to be a partial agonist of the androgen receptor (AR) in an androgen responsive element (ARE)-luciferase reporter assay. YK11 accelerates nuclear translocation of AR. Furthermore, YK11 does not induce amino/carboxyl-terminal (N/C) interaction and prevents 5α-dihydrotestosterone (DHT)-mediated N/C interaction. Thus, YK11 activates AR without causing N/C interaction, which may in turn be responsible for the partially agonistic nature of YK11 observed in the ARE-luciferase reporter system. YK11 acts as a gene-selective agonist of AR in MDA-MB 453 cells. The effect of YK11 on gene expression relative to that of androgen agonist varies depending on the gene context. YK11 activated the reporter gene by inducing the translocation of the AR into the nuclear compartment, where its amino-terminal domain (NTD) functions as a constitutive activator of AR target genes. The results suggest that YK11 might act as selective androgen receptor modulator (SARM) [11062].

**Delta6-methyltestosterone**

Since a few years more and more products have appeared on the market for dietary supplements containing steroids that had never been marketed as approved drugs, mostly without proper labeling of the contents. Syntheses and few data on pharmacological effects are available dated back mainly to the 1950s or 1960s. Only little knowledge exists about
effects and side effects of these steroids in humans. One study reports the identification of delta6-methyltestosterone in a product named "Jungle Warfare", which was obtained from a web-based supplement store. The main urinary metabolites, 17alpha-hydroxy-17beta-methylandrosta-4,6-dien-3-one (delta6-epimethyl-testosterone), 17alpha-methyl-5beta-androstane-3alpha,17beta-diol (3alpha,5beta-THMT), and 17beta-methyl-5beta-androstane-3alpha,17alpha-diol, as well as the parent compound excreted after a single oral administration were monitored by GC-MS/MS. delta6-Epimethyltestosterone and 3alpha,5beta-THMT served for long-term detection (still present in the 181-189 h urine). 17alpha-Methyltestosterone and its 17-epimer were not detected in the urines (LOD 0.3ng/mL). The highest concentrations were found in the 14-20.5h urine for delta6-epimethyltestosterone (600 ng/mL), and 3alpha,5beta-THMT (240 ng/mL) and in the 36-44.5h urine for 17beta-methyl-5beta-androstane-3alpha,17alpha-diol (7 ng/mL). For reference methyltestosterone and epimethyltestosterone were dehydrogenated with chloranil. The characterization of the products was performed by GC-MS/MS and NMR [12188].

Methylstenbolone

The use of "nutritional supplements" containing unapproved substances has become a regular practice in amateur and professional athletes. This represents a dangerous habit for their health once no data about toxicological or pharmacological effects of these supplements are available. Most of them are freely commercialized online and any person can buy them without medical surveillance. Usually, the steroids intentionally added to the "nutritional supplements" are testosterone analogues with some structural modifications. In this study, the analyzed product was bought online and a new anabolic steroid known as methylstenbolone (2,17alpha-dimethyl-17beta-hydroxy-5alpha-androst-1-en-3-one) was detected, as described on label. Generally, anabolic steroids are extensively metabolized, thus in-depth knowledge of their metabolism is mandatory for doping control purposes. For this reason, a human excretion study was carried out with four volunteers after a single oral dose to determine the urinary metabolites of the steroid. Urine samples were submitted to enzymatic hydrolysis of glucuron conjugated metabolites followed by liquid-liquid extraction and analysis of the trimethylsilyl derivates by gas chromatography coupled to tandem mass spectrometry. Mass spectrometric data allowed the proposal of two plausible metabolites: 2,17alpha-dimethyl-16epsilon,17beta-dihydroxy-5alpha-androst-1-en-3-one (S1), 2,17alpha-dimethyl-3alpha,16epsilon,17beta-trihydroxy-5alpha-androst-1-ene (S2). Their electron impact mass spectra are compatible with 16-hydroxylated steroids O-TMS derivatives presenting diagnostic ions such as m/z 231 and m/z 218. These metabolites were detectable after one week post administration while unchanged methylstenbolone was only detectable in a brief period of 45 h [12189].

Methyl-1-testosterone (M1T)

Various products containing rarely characterized anabolic steroids are nowadays marketed as dietary supplements. Herein, the designer steroid methyl-1-testosterone (M1T) (17beta-hydroxy-17alpha-methyl-5alpha-androst-1-en-3-one) was identified, and its biological activity, potential adverse effects, and metabolism were investigated. The affinity of M1T toward the androgen receptor (AR) was tested in vitro using a yeast AR transactivation assay. Its tissue-specific androgenic and anabolic potency and potential adverse effects were studied in a Hershberger assay (sc or oral), and tissue weights and selected molecular markers were investigated. Determination of M1T and its metabolites was performed by gas chromatography mass spectrometry. In the yeast AR transactivation assay, M1T was characterized as potent androgen. In rats, M1T dose-dependently stimulated prostate and
levator ani muscle weight after sc administration. Oral administration had no effect but stimulated proliferation in the prostate and modulated IGF-I and AR expression in the gastrocnemius muscle in a dose-dependent manner. Analysis of tyrosine aminotransferase expression provided evidence for a strong activity of M1T in the liver (much higher after oral administration). In rat urine, 17alpha-methyl-5alpha-androstane-3alpha,17beta-diol, M1T, and a hydroxylated metabolite were identified. In humans, M1T was confirmed in urine in addition to its main metabolites 17alpha-methyl-5alpha-androst-1-ene-3alpha,17beta-diol and 17alpha-methyl-5alpha-androstane-3alpha,17beta-diol. Additionally, the corresponding 17-epimers as well as 17beta-hydroxymethyl-17alpha-methyl-18-nor-5alpha-androsta-1,13-dien-3-one and its 17-epimer were detected, and their elimination kinetics was monitored. It was demonstrated that M1T is a potent androgenic and anabolic steroid after oral and sc administration. Obviously, this substance shows no selective AR modulator characteristics and might exhibit liver toxicity, especially after oral administration [12190].

It has been produced multiple proofs that the designer steroid methyl-1-testosterone (M1T) is an active anabolic/androgenic steroid. They set out to prove this point from the perspective of a multifaceted strategy from the source, the supplement M1T. The mass spectrum of the bis-TMS derivative was determined, followed by a metabolic study in rats in which the same compound and a large number of its metabolites were detected. In vitro activity was determined in a yeast androgen screen with an EC50 consistent with that of the more potent androgen, dihydrotestosterone (3 x 10^-9 M). The compound was strongly anabolic (in a dose-dependent manner) to increase the weight of the prostate, seminal vesicles, and levator ani muscle in orchidectomized rats, according to standard androgen assays in use for more than 60 years. Multiple metabolites were found in the urine of rats and men administered M1T yielding a signature for doping detection in athletes as well as a notion of the kinetics of metabolism and excretion. Assiduously following detailed protocols permitted the attainment of unassailable data to prove that M1T is an anabolic steroid both in vitro and in vivo and to determine a testing procedure that meets WADA standards [12113].

Laboratory techniques

New anabolic steroids show up occasionally in sports doping and in veterinary control. The discovery of these designer steroids is facilitated by findings of illicit preparations, thus allowing bioactivity testing, structure elucidation using NMR and mass spectrometry, and final incorporation in urine testing. However, as long as these preparations remain undiscovered, new designer steroids are not screened for in routine sports doping or veterinary control urine tests since the established GC/MS and LC/MS/MS methods are set up for the monitoring of a few selected ions or MS/MS transitions of known substances only. In one study, the feasibility of androgen bioactivity testing and mass spectrometric identification is being investigated for trace analysis of designer steroids in urine. Following enzymatic deconjugation and a generic solid-phase extraction, the samples are analyzed by gradient LC with effluent splitting toward two identical 96-well fraction collectors. One well plate is used for androgen bioactivity detection using a novel robust yeast reporter gene bioassay yielding a biogram featuring a 20-s time resolution. The bioactive wells direct the identification efforts to the corresponding well numbers in the duplicate plate. These are subjected to high-resolution LC using a short column packed with 1.7-microm C18 material and coupled with electrospray quadrupole time-of-flight mass spectrometry (LC/QTOFMS) with accurate mass measurement. Element compositions are calculated and used to interrogate electronic substance databases. The feasibility of this approach for doping control is demonstrated via the screening of human urine samples spiked with the designer anabolic steroid tetrahydrogestrinone. Application of the proposed methodology, complementary to the established targeted urine screening for known anabolics, will increase the chance of finding unknown emerging designer steroids, rather then being solely dependent on findings
Reductase inhibitors

5alpha-Reductase inhibitors (or 5-alpha-reductase inhibitors) are a group of drugs with antiandrogenic activity, used in the treatment of benign prostatic hyperplasia and androgenic (or androgenetic) alopecia. These drugs decrease the levels of available 5alpha-reductase prior to testosterone’s binding with the enzyme, thus reducing levels of dihydrotestosterone that derives from such a bond. 5alpha-Reductase inhibitors are clinically used in the treatment of conditions that are exacerbated by dihydrotestosterone. These indications may include mild-to-moderate benign prostatic hyperplasia and lower urinary tract symptoms and androgenic (or androgenetic) alopecia. In general, adverse drug reactions are dose-dependent and include impotence, decreased libido, decreased ejaculate volume, depression, and anxiety. In benign prostatic hyperplasia, dihydrotestosterone acts as a potent cellular androgen and promotes prostate growth; therefore, inhibiting the enzyme reduces the excessive prostate growth. In alopecia, male-pattern baldness is one of the effects of androgenic receptor activation. Thus, reducing the levels of dihydrotestosterone reduces alopecia. The aim of one study was to determine the capacity of some progesterone derivatives, to inhibit the conversion of labeled androstenedione to dihydrotestosterone (DHT) in prostate nuclear membrane fractions, where the 5alpha-reductase activity is present. The enzyme 5alpha-reductase catalyzes the 5alpha-reduction of 4-dione whereas the 17beta-hydroxysteroid dehydrogenase catalyzes the transformation of 4-dione to testosterone or 5alpha-dione to dihydrotestosterone (DHT). Moreover, we also investigated the role of unlabeled 5alpha-dione in these pathways. In order to determine the inhibitory effect of different concentrations of the progesterone derivatives in the conversion of 4-dione to DHT, homogenates of human prostate were incubated with 4-dione, NADPH and increasing concentrations of non-labeled 5α-dione. The incubating mixture was extracted and purified using thin layer chromatography. The fraction of the chromatogram corresponding to the standard of DHT was separated and the radioactivity determined. The results showed that the presence of 4-dione plus unlabelled 5alpha-dione produced similar levels of DHT as compared to 4-dione. On the other hand, the results indicated that 17alpha-hydroxyprog-4-ene-3,20-dione 5 and 4-bromo-17alpha-hydroxyprog-4-ene-3,20-dione 7b, were the most potent steroids to inhibit the conversion of 4-dione to DHT, showing IC50 values of 2 and 1.6 nM, respectively [11453].

Finasterid

The increasing use of alpha-reductase inhibitors for treatment of male pattern baldness has led to positive urine samples of athletes for finasteride, the main metabolite. Finasteride is a banned substance listed under S5. Diuretics and masking agents in the doping control regulations. A German football player was suspended by the supreme court of the German Soccer Federation for six months with additional fine after a positive result for finasteride. In this case, the laboratory explicitly used more sensitive analytical methods which could not identify any traces of anabolic steroids in the sample – a finding that is not covered by the World Anti-Doping Code. This case illustrates several critical medicolegal aspects which have to be addressed, including the questions of a psychological disease and the eligibility for a therapeutic use exemption in case of male pattern baldness [06002].

It was presented an unusual case of secondary infertility after prolonged use of low-dose finasteride for androgenetic alopecia in a 40-year-old man. It was detected sperm DNA damage in the patient. Despite such a long-term use, it was observed that impairment in
sperm DNA fragmentation index regressed after the drug was discontinued. Consequently, pregnancy occurred and resulted in live birth [12184].

Finasteride and dutasteride are 5alpha-reductase inhibitors included in the World Anti-Doping Agency's list of banned substances. Two highly sensitive and selective ELISA assays were developed for these compounds. Polyclonal rabbit antibodies were raised using synthesized haptons and other commercial products. The best immunoassay obtained, based on an antibody-coated format, showed a limit of detection of 0.01 microg/L and an IC_{50} of 0.75 microg/L for finasteride (cross-reactivity with dutasteride <4%). The second assay allowed finasteride and dutasteride determination, with limits of detection of 0.013 and 0.021 microg/L, and IC_{50} values 0.18 and 1.18 microg/L, respectively. Both assays were highly selective to a set of anabolic steroids, but they showed 37 percent and 30 percent cross-reactivity with the major urinary metabolite of finasteride, allowing its determination. The developed ELISA had better sensitivity than HPLC/MS/MS method and was applied as a screening technique to quantify dutasteride, finasteride, and its main metabolite in human urine without sample pre-treatment. Moreover, the analysis of dutasteride's excretion urines by ELISA was used to obtain its human excretion rate, essential to improve the analytical strategies about this type of drugs (permitted as medicines and prohibited in sport) and to establish an effective anti-doping policy [10093].

Androgenetic alopecia is the most common form of alopecia in men. MEDLINE, EMBASE, CINAHL, Cochrane Registers, and LILACS were searched for randomized controlled trials reported in any language that evaluated the efficacy and safety of finasteride therapy in comparison to treatment with placebo in adults with androgenetic alopecia. Outcome measures included patient self-assessment, hair count, investigator clinical assessment, global photographic assessment, and adverse effects at short term (≤12 months) and long term (≥24 months). Heterogeneity was explored by testing a priori hypotheses. Twelve studies fulfilled the eligibility criteria (3927 male patients), 10 of which demonstrated a Jadad score of 3 or more. The proportion of patients reporting an improvement in scalp hair was greater with finasteride therapy than with placebo treatment in the short term and in the long term both results were considered to have moderate-quality evidence. The number needed to treat for 1 patient to perceive himself as improved was 5.6 in the short term and 3.4 in the long term. Moderate-quality evidence suggested that finasteride therapy increased the mean hair count from baseline in comparison to placebo treatment, expressed as a percentage of the initial count in each individual, at short term and at long term. Also, the proportion of patients reported as improved by investigator assessment was greater in the short term; number needed to treat, 3.7 (moderate-quality evidence). Moderate-quality evidence suggested an increase in erectile dysfunction and a possible increase in the risk of any sexual disturbances. The risk of discontinuing treatment because of sexual adverse effects was similar to that of placebo. It was concluded that moderate-quality evidence suggests that daily use of oral finasteride increases hair count and improves patient and investigator assessment of hair appearance, while increasing the risk of sexual dysfunction [10458].

Finasteride (FIN), a widely used medication for the treatment of androgen-dependent diseases, blocks the conversion of testosterone to a more potent androgen, dihydrotestosterone (DHT). In this study, we investigated a dosing time-dependent effect and safety of FIN in rats. Androgen receptor (AR) mRNA and nuclear protein levels exhibited clear daily rhythms with the peak during the dark period in the prostate and during the light period in the liver. Repeated oral administration of FIN (5 or 100 mg/kg) at 3 h after lights on (HALO) for 2 weeks decreased serum DHT concentration throughout a 24-h period, whereas the dosing of the agent at 15 HALO decreased its level only transiently even in the higher dose group. FIN caused laboratory abnormalities in the 3 HALO group but not in the 15 HALO group. However, the effect of FIN on the prostate weight was not influenced by the
dosing time. These results suggest that the safety, but not effect, of FIN depends on its
dosing time in rats. The dosing of FIN in the active period might be a rational dosage
regimen, which is needed to be confirmed in human subjects [11105].

5alpha-Reductase inhibitors such as finasteride are prohibited in sports according to the
World Anti-Doping Agency. This class of drugs is used therapeutically to treat benign
prostatic hyperplasia, as well as male baldness, by decreasing 5alpha-reductase activity.
Accordingly, metabolic pathways of endogenous as well as synthetic steroids are influenced,
which complicates the evaluation of steroid profiles in sports drug testing. The possibility of
manipulating steroid excretion profiles and, presumably, to mask steroid abuse was
investigated in 5 administration studies with use of finasteride at different doses, with and
without coadministration of 19-norandrostenedione. The evaluation of urinary steroid profiles
demonstrated the intense effect of finasteride on numerous crucial analytical parameters, in
particular the production of 5alpha-steroids such as androsterone and 5alpha-androstane-3alpha,17beta-diol, which was significantly reduced. In addition, the excretion of the main
metabolite of norandrostenedione, norandrosterone, was significantly suppressed, by up to
84 percent, in elimination studies. For doping-control analysis the use of 5alpha-reductase
inhibitors causes considerable problems because steroid profile parameters, which are
commonly considered stable, are highly affected and complicate the detection of steroid
abuse. In addition, the suppression of production and renal excretion of 5alpha-steroids such
as 19-norandrosterone generated from anabolic agents such as 19-norandrostenedione may
lead to false-negative doping-control results, because urine specimens are reported positive
only when a threshold level of 2 ng/mL is exceeded. Finally, a method for the determination
of the major urinary metabolite of finasteride (carboxy-finasteride) in routine doping-control
screening with use of liquid chromatography-tandem mass spectrometry is described,
allowing the detection of carboxy-finasteride for up to 94 hours in urine specimens collected
after an oral administration of 5 mg of finasteride [07097].

Finasteride (FIN), a widely used medication for the treatment of androgen-dependent
diseases, blocks the conversion of testosterone to a more potent androgen,
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regimen, which is needed to be confirmed in human subjects [11331].

Aromatase inhibitors

Aromatase is the rate-limiting enzyme in estrogen biosynthesis. As a cytochrome P450, it
utilizes electrons from NADPH-cytochrome P450 reductase (CPR) to produce estrogen from
androgen. Estrogen is a key factor in the promotion of hormone-dependent breast cancer
growth. Aromatase inhibitors (AIs) are drugs that block estrogen synthesis, and are widely
used to treat estrogen-dependent breast cancer. Structure-function experiments have been
performed to study how CPR and AIs interact with aromatase to further the understanding of
how these drugs elicit their effects. Studies have revealed a strong interaction between
aromatase and CPR, and that the residue K108 is situated in a region important to the interaction of aromatase with CPR. The published X-ray structure of aromatase indicates that the F221, W224 and M374 residues are located in the active site. The site-directed mutagenesis experiments confirm their importance in the binding of the androgen substrate as well as AIs, but these residues interact differently with steroidal inhibitors (exemestane) and non-steroidal inhibitors (letrozole and anastrozole). Furthermore, the results predict that the residue W224 also participates in the mechanism-based inhibition of exemestane, as time-dependent inhibition is eliminated with mutation on this residue. Together with previous research from our laboratory, the study confirms that W224, E302, D309 and S478 are important active site residues involved in the suicide mechanism of exemestane against aromatase [11335].

Aromatase inhibitors are a rapidly growing class of drugs including both steroidal and nonsteroidal mechanism-based inhibitors. The steroidal agents are mostly androstenedione analogs like testolactone, formestane (Lentaron), exemestane (Aromasin), and atamestane. The nonsteroidal agents are fadrozole, letrozole (Femara), anastrozole (Arimidex), vorozole (Rivizor), and finrazole [06127].

Aromatase inhibitors are a class of drugs used in the treatment of breast cancer and ovarian cancer in postmenopausal women. They may also be used off-label to treat or prevent gynaecomastia in men. Aromatase is the enzyme which synthesizes estrogen. As breast and ovarian cancers require estrogen to grow, AIs are taken to either block the production of estrogen or block the action of estrogen on receptors. Aromatase inhibitors work by inhibiting the action of the enzyme aromatase, which converts androgens into estrogens by a process called aromatization. As breast tissue is stimulated by estrogens, decreasing their production is a way of suppressing recurrence of the breast tumor tissue. The main source of estrogen is the ovaries in premenopausal women, while in post-menopausal women most of the bodies estrogen is produced in the conversion of androgens to estrogen by the aromatase enzyme in peripheral tissues (outside the CNS), and also a few CNS sites in various regions within the brain. Estrogen is produced and acts locally via action of the aromatase enzyme in these tissues, but any circulating estrogen, which exerts systemic estrogenic effects in men and women, is the result of estrogen escaping local metabolism and spreading to the circulatory system.

The purpose of this study was to determine the effects of an alleged aromatase and 5-alpha reductase inhibitor (AI) on strength, body composition, and hormonal profiles in resistance-trained men. Thirty resistance-trained men were randomly assigned in a double-blind manner to ingest 500 mg of either a placebo or AI once per day for 8 wk. Participants participated in a 4-d/wk resistance-training program for 8 wk. At Weeks 0, 4, and 8, body composition, 1-repetition-maximum (1RM) bench press and leg press, muscle endurance, anaerobic power, and hormonal profiles were assessed. Significant Group × Time interaction effects occurred over the 8-wk period for percent body fat, total testosterone, and bioavailable testosterone. Significant main effects for time were noted for bench- and leg-press 1RM, lean body mass, and estradiol. No significant changes were detected among groups for Wingate peak or mean power, total body weight, dihydrotestosterone, hemodynamic variables, or clinical safety data. The authors concluded that 500 mg of daily AI supplementation significantly affected percent body fat, total testosterone, and bioavailable testosterone compared with a placebo in a double-blind fashion [10461].

Exemestane
The representative of aromatase inhibitors referred to as exemestane and particularly its major metabolite 17β-hydroxyexemestane were implemented in routine doping controls using a modified GC-MS-based method [12016].

Exemestane is an irreversible aromatase inhibitor used for anticancer therapy. Unfortunately, this drug is also misused in sports to avoid some adverse effects caused by steroids administration. For this reason exemestane has been included in World Anti-Doping Agency prohibited list. Usually, doping control laboratories monitor prohibited substances through their metabolites, because parent compounds are readily metabolized. Thus metabolism studies of these substances are very important. Metabolism of exemestane in humans is not clearly reported and this drug is detected indirectly through analysis of its only known metabolite: 17β-hydroxyexemestane using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and gas chromatography coupled to mass spectrometry (GC-MS). This drug is extensively metabolized to several unknown oxidized metabolites. For this purpose LC-MS/MS has been used to propose new urinary exemestane metabolites, mainly oxidized in C6-exomethylene and simultaneously reduced in 17-keto group. Urine samples from four volunteers obtained after administration of a 25mg dose of exemestane were analyzed separately by LC-MS/MS. Urine samples of each volunteer were hydrolyzed followed by liquid-liquid extraction and injected into a LC-MS/MS system. Three unreported metabolites were detected in all urine samples by LC-MS/MS. The postulated structures of the detected metabolites were based on molecular formulae composition obtained through high accuracy mass determination by liquid chromatography coupled to hybrid quadrupole-time of flight mass spectrometry (LC-QTOF MS) (all mass errors below 2 ppm), electrospray (ESI) product ion spectra and chromatographic behavior [11452].

**Anastrozole and exemestane**

Anastrozole (2,2’-[5-(1H-1,2,4-triazol-1-ylmethyl)-1,3-phenylene]bis(2-methylpropionitrile)) and exemestane (6-methylenandrostan-1,4-diene-3,17-dione) are therapeutically used to treat hormone-sensitive breast cancer in postmenopausal women. For doping purposes they may be used to counteract adverse effects of an extensive abuse of anabolic androgenic steroids (gynaecomastia) and to increase plasma testosterone concentrations. Excretion study urine samples and spot urine samples from women suffering from metastatic breast cancer, being treated with anastrozole or exemestane, were collected and analyzed to develop/optimize a detection system for anastrozole and exemestane to allow the identification of athletes who do not comply with the internationally prohibited use of these cancer drugs. The assay was based on liquid-liquid extraction after enzymatic hydrolysis following liquid chromatography/tandem mass spectrometry (LC/MS/MS). Anastrozole, exemestane and its main metabolite (17-dihydroexemestane) were identified in urine by comparison of mass spectra and retention times with respective reference substances. An assay validation for the analysis of anastrozole and exemestane was performed regarding lower limits of detection (anastrozole: 0.02 ng/mL; exemestane: 3.1 ng/mL; and dihydroexemestane: 0.5 ng/mL) [06128].

**Formestane**

Aromatase is the enzyme that synthesizes estrogen. Aromatase inhibitors (AIs) are a class of drugs used in the treatment of breast cancer and ovarian cancer in postmenopausal women. AIs may also be used off-label to treat or prevent gynaecomastia in men. The aromatase inhibitor formestane (4-hydroxy-androst-4-ene-3,17-dione) is prohibited in sports by the World Anti-Doping Agency (WADA). Formestane possesses only weak androgenic properties and is presumed to be employed in order to suppress estrogen production during
the illicit intake of anabolic steroids by athletes. Former studies additionally showed that formestane is an endogenous steroid produced in low amounts. According to the regulations of WADA, urinary concentrations above 100 ng/mL are assumed to be due to ingestion of formestane. To distinguish between endogenous or exogenous sources of urinary formestane, isotope ratio mass spectrometry (IRMS) is the method of choice. Therefore, a method to determine the carbon isotope ratio (CIR) of formestane in urine samples was developed and validated. Routine samples (n=42) showing concentrations of formestane above 5 ng/mL were investigated and enabled elucidation of the CIR of endogenous formestane and subsequent the calculation of a reference limit. A reference population encompassing (n=90) males and females was investigated regarding endogenous concentrations of formestane. An excretion study with one male volunteer was conducted to test and validate the developed method and to identify possible impact of formestane administration on other endogenous steroids. By CIR determination of formestane it is clearly possible to elucidate its endogenous or exogenous source. Taking into account the CIR of other target analytes like testosterone, a differentiation between formestane and androstenedione intake is possible. In 2011, the first exogenous formestane below the WADA threshold could be detected by means of the developed IRMS method [12196].

**Metabolism of keto and hydroxy steroids**

4-OH-androstenedione (formastane)

4-Hydroxy-androst-4-ene-3,17-dione (formestane) is an aromatase inhibitor and has shown anti-tumour activity in post-menopausal cancer. It is prohibited in sports by WADA and classified as an anti-estrogenic agent, and intensively subjected to phase I metabolism. Among the major identified metabolites are 4-hydroxy-testosterone, 3,17-dihydroxy-androstan-4-ones; 3alpha,4beta-dihydroxy-5alpha-androstane-17-one, 3beta,4beta-dihydroxy-5alpha-androstane-17-one, 4beta-hydroxy-5alpha-androstan-3,17-dione and 3beta-hydroxy-5alpha-androstan-4,17-dione. Formestane itself was also identified as a metabolite of androst-4-ene-3,17-dione [06004].

4-OH-testosterone

4-Hydroxy-testosterone (17beta,4-dihydroxy-4-androstene-3-one) is a steroid sold as a nutritional supplement in the United States (Testobol®) and regarded as a prohibited substance by WADA. This substance is excreted as the parent compound as well as 4-hydroxy-androst-4-ene-3,17-dione [06004].

6-Oxo-androstenedione

6-Oxo-androstenedione (androst-4-ene-3,6,17-trione) is marketed as an aromatase inhibitor and sold as a nutritional supplement. Analysis of urine samples collected after administration of 6-oxo-androstenedione resulted in the detection of the parent compound, 6alpha-OH-testosterone (minor metabolite), 6alpha-OH-etiocholanolone and 6alpha-OH-androstenedione (major metabolites). The parent compound was excreted 88 percent glucuronidated and the other metabolites were exclusively excreted as glucuronides [06004].

7-Keto-dehydroepiandrosterone

7-Keto-dehydroepiandrosterone has been characterized as a metabolite of dehydroepiandrosterone in in vitro studies and was detected in human urine samples. 7-Keto-dehydroepiandrosterone and 3-acetyl-7-keto-dehydroepiandrosterone have been marketed as nutritional supplements as part of a replacement therapy to increase androgen concentrations and improve the feeling of well being in males and females of advanced age. In vivo, 7-keto-dehydroepiandrosterone and 3-acetyl-7-keto-dehydroepiandrosterone are
metabolised to 7beta-hydroxy-epiandrosterone and 7alpha-hydroxy-epiandrosterone. Additionally, elevated concentrations of 7-keto-dehydroepiandrosterone and 7-keto-androsterone as well as 7epsilon-hydroxy-androstenedione were also found [06004].

**Oxabolone**

Oxabolone (4,17beta-dihydroxyestr-4-en-3-one cyclopentylpropionate) is a cyclopentylpropionate ester of 4-hydroxy-19-nortestosterone. The myotropic:androgenic dissociation of the unesterified steroid is 4.5, but with a smaller duration of activity than the esterified analogue, which has a myotropic:androgenic dissociation of 6.0. The main metabolite of 4-hydroxy-nortestosterone has been identified as 4-hydroxy-norandrostenedione (4-hydroxy-estr-4-ene-3,17-dione). Besides this major metabolite and 4-hydroxy-19-nortestosterone, several other metabolites including 4-hydroxy-estra 3,17-dione, 3alpha,4-dihydroxy-5alpha-androstan-17-one, 3alpha,4-dihydroxy-5beta-estran-17-one and 3beta,4-dihydroxy-5alpha-estran-17-one were detected [06004].

**Testolactone**

Testolactone (D-homo-17alpha-oxa-androsta-1,4-diene-3,17-dione) a prohibited substance according to the International Olympic Committee in the class of anabolic steroids. However, evidence of its anabolic activity is lacking and under the current WADA-regulations testolactone is classified as an aromatase inhibitor. The A-ring of testolactone is particularly subjected to intensive metabolism and several metabolites have been identified of which 4,5-dihydrotestolactone seems to be the most prominent one [06004].

**Dehydrogenase inhibitor**

All redox reactions take place in the extramembranous portion of NADH dehydrogenase. NADH initially binds to NADH dehydrogenase, and transfers two electrons to the flavin mononucleotide (FMN) prosthetic group of complex I, creating FMNH2. The electron acceptor – the isoalloxazine ring – of FMN is identical to that of FAD. The electrons are then transferred through the second prosthetic group of NADH dehydrogenase via a series of iron-sulfur (Fe-S) clusters, and finally to coenzyme Q (ubiquinone). Despite more than 50 years of study of NADH dehydrogenase, no inhibitors blocking the electron flow inside the enzyme have been found. Hydrophobic inhibitors like rotenone or piericidin most likely disrupt the electron transfer between the terminal FeS cluster N2 and ubiquinone. It has been shown that long-term systemic inhibition of complex I by rotenone can induce selective degeneration of dopaminergic neurons. NADH dehydrogenase is also blocked by adenosine diphosphate ribose – a reversible competitive inhibitor of NADH oxidation by binding to the enzyme at the nucleotide binding site. Both hydrophyllic NADH and hydrophobic ubiquinone analogs act at the beginning and the end of the internal electron-transport pathway, respectively. 17beta-Hydroxysteroid dehydrogenase type 2 (17beta-HSD2) catalyzes the oxidation of the highly potent steroids: the estrogen estradiol (E2) and the androgen testosterone (T) to the less active estrone and androstenedione, respectively. Inhibition of this enzyme may help maintain the local E2 level in bone tissue when the circulating E2 level drops and is therefore a novel and promising approach for the treatment of osteoporosis. In this work, a series of new nonsteroidal and achiral 17beta-HSD2 inhibitors, namely N-benzyl-diphenyl-3(or 4)-carboxamide and N-benzyl-5-phenyl-thiophene-2-carboxamide was designed and the compounds were synthesized in a two to three steps reaction. A small library was built applying parallel synthesis. Highly potent 17beta-HSD2 inhibitors could be identified in the thiophene-2-carboxamide class with IC50 in the low nanomolar range. These compounds also showed a good selectivity profile toward 17beta-HSD1 and toward the estrogen receptors alpha and beta. The most interesting 17beta-HSD2 inhibitor identified in
this study is the 5-(2-fluoro-3-methoxyphenyl)-N-(3-hydroxybenzyl)-N-methylthiophene-2-carboxamide 6 week displaying an IC$_{50}$ of 61 nM and a selectivity factor of 73 toward 17beta-HSD1 [11454].

**Transsexuality**

Sex segregation in competitive sports is regarded as fair. Before puberty boys and girls do not differ in height, muscle and bone mass. Testosterone exposure during puberty leads to an ultimate average greater height in men of 12-15 cm, longer and larger bones and muscle mass and strength and higher hemoglobin levels. Postpubertal androgen ablation reverses, at least in part, previous anabolic effects of testosterone on muscle, bone mineral density and hemoglobin but the long bones remain longer and wider. Testosterone administration dose dependently increases muscle mass and maximal voluntary strength. Therefore, exogenous androgens, being performance enhancing drugs, are banned for all athletes. An issue is the participation in competitive sports of people with errors of sexual differentiation and particularly transsexuals who have been sex-reassigned. In view of the effects of testosterone a clear demarcation is whether sex reassignment has taken place before or after hormonal puberty. Pubertal effects of testosterone are in part reversible but there is no reliable evidence as to its completeness. The International Olympic Committee (IOC) has taken an inevitably arbitrary decision with regard to participation of sex-reassigned transsexuals in elite sports: sex reassignment must have taken place at least two years earlier, hormone treatment must be appropriate for the reassigned sex and the reassigned sex must be legally recognized. The IOC policy is not binding for other organisations [08155].
SELECTIVE ANDROGEN RECEPTOR MODULATOR (SARM)

The elucidation of the metabolism of new therapeutics is a major task for pharmaceutical companies and of great interest for drug testing laboratories. The latter in particular need to determine the presence or absence of drugs or their metabolic products in urine to test for a misuse of these compounds. Commonly, in vitro or animal models are used to mimic the human metabolism and produce potential targets in amounts allowing for method development. Selective androgen receptor modulators (SARMs) have become a major field of clinical research enabling the tissue-selective stimulation of androgen receptors. The treatment of debilitating diseases, osteoporosis and frailty are primary goals and promising results have been obtained from clinical trials.

Androgen receptor (AR) plays a critical role in the function of several organs including primary and accessory sexual organs, skeletal muscle, and bone, making it a desirable therapeutic target. Selective androgen receptor modulators (SARMs) bind to the AR and demonstrate osteo- and myo-anabolic activity; however, unlike testosterone and other anabolic steroids, these nonsteroidal agents produce less of a growth effect on prostate and other secondary sexual organs. SARMs provide therapeutic opportunities in a variety of diseases, including muscle wasting associated with burns, cancer, or end-stage renal disease, osteoporosis, frailty, and hypogonadism [08196].

The selective androgen receptor modulators represent a novel class of drugs with tissue-specific agonistic and antagonistic properties, but are prohibited in sports from January 2008 according to the World Anti-Doping Agency. Preventive approaches to restrict the use of SARMs include early implementation of target analytes into doping control screening assays. Five model SARMs were synthesized, four of which are analogs to prostate-specific androgen receptor antagonists with a 5,6-dichloro-benzimidazole nucleus. The fifth SARM is a muscle-tissue specific agonist with a bicyclic hydantoin structure (BMS-564929). Dissociation pathways after negative electrospray ionization were studied using an LTQ-Orbitrap mass analyzer, and diagnostic product ions and common fragmentation patterns were employed to establish a screening procedure that target the intact SARMs as well as putative metabolic products. Sample preparation based on solid-phase extraction and subsequent LC-MS/MS measurement allowed for detection limits of 1-20 ng/mL, intra- and interday precisions of between 2.4 and 13.2 percent and between 6.5 and 24.2 percent, respectively. Recoveries varied from 89 to 106 percent, and tests for ion suppression or enhancement effects were negative for all analytes [08197].

Nonsteroidal selective androgen receptor modulators (SARMs) are an emerging class of drugs for treatment of various diseases including osteoporosis and muscle wasting as well as the correction of age-related functional decline such as muscle strength and power. Several SARMs, which have advanced to preclinical and clinical trials, are composed of diverse chemical structures including arylpropionamide-, bicyclic hydantoin-, quinoline-, and tetrahydroquinoline-derived nuclei. Since January 2008, SARMs have been categorized as anabolic agents and prohibited WADA. Suitable detection methods for these low-molecular weight drugs were based on mass spectrometric approaches, which necessitated the elucidation of dissociation pathways in order to characterize and identify the target analytes in doping control samples as well as potential metabolic products and synthetic analogs. Fragmentation patterns of representatives of each category of SARMs after electrospray ionization and collision-induced dissociation (CID) as well as electron ionization (EI) have been discussed The complexity and structural heterogeneity of these drugs is a daunting challenge for detection methods [08198].
Androgen receptor (AR) ligands are important for the development and function of several tissues and organs. However, the poor oral bioavailability, pharmacokinetic properties, and receptor cross-reactivity of testosterone, coupled with side effects, place limits on its clinical use. Selective AR modulators (SARMs) elicit anabolic effects in muscle and bone, sparing reproductive organs like the prostate. However, molecular mechanisms underlying the tissue selectivity remain ambiguous. It was performed a variety of in vitro studies to compare and define the molecular mechanisms of an aryl propionamide SARM, S-22, as compared with dihydrotestosterone (DHT). Studies indicated that S-22 increased levator ani muscle weight but decreased the size of prostate in rats. Analysis of the upstream intracellular signaling events indicated that S-22 and DHT mediated their actions through distinct pathways. Modulation of these pathways altered the recruitment of AR and its cofactors to the PSA enhancer in a ligand-dependent fashion. These studies reveal novel differences in the molecular mechanisms by which S-22, a nonsteroidal SARM, and DHT mediate their pharmacological effects [08199].

Selective androgen receptor modulators represent an emerging class of therapeutics to counteract various diseases such as osteoporosis and muscle wasting. Numerous drug candidates have been developed and investigated including a group that comprises a tricyclic tetrahydroquinoline nucleus such as 2-methyl-2-(8-nitro-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]chinolin-4-yl)propan-1-ol. Due to their novelty and medicinal purpose, these compounds also possess great potential for misuse in sports, and studies on the mass spectrometric behavior of three synthesized model substances and drug candidates were conducted to provide information on typical dissociation pathways following electrospray ionization and collision-induced dissociation. Product ion mass spectra derived from protonated molecules were studied using high resolution/high accuracy orbitrap mass spectrometry, and characteristic fragmentation routes and product ions were elucidated. Major and general findings include the elimination of a hydroxyl radical from [M+H](+)+, the elimination of the 2-substituted side chain, and the gas-phase rearrangement of the investigated tricyclic tetrahydroquinolines to 6-nitroquinoline yielding a common product ion at m/z 175. Knowledge of these dissociation pathways supports the identification of related substances as well as metabolic products, which is of utmost importance to drug testing laboratories. The compounds were implemented into existing screening procedures, and detection limits (0.2-0.6 ng/mL), recoveries (92-97 %), and intraday and interday precision (<22 %) have been evaluated [08200].

The potential for misuse of SARMs in sport is great and drug testing methods based on liquid chromatography were established for different classes including arylpropionamide-, 2quinolinone- and bicyclic hydantoin-derived compounds. As gas chromatography and mass spectrometry (GC-MS) are still important analytical tools in sports drug testing, a method to determine 2-quinolinone- and bicyclic hydantoin-derived SARMs established. Spiked urine samples were subjected to routine doping control protocols including enzymatic hydrolysis, liquid-liquid extraction, concentration and derivatisation to trimethylsilylated analogues followed by GC-MS analysis. The method was validated for the items specificity, lower limit of detection (0.2-10 ng/mL), recovery (83-85 %), intraday and interday precision (9-15 % and 13-18 %, respectively), which demonstrates the suitability of conventional GC-MS systems to determine representatives of an emerging class of compounds in doping control specimens [08201].

Anabolic hormones, including testosterone, have been suggested as a therapy for aging-related conditions, such as osteoporosis and sarcopenia. These therapies are sometimes associated with severe androgenic side effects. The past decade has witnessed an unprecedented discovery effort to develop selective androgen receptor modulators (SARMs)
that improve physical function and bone health without adversely affecting the prostate and cardiovascular outcomes. SARMs have the potential to mimic the desirable central and peripheral androgenic anabolic effects of testosterone without having its side effects. In this study we evaluated the effects of LGD2941, in comparison to testosterone, on mRNA expression of selected target genes in whole blood in an non-human model. The regulated genes can act as potential blood biomarker candidates in future studies with androgen receptor ligands. Cynomolgus monkeys (Macaca fascicularis) were treated either with testosterone or LGD2941 for 90 days in order to compare their effects on mRNA expression in blood. Blood samples were taken before SARM application, on day 16 and on day 90 of treatment. Gene expression of 37 candidate genes was measured using quantitative real-time RT-PCR (qRT-PCR) technology. Our study shows that both testosterone and LGD2941 influence mRNA expression of 6 selected genes out of 37 in whole blood. The apoptosis regulators CD30L, Fas, TNFR1 and TNFR2 and the interleukins IL-12B and IL-15 showed significant changes in gene expression between control and the treatment groups and represent potential biomarkers for androgen receptor ligands in whole blood [09143].

One review described the historical evolution, the rationale for SARM development, and the mechanisms of testosterone action and SARM selectivity. Although steroidal SARMs have been around since the 1940s, a number of nonsteroidal SARMs that do not serve as substrates for CYP19 aromatase or 5alpha-reductase, act as full agonists in muscle and bone and as partial agonists in prostate are in development. The differing interactions of steroidal and nonsteroidal compounds with androgen receptor (AR) contribute to their unique pharmacologic actions. Ligand binding induces specific conformational changes in the ligand-binding domain, which could modulate surface topology and protein-protein interactions between AR and coregulators, resulting in tissue-specific gene regulation. Preclinical studies have demonstrated the ability of SARMs to increase muscle and bone mass in preclinical rodent models with varying degree of prostate sparing. Phase I trials of SARMs in humans have reported modest increments in fat-free mass [09144].

It was described the discovery of ACP-105 (1), a novel and potent nonsteroidal selective androgen receptor modulator (SARM) with partial agonist activity relative to the natural androgen testosterone. Compound 1 was developed from a series of compounds found in a HTS screen using the receptor selection and amplification technology (R-SAT). In vivo, 1 improved anabolic parameters in a 2-week chronic study in castrated male rats. In addition to compound 1, a number of potent antiandrogens were discovered from the same series of compounds whereof one compound, 13, had antagonist activity at the AR T877A mutant involved in prostate cancer [09145].

Hundreds of steroidal analogs have been prepared with a superior oral bioavailability, which should also possess reduced undesirable effects. However, only a few entered the pharmaceutical market due to severe toxicological incidences that were mainly attributed to the lack of tissue selectivity. Prominent representatives of anabolic-androgenic steroids (AAS) are for instance methyltestosterone, metandienone and stanozolol, which are discussed as model compounds with regard to general pharmacological aspects of synthetic AAS. Recently, nonsteroidal alternatives to AAS have been developed that selectively activate the androgen receptor in either muscle tissue or bones. These so-called selective androgen receptor modulators (SARMs) are currently undergoing late clinical trials (IIb) and were prohibited by the World Anti-Doping Agency from January 2008. Their entirely synthetic structures are barely related to steroids, but particular functional groups allow for the tissue-selective activation or inhibition of androgen receptors and, thus, the stimulation of muscle growth without the risk of severe undesirable effects commonly observed in steroid replacement therapies. Hence, these compounds possess a high potential for misuse in sports and will be the subject of future doping control assays [10097].
Selective androgen receptor modulators (SARMs) are potent anabolic agents with tissue-selective properties. Due to their potential misuse in elite sport, the World Anti-Doping Agency (WADA) has prohibited SARMs since 2008, and although no representative drug candidate has yet received full clinical approval, recent findings of SARMs illegally sold via the internet have further supported the need to efficiently test for these compounds in doping controls. In one communication, the mass spectrometric characterization of urinary metabolites of the SARM Andarine (also referred to as S-4) compared with earlier in vitro and animal studies is reported. Liquid chromatography interfaced to high-resolution/high-accuracy (tandem) mass spectrometry was used to identify phase I and II metabolites, confirming the predicted target analytes for sports drug testing purposes including the glucuronic acid conjugates of the active drug, its monohydroxylated and/or deacetylated product, the hydrolysis product resulting from the removal of the compound’s B-ring, as well as the sulfate of the monohydroxylated and the deacetylated phase I metabolite. The obtained data will support future efforts to effectively screen for and confirm the misuse of the non-approved drug candidate Andarine [10348].

Glucocorticoids are the most widely used antiinflammatory drugs in the world. However, prolonged use of glucocorticoids results in undesirable side effects such as muscle wasting, osteoporosis, and diabetes. Skeletal muscle wasting, which currently has no approved therapy, is a debilitating condition resulting from either reduced muscle protein synthesis or increased degradation. The imbalance in protein synthesis could occur from increased expression and function of muscle-specific ubiquitin ligases, muscle atrophy F-box (MAFbx)/atrogen-1 and muscle ring finger 1 (MuRF1), or decreased function of the IGF-I and phosphatidylinositol-3 kinase/Akt kinase pathways. It was examined the effects of a nonsteroidal tissue selective androgen receptor modulator (SARM) and testosterone on glucocorticoid-induced muscle atrophy and castration-induced muscle atrophy. The SARM and testosterone propionate blocked the dexamethasone-induced dephosphorylation of Akt and other proteins involved in protein synthesis, including Forkhead box O (FoxO). Dexamethasone caused a significant up-regulation in the expression of ubiquitin ligases, but testosterone propionate and SARM administration blocked this effect by phosphorylating FoxO. Castration induced rapid myopathy of the levator ani muscle, accompanied by up-regulation of muscle atrophy F-box and muscle ring finger 1 and down-regulation of IGF-I, all of which was attenuated by a SARM. The results suggest that levator ani atrophy caused by hypogonadism may be the result of loss of IGF-I stimulation, whereas that caused by glucocorticoid treatment relies almost solely on up-regulation of muscle atrophy F-box and muscle ring finger 1. The studies provide the first evidence that glucocorticoid- and hypogonadism-induced muscle atrophy are mediated by distinct but overlapping mechanisms and that SARMs may provide a more effective and selective pharmacological approach to prevent glucocorticoid-induced muscle loss than steroidal androgen therapy [10349].

An alternative route based on electrochemical reactions of drugs was reported to allow for the generation of selected metabolites. The utility of this approach for doping control purposes was demonstrated with a novel class of anabolic agents termed selective androgen receptor modulators (SARMs). An arylpropionamide- derived drug candidate was subjected to electrochemical "metabolism" and a major phase-I- metabolite, resulting from the elimination of a substituted phenol residue as identified in in vitro experiments, was generated and characterised using liquid chromatography/nuclear magnetic resonance spectroscopy and high resolution/high accuracy mass spectrometry. The metabolite was included in routine doping control procedures based on liquid chromatography/tandem mass spectrometry and has served as a reference compound for 5000 doping control specimens [08202].
Selective androgen receptor modulators (SARMs) represent an emerging class of drugs likely to be abused in sport. For clinical applications, these substances provide a promising alternative to testosterone-replacement therapies and their advantages include oral bioavailability, androgen receptor specificity, tissue selectivity, and the absence of steroid-related side effects. Although not yet commercially available, since January 2008 SARMs have been included on the prohibited list issued yearly by the World Anti-Doping Agency (WADA), so control laboratories need to update their procedures to detect either the parent drugs or their metabolites. Within this context, two quinolinone SARM models were synthesized and automatically characterized to update the existing routine screening procedures. The conditions for the new target analytes are compatible with the existing laboratory protocols used for both in-competition and out-of-competition controls and can be included in them. Validation parameters according to ISO 17025 and WADA guidelines were successfully determined. For analytical determinations, spiked urine samples were hydrolyzed and extracted at pH 9.6 with 10 mL of tert-butyl methyl ether. Then, the analytes were subsequently converted into trimethylsilyl derivatives and detected by gas chromatography-mass spectrometry. The absence of interferents, together with excellent repeatability of both retention times and the relative abundances of diagnostic ions, allowed proper identification of all SARM analytes. The analytes' quantification was linear up to 500 ng/mL and precision criteria were satisfied (coefficient of variation less than 25 percent at 10 ng/mL). The limits of detection were 1 ng/mL for both SARMs, whereas recovery values were between 96 and 99 percent. The validated method can be efficiently used for urine screening of the 2-quinolinone-derived SARMs tested [11111].

Selective androgen receptor modulators (SARM) are a prominent group of compounds for being misused in sports owing to their advantageous anabolic properties and reduced side effects. To target the preventive doping control analysis in relevant compounds, the challenge is to predict the metabolic fate of a new compound. For aryl-propionamide-derived SARM, an in vitro assay employing microsomal and S9 human liver enzymes was developed to simulate phase-I and phase-II metabolic reactions. In vitro metabolic profiles and the structure-metabolic relationship were compared between four structurally modified substrates. Accurate mass measurements were used to characterize the synthesized metabolites, and also collision-induced dissociation was examined to suggest the methodological approach to monitor the prohibited use of aryl-propionamide-derived drug candidates. Subsequent phase-I and phase-II metabolic reactions were successfully combined in one in vitro assay. The main routes of phase-I modifications involved the hydrolysis of ether linkage, monohydroxylation, and hydrolytic cleavage of the amide bond. Nitro-reduction and deacetylation were reactions observed for substrates possessing the corresponding functionality. SARM metabolites were analyzed in negative ion electrospray ionization and detected as deprotonated species [M-H](−). The main metabolic modifications were observed to occur in the B-ring side, and collision-induced dissociation resulted in the product ions originating from the A-ring side of the compound. These structure-specific ions may be monitored as target ions in the routine doping control [07099].

New anabolic agents termed selective androgen receptor modulators (SARMs) have a structural diversity (they encompass at least six chemical categories). It has necessitated the extension of doping control assays through the introduction of new target analytes as well as precursor ion scanning in order to make sure that this emerging class of drugs is comprehensively screened for. Detection assays for arylpropionamide-derived SARMs were reported for between 1 and 50 ng per mL of urine. Screening assays are of particular importance in doping controls, as they provide the information necessary to consider a sample suspicious for drug abuse. They have been updated and extended frequently based on new requirements as well as new findings, such as additional long-term metabolites for
improved retrospectives, as recently reported for methandienone. However, the requirements of obligatory confirmation analyses are strict and have also led to the development of alternative sample preparation procedures for LC-MS/MS measurements that are specifically optimized for just a few analytes [07050].

Selective androgen receptor modulators (SARMs) represent a class of emerging drugs with high potential for misuse in sports, and therefore members of this group are banned as anabolic agents by the World Anti-Doping Agency. Preventive approaches to restrict their use include early implementation of target analytes into doping control screening assays and evaluation of the mass spectrometric behavior of these drugs to allow their unequivocal identification as well as the characterization of structurally related compounds and metabolic products. Four model SARMs with the 6-alkylamino-2-quinolinone structure, including the advanced drug candidate LGD-2226, were synthesized. Fragmentation pathways after positive electrospray ionization and collision-induced dissociation were studied using an LTQ Orbitrap mass analyzer, and diagnostic product ions and common dissociation pathways were employed to establish a screening procedure targeting intact quinolinone-based SARMs as well as putative metabolic products such as dealkylated analogues. Therefore, features of a triple quadrupole mass analyzer such as multiple reaction monitoring and precursor ion scanning were utilized. Sample preparation based on commonly employed liquid-liquid extraction and subsequent liquid chromatographic/tandem mass spectrometric measurement allowed for detection limits of 0.01-0.2 ng/mL, and intra- and interday precisions between 3.2 and 8.5 percent and between 6.3 and 16.6 percent, respectively. Recoveries varied from 81 to 98 percent, and tests for ion suppression or enhancement effects were negative for all analytes [07100].

Selective androgen receptor modulators (SARMs) bind to the androgen receptor and demonstrate anabolic activity in a variety of tissues; however, unlike testosterone and other anabolic steroids, these nonsteroidal agents are able to induce bone and muscle growth, as well as shrinking the prostate. The potential of SARMS is to maximise the positive attributes of steroidal androgens as well as minimising negative effects, thus providing therapeutic opportunities in a variety of diseases, including muscle wasting associated with burns, cancer, end-stage renal disease, osteoporosis, frailty and hypogonadism. One review summarised androgen physiology, the current status of SARMS and potential therapeutic indications for this emerging class of drugs [06129].

The androgen receptor mediates the androgenic and anabolic activity of the endogenous steroids testosterone and 5alpha-dihydrotestosterone. Current knowledge of the androgen receptor protein structure, and the molecular mechanisms surrounding the binding properties and activities of agonists and antagonists has led to the design and development of novel nonsteroidal ligands with selected tissue-specific androgen receptor agonist and antagonist activities. The activity of these compounds, termed selective androgen receptor modulators (SARMs), is directed toward the maintenance or enhancement of anabolic effects on bone and muscle with minimal androgenic effects on prostate growth. SARMs are of potential therapeutic value in the treatment of male hypogonadism, osteoporosis, frailty and muscle wasting, burn injury and wound healing, anemia, mood and depression, benign prostatic hyperplasia and prostate cancer [06130].

Among the class of non-steroidal anabolic agents, selective androgen receptor modulators (SARMs) were studied regarding their in vitro as well as in vivo metabolism. The urgency of these studies was underlined by reports demonstrating once more the easy availability of authentic SARMs via Internet-based suppliers despite the fact that SARMs have not yet received clinical approval [12016].
Anabolic agents have been top-ranked for many years among statistics of adverse analytical findings compiled by the World Anti-Doping Agency (WADA). Besides archetypical anabolic-androgenic steroids (AAS), alternative substances with similar effects concerning bone and muscle anabolism have been therapeutically pursued. A prominent emerging class of drugs is the chemically heterogeneous group of selective androgen receptor modulators (SARMs), some of which have been detected in doping control samples between 2009 and 2012 despite missing clinical approval. In order to support the momentum of expanding the preventive and proactive measures among anti-doping laboratories, the analytical characterization of substances with misuse potential is of great importance. In the present study, the SARM drug candidates RAD140 (comprising a 5-phenyloxadiazole nucleus) and ACP-105 (bearing an N-substituted tropanol pharmacophore) were studied regarding their mass spectrometric behavior under ESI-MS(ESI/MS) and EI-MS(ESI/MS) conditions. Reference material was synthesized according to established protocols and dissociation pathways of RAD140 and ACP-105 were elucidated with liquid chromatography/electrospray ionization quadrupole/time-of-flight or iontrap/orbitrap and gas chromatography/electron ionization quadrupole/time-of-flight high resolution/high accuracy mass spectrometry. Fragmentation pathways to diagnostic product ions of RAD140 (e.g., m/z 223 and 205 using ESI-MS/MS and m/z 421 and 349 using EI-MS/MS) and ACP-105 (such as m/z 233 and 193 or 231 and 217 for ESI-MS/MS and EI-MS/MS measurements, respectively) were proposed as substantiated by determined elemental compositions and MS(n) experiments as well as comparison to spectra of a structural analog. Notably, for the formation of the characteristic fragment ion at m/z 421 of RAD140, the comparably seldom intramolecular migration of a trimethylsilyl residue triggered by electron ionization was suggested as corroborated by all of the above-mentioned analytical means. The obtained data will support future sports drug testing methods and facilitate and accelerate the implementation of this analyte and related compounds or metabolites in both GC/MS(ESI/MS) and LC/MS(ESI/MS)-based routine doping control procedures [13268].
compounds or metabolites in both GC/MS/(MS)- and LC/MS/(MS)-based routine doping control procedures [13269].

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Background

Androgens are essential for male development and the maintenance of male secondary characteristics, such as bone mass, muscle mass, body composition, and spermatogenesis. Testosterone and structurally related anabolic steroids have been used to treat hypogonadism, muscle wasting, osteoporosis, cancer cachexia, and anemia, and been used for male contraception, and hormone replacement therapy in aging men or age-related frailty; while antiandrogens may be useful for treatment of conditions like acne, alopecia (male-pattern baldness), hirsutism, benign prostatic hyperplasia (BPH) and prostate cancer. However, the clinical application of the steroidal AR ligands has been limited by poor oral bioavailability, potential hepatotoxicity, lack of tissue selectivity, and occasionally, cross reaction with other steroid receptors. Also, structural modification of the steroidal ligands is somewhat limited by the steroid skeleton. Therefore, nonsteroidal AR ligands with improved pharmacological and pharmacokinetic properties have been developed to overcome these problems. The known AR ligands can be classified as steroidal or nonsteroidal based on the structure or as agonist and antagonist (antiandrogen) based on their ability to activate or inhibit transcription of AR target genes. Endogenous androgens, including testosterone and dihydrotestosterone (DHT), are steroidal agonists. Structural modifications of the endogenous steroids led to the development of various synthetic steroids, including agonists and antagonists. Nonsteroidal ligands were also proposed to achieve high AR specificity,
improved oral bioavailability, improved tissue selectivity, and more flexible structural modifications. Nonsteroidal AR ligands with improved pharmacological and pharmacokinetic properties have been developed to overcome these problems. One review focused on the pharmacokinetics, metabolism, and pharmacology of clinically used and emerging nonsteroidal AR ligands, including antagonists, agonists, and selective androgen receptor modulators [06049].

**Steroidal ligands and their clinical applications**

Testosterone and DHT are endogenous androgens. There are three modes of action of testosterone. It may directly act through AR in target tissues where 5alpha-reductase is not expressed, be converted to 5alpha-DHT (5-10 %) by 5alpha-reductase before binding to AR, or be aromatized to estrogen (0.2 %) and act through the estrogen receptor. DHT binds to AR with higher affinity, and has two to ten fold higher potency than testosterone in androgen-responsive tissues. On the other hand, estrogen plays a major role in regulating metabolic processes, mood and cognition, cardiovascular disease, sexual function including libido, and bone turnover in men. Testosterone is the major androgen that acts in FDHT-independent tissues, such as skeletal muscle, where 5alpha-reductase is not expressed or expressed at a very low level and it directly regulates skeletal muscle growth, bone formation, fat distribution, and sexual function. Following oral administration, the plasma half life of testosterone is less than 30 min, due to extensive metabolism. Approximately 90 percent of an oral dose of testosterone is metabolized before it reaches the systemic circulation. To improve the bioavailability, most of the testosterone preparations are delivered through transdermal patch or intramuscular injections. Alkylation or esterification at the 17 position was widely used in structural modification of the steroid skeleton to markedly slow down the hepatic metabolism and increase the oral bioavailability or duration of testosterone action. However, 17alpha-alkylated steroidal androgens are more likely to cause hepatotoxicity, the most serious side effect of the synthetic steroids. On the other hand, complete separation of androgenic and anabolic activity has not been accomplished with synthetic steroids. The androgenic activities of the synthetic steroids often cause undesirable side effects during therapy. Due to the structural similarity in the steroid skeleton, steroidal AR ligands also tend to cross react with other steroid receptors, which is also associated with adverse effects (i.e. gynecomastia) [06049].

Classically, testosterone is used to treat male hypogonadism, protein wasting diseases associated with cancer, burns, traumas, or Acquired Immunodeficiency Syndrome (AIDS), anemia secondary to chronic renal failure, aplastic anemia, hereditary angioedema, or as a component of hormonal male contraception. Recently, hormone replacement therapy (HRT) in aging males has also been proposed to improve body composition, bone and cartilage metabolism, certain domains of brain function, and even decrease cardiovascular risk. For most clinical applications, testosterone is usually given as longer acting esters through intramuscular injections, surgical implantation for implants and pellets, or transdermal delivery, such as patches and gels. In general, these administration routes are not very convenient, and are sometimes associated with fluctuation in serum testosterone levels, skin rashes and irritation. Pharmacologically, exogenous testosterone works well for male hypogonadism related to deficiency of endogenous hormone production, including primary (testicular), secondary (hypothalamic or pituitary), and age-related hypogonadism. However, when testosterone is used for age-related hypogonadism (HRT in aging men), the potential risk in the prostate becomes a major concern of long term treatment. Besides hypogonadism, testosterone is mainly used for the treatment of disease related muscle wasting and male hormonal contraception. When supraphysiologic concentrations of testosterone is used for
male contraception, steroid-related side effects, including decreases in HDL cholesterol, increases in hematologic parameters such as hemoglobin and hematocrit, increased body weight, and acne, are the major drawbacks of the treatment. Androgen can also be used as anabolic reagent to treat muscle wasting. Commonly used anabolic steroids include nandrolone decanoate and oxandrolone, although nandrolone decanoate is known to be associated with hepatotoxicity and side effects on the blood lipid profile. Muscle is not the only anabolic tissue. It has also been proposed that testosterone can be used as an anabolic reagent to treat osteoporosis, since androgens seem to have direct anabolic effects in bone, and the anabolic effects in skeletal muscle mass and strength could also be beneficial to the treatment of osteoporosis. However, the anabolic effects associated with most steroidal androgens become major concerns for therapy, particularly in aging men and women. On the other hand, both antiandrogens and 5alpha-reductase inhibitors are used to block androgen action in prostate cancer, BPH, and acne. The application of these steroidal antiandrogens, like cyproterone acetate and spironolactone, has been limited by the weak antagonist activities or crossreaction with other steroid receptors. Also, due to the lack of tissue selectivity, complete androgen blockage with antiandrogens also cause severe side effects related to androgen deficiency (e.g. loss of libido, hot flashes, impotence, and increased incidence of osteoporosis). In summary, steroidal AR ligands, including agonists and antagonists, are used in the treatment of a variety of androgen disorders. However, the side effects related to the lack of tissue selectivity, hepatotoxicity, and inconvenience of delivery limits the more widespread therapeutic applications of androgens. Several structural classes of nonsteroidal AR antagonists have been discovered. In summary, the development of nonsteroidal AR ligands will continue, with particular focus on the search for ligands that are AR specific, metabolically stable, safe, and tissue selective. A better understanding of the mechanism of action of the known nonsteroidal AR ligands will help design the next generation of ligands with improved target specificity and tissue selectivity that could greatly benefit the treatment of many diseases [06049].

**Arylpropionamide-based SARMs**

Drugs that promote anabolic processes with limited undesirable effects are of considerable therapeutic interest; some notable examples include those for the treatment of cancer cachexia and muscle-wasting diseases. Anabolic properties are not only therapeutically beneficial to critically ill and debilitated patients, but are also desirable to athletes seeking artificial enhancements in endurance, strength and accelerated recovery. The use of anabolic agents in the clinical setting is being reconsidered with the emergence of a new class of drugs referred to as SARMs (selective androgen receptor modulators). SARMs have the potential to complement or even replace anabolic androgenic steroidal use with the benefit of a reduction of the undesirable side effects associated with steroid administration alone. Arylpropionamide-based SARMs such as andarine (S-4) and S-22 have shown promising therapeutic properties and have attracted the interest of elite and amateur athletes despite the absence of clinical approval, and evidence for trafficking and misuse in sport has been obtained by doping control authorities. In this communication, the elucidation of urinary metabolites of the SARM drug candidate S-22 is compared with earlier in vitro metabolism studies. Following oral administration of illicit S-22, urine samples were collected after 62 and 135 h and analyzed for the active drug and its major metabolic products. Liquid chromatography interfaced with high-resolution/high-accuracy (tandem) mass spectrometry was used to identify and/or confirm the predicted target analytes for sports drug testing purposes. S-22 was detected in both specimens accompanied by its glucuronic acid conjugate. This was the B-ring hydroxylated derivative of S-22 plus the corresponding glucuronide (with the phase-II metabolites being the more abundant analytes). In addition,
the samples collected 62 h post-administration also contained the phase-I metabolite hydroxylated at the methyl residue (C-20) and the B-ring depleted degradation product “dephenylated” S-22) together with the corresponding carboxy analog that was previously reported for canine metabolism. The obtained data supports future efforts to effectively screen for and confirm the misuse of the non-approved S-22 drug candidate in doping controls [11334].

**SARMs produced by fungus**

Selective androgen receptor modulators (SARMs) are a group of substances that have potential to be used as doping agents in sports. Being a relatively new group not available on the open market means that no reference materials are commercially available for the main metabolites. In one study, the in vitro metabolism of SARMs by the fungus Cunninghamamella elegans has been investigated with the purpose of finding out if it can produce relevant human and equine metabolites. Three different SARMs, S1, S4 and S24, were incubated for 5 days with C. elegans. The samples were analysed both with and without sample pretreatment using ultra performance liquid chromatography coupled to high resolution mass spectrometry. All the important phase I and some phase II metabolites from human and horse were formed by the fungus. They were formed through reactions such as hydroxylation, deacetylation, O-dephenylation, nitro-reduction, acetylation and sulfonation. 4.

The study showed that the fungus produced relevant metabolites of the SARMs and thus can be used to mimic mammalian metabolism. Furthermore, it has the potential to be used for future production of reference material [13271].

**Andarine**

Selective androgen receptor modulators (SARMs) are a relatively new class of non-steroidal compounds that possess potent anabolic activity. They were developed following the identification of androgen receptor antagonists such as bicalutamine in 1998, as an alternative to anabolic steroids for treating a wide range of clinical conditions including cancer cachexia, muscle wasting, osteoporosis, androgen deficiency, and prostate cancer. SARMs are a heterogeneous group of compounds that are usually classified based on core structures such as aryl propionamide, quinolinone, tetrahydroquinoline, bicyclic hydantoin, and numerous others. SARMs have high muscle and bone tissue selectivity and are not substrates for alpha-reductase and aromatase enzymes. This results in less androgenic and estrogenic effects compared to anabolic steroids. Indeed, preclinical animal studies have demonstrated that SARMs can prevent bone loss and reduce body fat with fewer undesirable anabolic steroid side-effects. Although none of the SARMs have been approved for human use, a limited number have completed or are currently being evaluated in multicentre clinical trials. S-4 [Andarine, S-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethyl-phenyl)-propionamide] a member of the aryl propionamide class of SARMs was evaluated in a phase I clinical trial, but the study had to be stopped due to adverse side-effects involving visual disturbances. S-22 (Ostarine®, GTx-024, enobosarm), another SARM in this class, is currently undergoing phase III clinical trials in patients with non-small lung cancer. Given the potential for misuse by athletes in power and strength sports, SARMs were placed on the World Anti-Doping Agency (WADA) list of prohibited substances in 2008. Although they are not available legally for medical purposes, a few SARMs have made their way on to the black market. For instance, S-4 is available for purchase in the United States via the internet. The S-22 SARM is also available via the Internet and has recently been identified in the urine of a 37-year-old male who exercises regularly. It is concerning that
someone would consider using these substances before they have been rigorously evaluated for safety in clinical trials. In the case of S-4, the adverse side-effects are known and should discourage anyone from using this substance. S-22 is still being evaluated and has common side-effects that include fatigue, anaemia, nausea, diarrhoea, headache, back pain, decreased total serum testosterone, and elevations in liver enzymes [13272]. Selective androgen receptor modulators (SARMs) comprise a new class of molecules that induce anabolic effects with fewer side effects than those of other anabolic agents. It was previously reported that the novel SARM S-101479 had a tissue-selective bone anabolic effect with diminished side effects in female animals. However, the mechanism of its tissue selectivity is not well known. In one report, it was shown that S-101479 increased alkaline phosphatase activity and androgen receptor (AR) transcriptional activity in osteoblastic cell lines in the same manner as the natural androgen ligand dihydrotestosterone (DHT); conversely, stimulation of AR dimerization was very low compared with that of DHT (34%). S-101479 increased bone mineral content in ovariectomized rats without promoting endometrial proliferation. Yeast two-hybrid interaction assays revealed that DHT promoted recruitment of numerous cofactors to AR such as TIF2, SRC1, beta-catenin, NCoA3, gelsolin and PROX1 in a dose-dependent manner. SARMs induced recruitment of fewer cofactors than DHT; in particular, S-101479 failed to induce recruitment of canonical p160 coactivators such as SRC1, TIF2 and notably NCoA3 but only stimulated binding of AR to gelsolin and PROX1. The results suggest that a full capability of the AR to dimerize and to effectively and unselectively recruit all canonical cofactors is not a prerequisite for transcriptional activity in osteoblastic cells and resulting anabolic effects in bone tissues. Instead, few relevant cofactors might be sufficient to promote AR activity in these tissues [13273].

New SARM compounds

A novel selective androgen receptor modulator scaffold was discovered as a byproduct obtained during synthesis of our earlier series of imidazolidin-2-ones. The resulting oxazolidin-2-imines are among the most potent SARMs known, with many analogues exhibiting sub-nM in vitro potency in binding and functional assays. Despite the potential for hydrolytic instability at gut pH, compounds of the present class showed good oral bioavailability and were highly active in a standard rodent pharmacological model [09146].

Laboratory techniques

Selective androgen receptor modulators (SARM) are a prominent group of compounds for being misused in sports owing to their advantageous anabolic properties and reduced side effects. To target the preventive doping control analysis in relevant compounds, the challenge is to predict the metabolic fate of a new compound. For aryl-propionamide-derived SARM, an in vitro assay employing microsomal and S9 human liver enzymes was developed to simulate phase-I and phase-II metabolic reactions. In vitro metabolic profiles and the structure-metabolic relationship were compared between four structurally modified substrates. Accurate mass measurements were used to characterize the synthesized metabolites, and also collision-induced dissociation was examined to suggest the methodological approach to monitor the prohibited use of aryl-propionamide-derived drug candidates. Subsequent phase-I and phase-II metabolic reactions were successfully combined in one in vitro assay. The main routes of phase-I modifications involved the hydrolysis of ether linkage, monohydroxylation, and hydrolytic cleavage of the amide bond. Nitro-reduction and deacetylation were reactions observed for substrates possessing the
corresponding functionality. SARM metabolites were analyzed in negative ion electrospray ionization and detected as deprotonated species [M-H](-). The main metabolic modifications were observed to occur in the B-ring side, and collision-induced dissociation resulted in the product ions originating from the A-ring side of the compound. These structure-specific ions may be monitored as target ions in the routine doping control [07101].

Anabolic agents have been among the most frequently detected drugs in amateur and professional sport. A novel class of therapeutics presumably complementing anabolic steroids in the near future includes so-called selective androgen receptor modulators (SARMs) that have been under clinical investigations for several years. Although not yet commercially available, their potential for misuse in sports is high. Four aryl-propionamide-derived SARMs were synthesized in order to establish a fast and robust screening procedure using liquid chromatography/electrospray ionization tandem mass spectrometry. Synthesized compounds were characterized by high-resolution/high-accuracy mass analysis employing a linear ion trap-Orbitrap hybrid mass spectrometer while routine analyses were conducted on a triple-quadrupole mass spectrometer. Characteristic product ions obtained by collision-induced dissociation were found at m/z 289 and 261 as well as m/z 269 and 241 representing the bisubstituted aniline residues of selected model compounds. Assay validation was performed regarding lower limit of detection (1 ng/mL), recovery (85-105 %), intraday precision (7.6-11.6 %) and interday precision (9.9-14.4 %), and precursor ion scan experiments on diagnostic product ions enabled the detection of a structurally related compound at 50 ng/mL [06131].
ESTROGENS AND FEMALE SPORTS

Women are at greater risk than men of sustaining certain kinds of injury and diseases of collagen-rich tissues. To determine whether a high level of oestradiol has an acute influence on collagen synthesis in tendons at rest and in response to exercise, one-legged kicking exercise was performed for 60 min at 67 percent of maximum power by healthy, young oral contraceptive (OC) users when circulating synthetic (ethinyl) oestradiol was high (n=11) and compared to similar women who had never used OCs when circulating endogenous oestrogen was low (n=12). Interstitial fluid was collected 24 h post-exercise through microdialysis catheters placed anterior to the patellar tendon in both legs and subsequently analysed for the amino-terminal propeptide of type I collagen (PINP), a marker of tendon collagen synthesis. To determine the long-term effect of OC usage, patellar tendon cross-sectional area (CSA) was measured by magnetic resonance imaging (MRI). A lower exercise-induced increase in tendon collagen synthesis was observed at higher than at lower levels. Furthermore, serum and the interstitial peritendinous tissue concentrations of insulin-like growth factor I (IGF-I) and IGF-binding proteins showed a reduced bioavailability at higher compared with results lower levels. No difference in patellar tendon CSA was observed between groups. In conclusion, the selective increase in tendon collagen synthesis when circulating endogenous oestrogen was low but not when circulating synthetic (ethinyl) oestradiol was high 24 h post-exercise is consistent with the hypothesis that oestradiol inhibits exercise-induced collagen synthesis in human tendon. The mechanism behind this is either a direct effect of oestradiol, or an indirect effect via a reduction in levels of free IGF-I. However, the data did not indicate any long-term effect on tendon size associated with chronic oral contraceptive use [08203].

The female hormones, estrogen and progesterone, fluctuate predictably across the menstrual cycle in naturally cycling eumenorhhoic women. Other than reproductive function, these hormones influence many other physiological systems, and their action during exercise may have implications for exercise performance. Although a number of studies have found exercise performance - and in particular, endurance performance - to vary between menstrual phases, there is an equal number of such studies reporting no differences. However, a comparison of the increase in the estrogen concentration (E) relative to progesterone concentration (P) as the E/P ratio (pmol/nmol) in the luteal phase in these studies reveals that endurance performance may only be improved in the mid-luteal phase compared with the early follicular phase when the E/P ratio is high in the mid-luteal phase. Furthermore, the late follicular phase, characterized by the pre-ovulatory surge in estrogen and suppressed progesterone concentrations, tends to promote improved performance in a cycling time trial and future studies should include this menstrual phase. Menstrual phase variations in endurance performance may largely be a consequence of changes to exercise metabolism stimulated by the fluctuations in ovarian hormone concentrations. The literature suggests that estrogen may promote endurance performance by altering carbohydrate, fat and protein metabolism, with progesterone often appearing to act antagonistically. Details of the ovarian hormone influences on the metabolism of these macronutrients are no longer only limited to evidence from animal research and indirect calorimetry but have been verified by substrate kinetics determined with stable tracer methodology in eumenorrhhoic women. One review thoroughly examined the metabolic perturbations induced by the ovarian hormones and, by detailed comparison, proposes reasons for many of the inconsistent reports in menstrual phase comparative research. Often the magnitude of increase in the ovarian hormones between menstrual phases and the E/P ratio appear to be important factors determining an effect on metabolism. However, energy demand and nutritional status may be confounding variables, particularly in carbohydrate metabolism. The review
specifically considers how changes in metabolic responses due to the ovarian hormones may influence exercise performance. For example, estrogen promotes glucose availability and uptake into type I muscle fibres providing the fuel of choice during short duration exercise; an action that can be inhibited by progesterone. A high estrogen concentration in the luteal phase augments muscle glycogen storage capacity compared with the low estrogen environment of the early follicular phase. However, following a carbo-loading diet will super-compensate muscle glycogen stores in the early follicular phase to values attained in the luteal phase. Estrogen concentrations of the luteal phase reduce reliance on muscle glycogen during exercise and although not as yet supported by human tracer studies, estrogen increases free fatty acid availability and oxidative capacity in exercise, favouring endurance performance. Evidence of estrogen's stimulation of 5'-AMP-activated protein kinase may explain many of the metabolic actions of estrogen. However, both estrogen and progesterone suppress gluconeogenic output during exercise and this may compromise performance in the latter stages of ultra-long events if energy replacement supplements are inadequate. Moreover, supplementing energy intake during exercise with protein may be more relevant when progesterone concentration is elevated compared with menstrual phases favouring a higher relative estrogen concentration, as progesterone promotes protein catabolism while estrogen suppresses protein catabolism [10098].

It was shown that isoflavones and exercise improve total and abdominal fat mass (FM) to a greater extent than does exercise alone in postmenopausal women, but not other cardiovascular disease (CVD) risk factors. Fat-free mass (FFM) showed a wide variability of responses, with 60 percent of women having increased FFM and 40 percent having decreased FFM. It was thus wondered if women who had decreased FFM could be considered as nonresponders (NRs) to exercise and if this masked a potential synergistic effect of phytoestrogens (PHY) and exercise. The aim of one study was to verify if PHYs enhance the response obtained after aerobic and resistance exercises in CVD risk profile in exercise responders. Among 21 women who participated in a 6-month exercise program and received PHY or placebo (PLA), 14 were exercise responders (PHY, n=8; PLA, n=6) whereas 7 were NRs. Body weight, waist circumference, FM, and FFM were assessed (dual-energy x-ray absorptiometry). Plasma glucose, insulin, sex hormone-binding globulin, and testosterone levels were obtained after a 12-hour overnight fast. Total energy intake was measured with a 3-day dietary record. All measurements were performed before and after the intervention. After exercise training, the PHY and PLA groups, but not the NR group, had significantly increased FFM. On the other hand, body weight, FM, and systolic and diastolic blood pressure decreased in the PHY group only. Furthermore, plasma insulin level and homeostasis model assessment significantly decreased while plasma sex hormone-binding globulin increased after training in the PHY group, whereas energy intake remained unchanged in both groups after the intervention. It was concluded PHYs combined with exercise compared with exercise alone seem to improve body composition and CVD risk profile in exercise-responder women [10350].

The identification of subtle menstrual cycle disturbances requires daily hormone assessments. In contrast, the identification of severe menstrual disturbances, such as amenorrhea and oligomenorrhea, can be established by clinical observation. The primary purpose of one study was to determine the frequency of subtle menstrual disturbances, defined as luteal phase defects (LPD) or anovulation, in exercising women, with menstrual cycles of 26-35 days, who engage in a variety of sports, both recreational and competitive. Secondly, the prevalence of oligomenorrhea and amenorrhea was also determined via measurement of daily urinary ovarian steroids rather than self report alone. Menstrual status was documented by daily measurements of estrone and pregnanediol glucuronide and luteinizing hormone across two to three consecutive cycles and subsequently categorized as ovulatory (Ovu), LPD, anovulatory (Anov), oligomenorrheic (Oligo) and amenorrheic (Amen).
in sedentary (Sed) and exercising (Ex) women. Among the menstrual cycles studied in the Sed group, the prevalence of subtle menstrual disturbances was only 4 percent; 96 percent of the observed menstrual cycles were ovulatory. This finding stands in stark contrast to that observed in the exercising group where only 50 percent of the observed menstrual cycles were ovulatory and as many as 50 percent were abnormal. Of the abnormal cycles in the exercising group, 29 percent were classified as LPD (short, inadequate or both) and 21 percent were classified as Anov. Among the cycles of exercising women with severe menstrual disturbances, 4 percent of the cycles were Oligo and 34 percent were Amen. No cycles of Sed women (0/20) displayed either Oligo or Amen. This study suggests that approximately half of exercising women experience subtle menstrual disturbances, i.e. LPD and anovulation, and that one third of exercising women may be amenorrheic. Estimates of the prevalence of subtle menstrual disturbances in exercising women determined by the presence or absence of short or long cycles does not identify these disturbances. In light of known clinical consequences of menstrual disturbances, these findings underscore the lack of reliability of normal menstrual intervals and self report to infer menstrual status [10099].

The continuous introduction of new products used as growth promoters in animal husbandry, for sports doping and as products for body-building requires residue laboratories to initiate research on developing a strategy for the identification of 'unknown' components. In this study, a strategy is presented for elucidating the identity, the structure and the possible effects of illegal estrogenic compounds in an unidentified water-based solution. To obtain complete information on the composition and activity of the unidentified product, a multidisciplinary approach was needed. A case-study is described with a “solution X” found during a raid. First, in vivo techniques (animal trials with mice, anatomical and histological research) were combined with in vitro techniques (the yeast estrogenic screen (YES)). In a later stage of the investigation, HPLC-fractionation, liquid chromatography-multiple mass spectrometry (LC-MSn) and gas chromatography-multiple mass spectrometry (GC-MSn) were used. Finally, the identity of “solution X” was confirmed in a very low concentration range (10 ng/L estrone and 400 ng/L ethinyloestradiol) [06132].

In contrast to age-matched men, endurance exercise training is not consistently associated with enhanced endothelial function in estrogen-deficient postmenopausal women. It was determined whether endurance exercise training improves endothelial function in postmenopausal women treated with estrogen. In a substudy, it was determined if oxidative stress is mechanistically linked to endothelial function adaptations to endurance exercise training. Brachial artery flow-mediated dilation (FMD) was measured in 36 sedentary, estrogen-deficient postmenopausal women (45-65 years) at study entry (baseline), after 12 weeks of either placebo, oral (1 mg/d) estradiol, or transdermal estradiol (0.05 mg/d) (randomized), and after an additional 12 weeks of continued estradiol or placebo treatment with concurrent endurance exercise training. In subgroups of women, FMD also was measured during the infusion of ascorbic acid at baseline and following estradiol/placebo plus endurance exercise training, and in seven habitually endurance-trained estrogen-deficient controls. FMD increased in the estrogen-treated groups after 12 weeks and remained unchanged in placebo. FMD further increased following 12 weeks of endurance exercise training in estrogen-treated, but not placebo-treated women. In the substudy, baseline FMD was similar between sedentary and endurance-trained controls. Ascorbic acid increased FMD at baseline in sedentary women and endurance-trained controls, and following endurance exercise training in placebo-treated, but not in estrogen-treated women. It was concluded that estrogen status appears to play an important modulatory role in improvements in endothelial function with endurance exercise training in postmenopausal women. The restored endurance exercise training adaptation in estrogen-treated postmenopausal women may be related to mitigation of oxidative stress [13277].
Ethnic variations

Previous studies have suggested that estrogen levels may be higher in African-American women (AAW) compared with Caucasian women (CW), but none have systematically examined estrogen secretion across the menstrual cycle or in relation to other reproductive hormones. The objective of one study was to compare estradiol (E2), progesterone (P), gonadotropins, androstenedione (a’dione), inhibins, and SHBG levels between AAW and CW across the menstrual cycle. Daily blood samples were collected from regularly cycling AAW (n=27) and CW (n=27) for a full menstrual cycle, and serial ultrasounds were performed. AAW and CW were of similar age (27) and body mass index (22.7 ± 0.4 kg/m²). All subjects grew a single dominant follicle and had comparable cycle (25-35 d) and follicular phase (11-24 d) lengths. E2 levels were significantly higher in AAW compared with CW with the most pronounced differences in the late follicular phase, midluteal phase, and late luteal phase. Although LH, FSH, inhibins A and B, P, a’dione, and SHBG were not different between the two groups, the a’dione to E2 ratio was lower in AAW. Estradiol is higher in AAW compared with CW across the menstrual cycle. Higher estradiol in the face of similar androstenedione and FSH levels suggests enhanced aromatase activity in AAW. Such differences may contribute to racial disparities in bone mineral density, breast cancer, and uterine leiomyomas [11455].

Urinary estrogens and androgens during pregnancy

Alterations in the maternal excretion of steroids during pregnancy are not restricted to the production of progesterone and estriol by the fetoplacental unit. Although there is a lack of longitudinal data on urinary androgen concentrations during pregnancy, some studies revealed that modifications in the excretions of androgens might be significant. Recently, several testosterone metabolites excreted as cysteine conjugates have been reported in human urine. It was conducted a longitudinal study on androgens conjugated with cysteine and major androgens and estrogens excreted as glucuronides in three pregnant women by mass spectrometric techniques. The urinary concentrations obtained in samples weekly collected during each of the three trimesters and samples collected before pregnancy were compared. Results showed a significant increase in urinary estrogens and norandrosterone and a moderate decrease in the urinary concentrations for most of the androgens. The most significant exception to this behavior was the rise observed for epitestosterone glucuronide when comparing basal levels with the first trimester. Cysteinyl conjugates of testosterone metabolites showed a different behavior. Whereas 4,6-androstanedione remained almost constant through the three trimesters, and delta6-testosterone decreased as the majority of androgens, the excretion profile of 1,4-androstanedione notably increased, reaching a maximum at the third trimester. Alterations in the steroid profile are used in doping control analysis for the screening of endogenous anabolic androgenic steroid misuse. In the study, the main parameters proposed for doping control have been determined for basal samples and samples collected in the first trimester and they have been compared. In spite of the limited number of cases, significant variations have been found in all pregnancies studied. These alterations have to be taken into consideration if anabolic steroids are included into the Athlete Biological Passport [13284].

Endogenous versus exogenous estrogens
Estrogens were prohibited in the food producing animals by European Union (96/22/EC directive) and added to the Report on Carcinogens in United States since 2002. Due to very low concentration in serum or urine (pg/mL), the method of control its abuse had not been fully developed. The endogenous estrogens were separated from urines of 18 adult men and women. The exogenous estrogens were chemical reference standards and over the counter preparations. Two patients of dysfunctional uterine bleeding (DUB) administered exogenous estradiol and the urines were collected for 72 h. The urinary estrogens were separated by high-performance liquid chromatography (HPLC) and confirmed. The exogenous and exogenous estrogens were analyzed by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) to determine the $^{13}$C/$^{12}$C ratio. Stable carbon isotope analysis could distinguish the endogenous and exogenous urinary estrogen in humans [13285].

Sex-hormone binding globulin

A generic high-throughput bioaffinity liquid chromatography-mass spectrometry (BioMS) approach was developed and applied for the screening and identification of known and unknown recombinant human sex hormone-binding globulin (rhSHBG)-binding designer steroids in dietary supplements. For screening, a semi-automated competitive inhibition binding assay was combined with fast ultrahigh-performance-LC-electrospray ionization-triple-quadrupole-MS (UPLC-QqQ-MS). 17β-Testosterone-D3 was used as the stable isotope label of which the binding to rhSHBG-coated paramagnetic microbeads was inhibited by any other binding (designer) steroid. The assay was performed in a 96-well plate and combined with the fast LC-MS, 96 measurements could be performed within 4 h. The concentration-dependent inhibition of the label by steroids in buffer and dietary supplements was demonstrated. Following an adjusted bioaffinity isolation procedure, suspect extracts were injected into a chip-UPLC(NanoTile)-Q-time-of-flight-MS system for full-scan accurate mass identification. Next to known steroids, 1-testosterone was identified in three of the supplements studied and the designer steroid tetrahydrogestrinone was identified in a spiked supplement. The generic steroid-binding assay can be used for high-throughput screening of androgens, estrogens, and gestagens in dietary supplements to fight doping. When combined with chip-UPLC-MS, it is a powerful tool for early warning of unknown emerging rhSHBG bioactive designer steroids in dietary supplements [13277].

Testosterone

To examine the androgen response to exercise in women under conditions of high (H) and low (L) estrogen (E2) levels 10 exercise trained eumenorrheic women (20 years, 59 kg, 22 % body fat, VO$_{2}$max 51 mL/kg/min) completed a 60 min treadmill run at about 70 percent of VO$_{2}$max during both the mid-follicular (L-E2, 70 % VO$_{2}$max) and mid-luteal (H-E2, 68 % VO$_{2}$max) phases of their menstrual cycle. Blood samples were taken pre-exercise (PRE), immediately post (POST), and 30 min into recovery (30R) from exercise and analyzed for total testosterone using ELISA assays. Statistical analysis of testosterone response indicated no significant interaction existed between high and low estrogen conditions across the blood sampling times. However, a main effect occurred for exercise with the post-testosterone concentration being greater than pre, although pre versus 30R was not different. All testosterone hormonal concentrations immediately post-exercise greatly exceeded the level of hemoconcentration observed during the L-E2 and H-E2 exercise sessions. Thus, prolonged aerobic exercise induces short-term elevations in testosterone in trained eumenorrheic women, which appears unrelated to estrogen levels and menstrual cycle phase. These increases may occur due to either increased androgen production and/or
decreased degradation rates of the hormone, and are not solely the result of plasma fluid shifts from the exercise [13278].

In hypopituitary men, oral delivery of unesterified testosterone in doses that result in a solely hepatic androgen effect enhances protein anabolism during GH treatment. In one study, it was aimed to determine whether liver-targeted androgen supplementation induces protein anabolism in GH-replete normal women. Eight healthy postmenopausal women received 2-week treatment with oral testosterone at a dose of 40 mg/day (crystalline testosterone USP). This dose increases portal concentrations of testosterone, exerting androgenic effects on the liver without a spillover into the systemic circulation. The outcome measures were whole-body leucine turnover, from which leucine rate of appearance (LRa, an index of protein breakdown) and leucine oxidation (Lox, a measure of irreversible protein loss) were estimated, energy expenditure and substrate utilization. It was measured the concentration of liver transaminases as well as of testosterone, SHBG and IGF1. Testosterone treatment significantly reduced LRa by 7.1 ± 2.5 percent and Lox by 14.6 ± 4.5 percent. The concentration of liver transaminases did not change significantly, while that of serum SHBG fell within the normal range by 16.8 ± 4.0 percent and that of IGF1 increased by 18.4 ± 7.7 percent. The concentration of peripheral testosterone increased from 0.4 ± 0.1 to 1.1 ± 0.2 nmol/l, without exceeding the upper normal limit. There was no change in energy expenditure and fat and carbohydrate utilization. It was concluded that hepatic exposure to unesterified testosterone by oral delivery stimulates protein anabolism by reducing protein breakdown and oxidation without inducing systemic androgen excess in women. It was conclude that a small oral dose of unesterified testosterone holds promise as a simple novel treatment of protein catabolism and muscle wasting [13279].

**Prolonged aerobic exercise**

It was examined the androgen response to exercise in women under conditions of high (H) and low (L) estrogen (E2) levels. Ten exercise trained eumenorrheic women (mean ± SD: 20.0 ± 2.2 years, 58.7 ± 8.3 kg, 22.3 ± 4.9 % body fat, VO2max 50.7 ± 9.0 mL/kg/min) completed a 60 min treadmill run at about 70 percent of VO2max during both the mid-follicular (L-E2, 69.7 ± 7.3 % VO2max) and mid-luteal (H-E2, 67.6 ± 7.9 % VO2max) phases of their menstrual cycle. Blood samples were taken pre-exercise (PRE), immediately post (POST), and 30 min into recovery (30R) from exercise and analyzed for total testosterone using ELISA assays. Results were analyzed using repeated measures ANOVA. Testosterone responses were (mean ± SD: L-E2, pre = 1.41 ± 0.21, post = 1.86 ± 0.21, 30R = 1.75 ± 0.32 nmol/L; H-E2, pre = 1.27 ± 0.23, post = 2.43 ± 0.56, 30R = 1.69 ± 0.34 nmol/L). Statistical analysis indicated no significant interaction existed between high and low estrogen conditions across the blood sampling times. However, a main effect occurred for exercise with the post-testosterone concentration being greater than pre, although pre versus 30R was not different. All testosterone hormonal concentrations immediately post-exercise greatly exceeded the level of hemoconcentration observed during the L-E2 and H-E2 exercise sessions. It was concluded that prolonged aerobic exercise induces short-term elevations in testosterone in trained eumenorrheic women, which appears unrelated to estrogen levels and menstrual cycle phase. These increases may occur due to either increased androgen production and/or decreased degradation rates of the hormone, and are not solely the result of plasma fluid shifts from the exercise [13280].

**Urinary steroids in exercise**

Some studies suggest that performing strength training may cause alterations on the hypothalamic pituitary axis, resulting in steroid hormone variations. Intense training has been associated to slow the concentrations of estrogens and progesterone in women. The main
The purpose of this study was to evaluate the effects of strength training on the urinary steroid concentrations across the menstrual cycle phases. Twenty healthy women, regularly menstruating and not using pharmacologic contraceptives, performed a strength training during 8 weeks. Participants worked out 3 sets × 10 repetitions, with 2 min recovery time between sets, at 70-75 % of one maximum strength repetition. Urine samples were taken in three different phases of the menstrual cycle (menstrual, follicular and luteal) and they were collected both before and after training. Testosterone, DHEA, cortisol, cortisone, estradiol and progesterone concentrations were determined by gas chromatography-mass spectrometry. The results showed a significant decline after training in the urinary excretion of estradiol, during the menstrual and follicular phase, and progesterone, during the menstrual and luteal phase. No significant difference was observed for other steroid hormones. These data demonstrated that strength training can play an important role in the estrogen and progesterone metabolism in women, decreasing their levels across the menstrual cycle [13281].

**Salivary testosterone in exercise**

Testosterone has been related to improved acute neuromuscular performance in athletic populations. It is a contention that testosterone may also contribute to improved volitional motivation and, when monitored longitudinally, may provide one proxy marker for readiness to perform. Twelve female netball players provided saliva samples prior to five standardized training sessions in which they completed a maximal-distance medicine ball throw, and then 3 sets of bench press and then back squat using a self-selected load perceived to equal a 3-repetition maximum load. Additional repetitions were encouraged when possible and total voluntary workload was calculated from the product of the load lifted and repetitions performed. Relative salivary testosterone levels as a group were correlated with bench press and squat self-selected workload, as well as maximal medicine ball throw performance. It was concluded that individual salivary testosterone, when viewed relatively over time, demonstrated strong relationships with self-selected workloads during an in-season training period in female netball players. As such, daily variations in testosterone may provide information regarding voluntary training motivation and readiness to perform in elite athletic populations. Psychological and behavioral aspects of testosterone may have the potential to enhance training adaptation by complementing the known anabolic and permissive properties of testosterone [13282].

**Hyperandrosteronism in women**

International sports governing bodies such as the International Association for Athletics Federation and the International Olympic Committee have recently revised their policies for inclusion of athletes competing in women’s international sports competitions. Previously, the focus was on verification of gender or femininity. The mishandling of Caster Semanya’s case brought the complex issues of fairness with regard to athletes with disorders of sexual development or hyperandrogenism into both public and private debates. The new International Association for Athletics Federation and International Olympic Committee policies for inclusion in women’s sporting events rest largely on the serum testosterone level, mandating that it be less than the lower limit of normal for men as the defining criteria. This report provides an overview of past problems and an update of the newly adopted policies for eligibility for competition in women’s events. Endocrinologists will play a key role in the evaluation and treatment of women athletes who have elevated androgen levels, regardless of the underlying cause [12200].
**Effects of menstrual cycle**

Anabolic androgenic steroids (AAS), synthetic testosterone derivatives that are used for ergogenic purposes, alter neurotransmission and behaviors mediated by GABA(A) receptors. Some of these effects may reflect direct and rapid action of these synthetic steroids at the receptor. The ability of other natural allosteric steroid modulators to alter GABA(A) receptor-mediated currents is dependent upon the phosphorylation state of the receptor complex. Here it was shown that phosphorylation of the GABA(A) receptor complex immunoprecipitated by beta2/beta3 subunit-specific antibodies from the medial preoptic area (mPOA) of the mouse varies across the estrous cycle; with levels being significantly lower in estrus. Acute exposure to the AAS, 17alpha-methyltestosterone (17alpha-MeT), had no effect on the amplitude or kinetics of inhibitory postsynaptic currents in the mPOA of estrous mice when phosphorylation was low, but increased the amplitude of these currents from mice in diestru, when it was high. Inclusion of the protein kinase C (PKC) inhibitor, calphostin, in the recording pipette eliminated the ability of 17alpha-MeT to enhance currents from diestrous animals, suggesting that PKC-receptor phosphorylation is critical for the allosteric modulation elicited by AAS during this phase. In addition, a single injection of 17alpha-MeT was found to impair an mPOA-mediated behavior (nest building) in diestru, but not in estrus. PKC is known to target specific serine residues in the beta3 subunit of the GABA(A) receptor. Although phosphorylation of these beta3 serine residues showed a similar profile across the cycle, as did phosphoserine in mPOA lysates immunoprecipitated with beta2/beta3 antibody (lower in estrus than in diestru or proestru), the differences were not significant. These data suggest that the phosphorylation state of the receptor complex regulates both the ability of AAS to modulate receptor function in the mPOA and the expression of a simple mPOA-dependent behavior through a PKC-dependent mechanism that involves the beta3 subunit and other sites within the GABA(A) receptor complex [12161].

One study examined the effects of menstrual cycle phase (MCP) upon sprinting and recovery as well as upon metabolic responses to such exercise. Eight females performed a repeated 30-s sprint on a non-motorised treadmill interspersed with a 2-min rest in three phases of the MCP, follicular (low 17beta-estradiol and progesterone), just prior to ovulation (midcycle trial, highest 17beta-estradiol concentration and low progesterone) and in the luteal phase (high 17beta-estradiol and high progesterone). MCP was verified later by radioimmunoassay of 17beta-estradiol and progesterone. Peak power output (PPO) and mean power output (MPO) were unaltered due to MCP for follicular, midcycle and luteal trials. Similarly, percentage recovery of PPO and MPO (the PPO or MPO during sprint 2 expressed as a percentage of the PPO or MPO during sprint 1) was also unchanged. Blood lactate, blood pH and plasma ammonia after sprinting and estimated plasma volume were also unaltered by MCP. These findings suggest that hormonal fluctuations due to MCP do not interfere with maximal intensity whole body sprinting and the metabolic responses to such exercise [10351].

Numerous studies from our and other laboratories have shown that women have a lower respiratory exchange ratio (RER) during exercise than equally trained men, indicating a greater reliance on fat oxidation. Differences in estrogen concentration between men and women likely play a role in this sex difference. Differing estrogen and progesterone concentrations during the follicular (FP) and luteal (LP) phases of the female menstrual cycle suggest that fuel use may also vary between phases. The purpose of the current study was to determine the effect of menstrual cycle phase and sex upon glucose turnover and muscle glycogen utilization during endurance exercise. Healthy, recreationally active young women (n=13) and men (n=11) underwent a primed constant infusion of [6,6-2H]glucose with muscle
biopsies taken before and after a 90-min cycling bout at 65 percent peak $O_2$ consumption. LP women had lower glucose rate of appearance rate of disappearance and metabolic clearance rate at 90 min of exercise and lower proglycogen macroglycogen and total glycogen utilization during exercise compared with FP women. Men had a higher RER glucose $Ra$, $Rd$, and $MCR$ during exercise compared with FP women, and men had a higher RER at 75 and 90 min of exercise, glucose $Ra$, $Rd$, and $MCR$ and a greater PG utilization compared with LP women. It was concluded that gender, and to a lesser extent menstrual cycle, influence glucose turnover and glycogen utilization during moderate-intensity endurance exercise [06133].

The purpose of one project was to compare the impact of the menstrual cycle on short-term, high intensity (power) performance in active females who either had normal menstrual cycles (NOC) or who were using oral contraceptives (OC). Subjects (7 NOC, 17 OC) completed a Margaria-Kalamen staircase test and a Wingate cycle test on 3 occasions: one for familiarization and the other two trials (random order) during menses (MEN) or luteal (LUT) phase. Phase was documented by urinary luteinizing hormone for the NOC. There were no significant differences between MEN and LUT in the NOC group on the Wingate test ($n=7$) for any of the following: peak power, peak power per kg body weight, anaerobic capacity, anaerobic capacity per kg body weight, power decline, power decline per kg body weight. Also there were no significant differences in power for the Margaria-Kalamen test ($n=6$). There were no significant differences between MEN and LUT in the OC group for any of the following variables calculated from the subjects’ performance on the Wingate test ($n=17$): peak power, peak power per kg body weight, anaerobic capacity, anaerobic capacity per kg body weight, power decline, power decline per kg body weight. Also there were no significant differences in power for the Margaria-Kalamen test ($n=11$). It was concluded that for a moderately active group of women, anaerobic power performance was not influenced by menstrual cycle phase in either NOC or OC users [06134].

**Effects of exercise on the female reproductive system and sex hormones**

The excess in physical activity could be closely linked to considerable negative consequences on the whole body. These dysfunctions called as "female athlete triad" by the American College of Sports Medicine (ACSM) include amenorrhea, osteoporosis and disorder eating. The female athlete triad poses serious health risks, both on the short and on the long term, to the overall well-being of affected individuals. Sustained low energy availability can impair health, causing many medical complications within skeletal, endocrine, cardiovascular, reproductive and central nervous system. On the contrary, several studies have shown, that physical activity improves cardiovascular risk factors, hormonal profile and reproductive function. These improvements include a decrease in abdominal fat, blood glucose, blood lipids and insulin resistance, as well as improvements in menstrual cyclicity, ovulation and fertility, decreases in testosterone levels and Free Androgen Index (FAI) and increases in sex hormone binding globulin (SHBG). Other studies reported that physical activity improved self-esteem, depression and anxiety. Thus, the aim of one review was to elucidate the effect of physical exercise on female reproductive system and viceversa the impact of hormonal status on physical activity and metabolism. In addition this review supports the idea that physical exercise is a helpful tool for the management of obesity, prevention of cardiovascular, metabolic diseases and female reproductive organs related diseases (e.g. breast cancer). When the excess in physical activity leads up to the female athlete triad, it is imperative to treat each component of the triad by employing both pharmacological and non pharmacological treatments [13286].
**Effects of strength training**

Some studies suggest that performing strength training may cause alterations on the hypothalamic pituitary axis, resulting in steroid hormone variations. Intense training has been associated to slow the concentrations of estrogens and progesterone in women. The main purpose of one study was to evaluate the effects of strength training on the urinary steroid concentrations across the menstrual cycle phases. Twenty healthy women, regularly menstruating and not using pharmacologic contraceptives, performed a strength training during 8 weeks. Participants worked out 3 sets × 10 repetitions, with 2 min recovery time between sets, at 70-75 percent of one maximum strength repetition. Urine samples were taken in three different phases of the menstrual cycle (menstrual, follicular and luteal) and they were collected both before and after training. Testosterone, DHEA, cortisol, cortisone, estradiol and progesterone concentrations were determined by gas chromatography-mass spectrometry. The results showed a significant decline after training in the urinary excretion of estradiol, during the menstrual and follicular phase, and progesterone, during the menstrual and luteal phase. No significant difference was observed for other steroid hormones. These data demonstrated that strength training can play an important role in the estrogen and progesterone metabolism in women, decreasing their levels across the menstrual cycle [12198].

**Comparison of baseline free testosterone between elite and non-elite female athletes**

To compare the baseline free testosterone (T) and cortisol (C) concentrations of elite and non-elite female athletes 18 females from different sports (track and field, netball, cycling, swimming, bob skeleton) were monitored over a 12-week period. Baseline measures of salivary free T and C concentrations were taken weekly prior to any training. The elites (n=9) and non-elites (n=9) were classified as international and national level competitors, respectively, with both groups matched by sport. The pooled free T concentrations of the elites (87 pg/ml) were significantly higher than the non-elites (41 pg/ml) and consistently so across all weekly time points. Pooled free C concentrations were also greater in the elite group (2.90 ng/ml) than the non-elites (2.32 ng/ml) (P < 0.01). It was concluded that the pooled baseline T and C measures were higher in elite female athletes than non-elites. Higher free T and C concentrations could indicate a greater capacity for physical performance at higher work rates, which is commensurate with the demands of elite sport. Speculatively, the T differences observed could influence female behavior and thereby help to regulate sporting potential [12199].

**Effect of stretching**

The aim of one study was to investigate whether variation in estrogen levels during the menstrual cycle influences susceptibility to exercise-induced muscle damage after stretch-shortening cycle exercise. Physically active women (n=18; age 20 years) participated in the research. The subjects performed one session of 100 maximal drop jumps on day 1 or 2 of the follicular phase and another identical session on day 1 or 2 of the ovulatory phase; the order of the sessions was randomized. Quadriceps femoris muscle peak torque evoked by electrical stimulation and maximal voluntary contraction, muscle pain, and CK activity were measured before and at various times up to 72 h after exercise. It was found that the high estrogen level during the ovulatory phase might be related to an earlier return to baseline muscle strength after strenuous stretch-shortening cycle exercise in that phase compared with the follicular phase. The estrogen effect appears to be highly specific to the damaged
site because the differences in most EIMD markers (CK, soreness, and low-frequency fatigue) between the two menstrual cycle phases were small [13287].

**Effect of estrogens on muscle regeneration**

Skeletal muscle regeneration efficiency declines with age for both men and women. This decline impacts on functional capabilities in the elderly and limits their ability to engage in regular physical activity and to maintain independence. Aging is associated with a decline in sex hormone production. Therefore, elucidating the effects of sex hormone substitution on skeletal muscle homeostasis and regeneration after injury or disuse is highly relevant for the aging population, where sarcopenia affects more than 30 percent of individuals over 60 years of age. While the anabolic effects of androgens are well known, the effects of estrogens on skeletal muscle anabolism have only been uncovered in recent times. Hence, the purpose of this review is to provide a mechanistic insight into the regulation of skeletal muscle regenerative processes by both androgens and estrogens. Animal studies using estrogen receptor (ER) antagonists and receptor subtype selective agonists have revealed that estrogens act through both genomic and non-genomic pathways to reduce leukocyte invasion and increase satellite cell numbers in regenerating skeletal muscle tissue. Although animal studies have been more conclusive than human studies in establishing a role for sex hormones in the attenuation of muscle damage, data from a number of recent well controlled human studies is presented to support the notion that hormonal therapies and exercise induce added positive effects on functional measures and lean tissue mass. Based on the fact that aging human skeletal muscle retains the ability to adapt to exercise with enhanced satellite cell activation, combining sex hormone therapies with exercise may induce additive effects on satellite cell accretion. There is evidence to suggest that there is a 'window of opportunity' after the onset of a hypogonadal state such as menopause, to initiate a hormonal therapy in order to achieve maximal benefits for skeletal muscle health. Novel receptor subtype selective ligands and selective estrogen and androgen receptor modulators (SERMs, SARMs) promise to reduce health risks associated with classical hormonal therapies, whilst maintaining the positive effects on muscle repair. Dietary supplements containing compounds with structural similarity to estrogens (phytoestrogens) are increasingly used as alternatives to classical hormone-replacement therapies (HRT), but the effects on skeletal muscle are currently largely unknown. Research has started to investigate the combined effects of exercise and alternative HRTs, such as soy isoflavones, on skeletal muscle regenerative processes to provide safer and more efficient therapies to promote muscle regeneration and maintenance of muscle mass and strength in the aging population [13282].

**Influence of IGF-1**

The aim of one study was to determine the relationships of bone mineral density (BMD) and content (BMC) with insulin-like growth factor-1 (IGF-1), IGF-binding protein-3 (IGFBP-3) and estradiol in pubertal female athletes. The participants were 170 healthy adolescent girls (13-15 years) who participated in competitive extramural athletic programs, i.e., sports games (n=49), track sprinting (n=24), rhythmic gymnastics (n=23), swimming (n=24) and cross-country skiing (n=17). The control group (n=33) consisted of girls who took part only in compulsory physical education classes at school. The whole-body BMD and femoral neck and lumbar spine BMD and BMC were measured using DXA, and the volumetric BMD was calculated. Venous blood samples to determine the concentration of IGF-1, IGFBP-3 and estradiol were drawn after an overnight fasting. After adjusting for age, body height and body
mass, the relationships among BMD variables, IGF-1 and the IGF-1/IGFBP-3 molar ratio remained significant only in the rhythmic gymnast group. BMDs at the femoral neck and lumbar spine were also significantly related to estradiol levels only in the rhythmic gymnast group. No relationships were found among the measured BMD, IGF axis and estradiol in other athletic groups. Only BMC at the femoral neck remained associated with the IGF-1/IGFBP-3 molar ratio in the rhythmic gymnast group after adjusting for age, body height and body mass. Stepwise multiple regression analysis indicated that IGF-1 and estradiol together explained 43 percent of total variance in the femoral neck BMD and IGF-1 alone 35 percent of the total variance in the femoral neck BMC only in the rhythmic gymnast group. It was concluded that femoral neck and lumbar spine BMD correlated with IGF-1, IGF-1/IGFBP-3 molar ratio and estradiol in rhythmic gymnasts. No relationships were found between bone parameters and the hormones used in other athletic groups [09139].

Influence of age on muscle mass in women

Menopause is associated with a natural decline in estrogen, that increases visceral fat mass, decreases bone mass density, muscle mass, and strength. One review examined the role of menopause transition and associated decrease in hormonal status with regards to those changes. Studying changes in muscle mass associated with menopause is important, because of the high number of postmenopausal women in developed countries and the related risk of physical incapacity. Among modifiable factors, low physical activity and protein intakes are the best contributors to sarcopenia and the loss of strength in postmenopausal women. On the other hand, some biological factors, namely oxidative stress, inflammation, estrogen and other hormone deficiency are predictors of these phenomena. Interestingly, some methods have the potential to attenuate the loss of muscle mass and strength such as exercise, and supplement intake [09140].

Gestrinone

Gestrinone is a synthetic steroid hormone with anti-estrogenic and anti-progesterone properties. It is used to treat endometriosis, shrink uterine fibroids and reduce menorrhagia; besides, it has been investigated for use as contraceptive. Also, due to its anabolic effects, it has been included in the banned list of performance enhanced drugs in sport. Polyclonal antibodies raised against bovine serum albumin coupled to gestrinone 3-carboxymethyloxime (3OCMO-G) were used to develop two highly sensitive and specific enzyme-linked immunosorbent assays for gestrinone. One of them, based on direct format, shows a detection limit (LD) of 0.09 +/- 0.03 ng/L. The second assay, hapten-protein coating format, can detect until (LD) 0.14 +/- 0.05 ng/L. Both immunoassays were also highly specific, showing negligible or no cross-reactivity to other anabolic steroids. The developed ELISAs detected lower amounts of gestrinone than those determined by the reference chromatographic HPLC/MS/MS methods. The direct format was applied to quantify this steroid in spiked human urine without sample pre-treatment, with recovery values between 76 and 122 percent [10464].

Estrogen blockers

Estrogen blockers include antiestrogens such as steroidal and nonsteroidal drugs that block estrogen receptor action and aromatase inhibitors that block the enzymatic synthesis of estradiol. The original class of estrogen blockers was antiestrogens. These are drugs that
bind to and block estrogen receptor-alpha and/or -beta. The original antiestrogens were the nonsteroidal drugs clomiphene (Clomid) and tamoxifen (Nolvadex). Subsequently newer estrogen receptor blockers have been developed as a class of partial or mixed estrogen agonists, often described by the marketing term specific estrogen receptor modulator (SERM). These agents display a mixture of estrogen agonist and antagonist properties that differ between tissues and between drugs [06127].

Effects in men

There are no valid clinical indications for estrogen blockers in men. Accepted off-label use for estrogen blockers would be limited to men with breast cancer, an exceptionally rare tumor. Some limited experimental uses for estrogen blockade in men have included delayed puberty, short stature, gynecomastia, spinal growth, and idiopathic male infertility. Given the lack of convincing evidence established for any of these experimental indications, it is unlikely that TUE for estrogen blockers in men would be justified, apart from exceptional circumstances. Because there are no proven or likely indications for estrogen blockers in men, there are few clinical studies on the effects of modern estrogen blockers in men. There is, however, abundant and consistent evidence that estrogen blockers increase blood testosterone concentrations in men. However, it is well established that in normal men antiestrogens such as clomiphene, tamoxifen, and raloxifene cause a reflex rise in pituitary gonadotrophin secretion and consequently in blood testosterone concentrations. This is attributable to their inhibition of testosterone-negative feedback on the hypothalamus, a process that involves local aromatization of testosterone within the brain. A similar increase in blood testosterone concentrations ranging from 5 to 20 nmol/L is reported with aromatase inhibitors such as testolactone, exemestane, and anastrozole. By virtue of their common mechanism of action in inhibiting that part of testosterone’s negative hypothalamic feedback due to aromatization, it is highly likely that all estrogen blockers would have similar class-wide effects, proportional to their estrogen-blocking effectiveness [06127].

It is notable that blood testosterone concentrations are markedly increased in mice with complete inactivation of aromatase (63) or estrogen receptors-α (64) but not -β (65), with consequences for androgen receptor-mediated effects on bone (65), prostate (63), and smooth muscle (66, 67), although skeletal muscle effects have not yet been reported. This predicts that more effective estrogen blockade in men would produce significant and sustained elevations of blood testosterone concentrations and likely myotrophic and ergogenic effects in men treated with such drugs. Hence, these observations make a strong case to ban estrogen blockers in men due to their class-wide ability to provoke reflex increase in endogenous pituitary LH and endogenous testosterone secretion [06127].

Experimental

Several combinations of effective treatments have been used in the search for higher response rates or more rapid responses than monotherapy to diminish treatment-resistant depression. One strategy is to combine olanzapine plus antidepressant drugs. In preclinical studies in male rats, olanzapine combined with fluoxetine produce antidepressant-like actions and increase the allopregnanolone levels in the brain. 17-beta estradiol also produces antidepressant-like actions by increasing allopregnanolone levels. However, the effects of combining olanzapine with 17-beta estradiol in the forced swimming test have not been tested before. Thus, systemic injections of vehicle plus olanzapine, or fluoxetine (20.0 mg/kg; 25.0 mg/kg) or 17-beta estradiol (10.0 microg/rat; 20.0 microg/rat) reduced immobility by increasing active behaviors, which were cancelled by finasteride (finasteride was used to
block the endogenous production of allospregnolone by the brain) in ovariectomized rats forced to swim. Subthreshold doses of olanzapine (2.5 mg/kg) combined with subthreshold doses of 17-beta estradiol (5.0 microg/rat) produced antidepressant-like actions, as did the combination subthreshold dose of olanzapine (2.5 mg/kg) plus the subthreshold dose of fluoxetine (15.0 mg/kg). Finasteride cancelled the antidepressant-like actions of the several combinations used. It is concluded that olanzapine alone or combined with fluoxetine or estradiol reduced immobility by increasing swimming. In conclusion, olanzapine produces antidepressant-like actions alone or in combination with estradiol. These antidepressant-like actions of this combination were cancelled by finasteride [09141].

**Oral contraceptives**

Oral contraceptive (OC) treatment has an inhibiting effect on protein synthesis in tendon and muscle connective tissue. It was aimed to investigate whether OC influence myofibrillar protein turnover in young women. OC-users (24 ± 2 years; Lindynette® n=7, Cilest® n=4) and non-OC-users (controls, 24 ± 4 years, n=12) performed one-legged kicking exercise. The next day, the myofibrillar protein fractional synthesis rate (FSR) was measured using stable isotopic tracers (13C-proline) while the subjects were fed standardized nutrient drinks. Simultaneously, a marker for myofibrillar protein breakdown, 3-methyl-histidine (3-MH), was measured in the interstitial fluid of the vastus lateralis. Measurements were performed in both legs. In general, myofibrillar protein FSR was significantly lower in OC-users (two-way analysis of variance), although the difference seemed to depend on the OC type. Interstitial 3-MH in the skeletal muscle was not different between groups and did not vary by OC type. Exercise did not change myofibrillar protein FSR or 3-MH concentrations. Serum androstenedione and bioavailability of testosterone were lower in OC-users. In conclusion, the results indicate that the use of OC has an inhibiting effect on myofibrillar protein synthesis and the magnitude of the effect may depend on the type of OC. In contrast, there was no effect of OC on myofibrillar protein breakdown in the fed state [09138].

Some reports suggest variation in physiological responses and athletic performance, for female athletes at specific phases of the menstrual cycle. However, inconsistent findings are common due to the inappropriate verification of menstrual cycle phase, small subject numbers, high intra- and interindividual variability in estrogen and progesterone concentration, and the pulsatile secretion of these hormones. Therefore, the oral contraceptive (OC) cycle may provide a more stable environment in which to evaluate the acute effect of reproductive hormones on physiological variables and exercise performance. To date, most of the OC research has compared differences between OC use and nonuse, and few researchers have examined within-cycle effects of the OC. It is also apparent that OC use is becoming far more prevalent in athletes; hence the effect of the different exogenous and endogenous hormonal profiles on athletic performance should be investigated. Research to date identifies potential for variation in aerobic performance, anaerobic capacity, anaerobic power and reactive strength throughout an OC cycle [09142].
In general, myofibrillar protein FSR was significantly lower in OC-users although the difference seemed to depend on the OC type. Interstitial 3-MH in the skeletal muscle was not different between groups and did not vary by OC type. Exercise did not change myofibrillar protein FSR or 3-MH concentrations. Serum androstenedione and bioavailability of testosterone were lower in OC-users. In conclusion, the results indicate that the use of OC has an inhibiting effect on myofibrillar protein synthesis and the magnitude of the effect may depend on the type of OC. In contrast, there was no effect of OC on myofibrillar protein breakdown in the fed state [11112].

The purpose of one study was to examine whether common team sport performance variables (anaerobic power, reactive strength and repeat sprint ability) are affected by acute hormonal fluctuation within a monophasic oral contraceptive (OC) cycle. Ten female team sport athletes completed performance tests at three time points of a single OC cycle, during the consumption phase (CONS), early (WITH1) and late in the withdrawal phase (WITH2). Tests included drop jumps (30 cm and 45 cm heights), a counter movement jump, a 10s cycle sprint test and a 5x 6s repeated sprint cycle test. Resting endogenous serum estradiol and progesterone concentrations were also measured. No significant differences were observed between phases for the counter movement jump and cycle tests (total work and peak power). Reactive strength measured from the 30cm drop height was significantly lower during WITH2 compared to both CONS and WITH1. Reactive strength measured from the 45cm drop height was significantly higher in CONS compared to both WITH1 and WITH2. Serum estradiol levels were greater during WITH2 compared to both WITH1 and CONS but there was no difference in serum progesterone levels. The results demonstrate that for female team sport athletes, only reactive strength varied significantly throughout an OC cycle, possibly due to the action of hormones on neuromuscular timing and the stretch-shortening cycle [07102].

The purpose was to examine effects of oral contraceptive (OC) use on plasma growth hormone (GH) responses to heavy resistance exercise. Sixty untrained women were placed into one of two groups: currently using OC (Ortho Tri-Cyclen) (n=25) or not currently using OC (NOC) (n=35). Participants performed an acute heavy resistance exercise test (AHRET; six sets of 10 repetition squats; 2min rest between sets) during days 2-4 of the follicular phase (NOC group) or of inactive oral contraceptive intake (OC group). Plasma was obtained before and immediately after AHRET and subsequently fractionated based on apparent molecular weight (>60kD, 30-60kD, and <30kD). GH was determined in unfractionated plasma and each plasma fraction using 4 methods. GH increased significantly in all fractions post-AHRET. OC use augmented immunological GH response to AHRET in unfractionated plasma and >60kD molecular weight subtraction. However, OC use only increased biological activity of GH in one of two bioassays. These data demonstrated that GH concentrations at rest and following exercise are assay-dependent [07103].

Thirteen female cyclists/triathletes using a monophasic oral contraceptive (OC) performed an endurance test (1-h cycle) at three time points of an OC cycle. Testing times were during the OC consumption phase (CONS), early in the OC withdrawal phase (WITH1) and late in the OC withdrawal phase (WITH2). Resting endogenous serum oestradiol and progesterone concentrations were measured. Power output, heart rate (HR), ventilation (Ve), oxygen consumption (VO2), respiratory exchange ratio (RER), rating of perceived exertion (RPE), blood lactate and blood glucose were measured throughout the 1-h test. Serum oestradiol levels were significantly greater during WITH2 compared to the CONS. No significant differences were present between the testing times for mean power output, HR, VO2, RER, RPE, and blood glucose concentration. Greater mean ventilation and oxygen consumption values were measured during CONS compared to WITH1 and WITH2 respectively and blood lactate values compared to WITH1 only. Despite variation in some physiological variables,
there was no difference in endurance performance throughout an OC cycle in endurance trained female athletes [07104].

It is now estimated that the prevalence of oral contraceptive use in athletic women matches that of women in the general population. The oral contraceptive pill (OCP) reduces cycle-length variability and provides a consistent 28-day cycle by controlling concentrations of endogenous sex hormones. The OCP is administered in three different forms that differ widely in chemical constitution and concomitant effects on the human body. As fluctuation in sex steroids are believed to be a possible causal factor in performance and exercise capacity, it is imperative to understand the effect of administering the various types of OCP on women. However, the research into oral contraceptives and exercise performance is not consistent. The type of OCP administered (monophasic, biphasic or triphasic), as well as the type and dose of estrogen and progestogen within, will have varying effects on exercise. To date, research in the area of oral contraceptives and exercise capacity is sparse and much has been plagued by poor research design, methodology and small sample size. It is clear from the research to date that more randomised clinical trials are urgently required to assess the array of OCP formulations currently available to women and their concomitant effect on health and exercise capacity. Therefore, the purpose of one article was to critically appraise the literature to date and to provide a current review of the physiological scientific knowledge base in relation to the OCP and exercise performance. In addition, methodological control, design and conduct will be considered with future areas of research highlighted [07105].

**Effect of bone mass**

To determine the effect of oral contraceptives (OC) on bone mass and stress fracture incidence in young female distance runners. One hundred fifty competitive female runners ages 18-26 yr were randomly assigned to OC (30 microg of ethinyl estradiol and 0.3 mg of norgestrel) or control (no intervention) for 2 years. Bone mineral density (BMD) and content (BMC) were measured yearly by dual x-ray absorptiometry. Stress fractures were confirmed by x-ray, magnetic resonance imaging, or bone scan. Randomization to OC was unrelated to changes in BMD or BMC in oligo/amenorrheic (n=50) or eumenorrheic runners (n=100). However, treatment-received analyses (which considered actual OC use) showed that oligo/amenorrheic runners who used OC gained about 1 percent per year in spine BMD and whole-body BMC, amounts similar to those for runners who regained periods spontaneously and significantly greater than those for runners who remained oligo/amenorrheic. Dietary calcium intake and weight gain independently predicted bone mass gains in oligo/amenorrheic runners. Randomization to OC was not significantly related to stress fracture incidence, but the direction of the effect was protective in both menstrual groups (hazard ratio 0.57) and the effect became stronger in treatment-received analyses. The trial's statistical power was reduced by higher-than-anticipated noncompliance. OC may reduce the risk for stress fractures in female runners, but our data are inconclusive. Oligo/amenorrheic athletes with low bone mass should be advised to increase dietary calcium and take steps to resume normal menses, including weight gain; they may benefit from OC, but the evidence is inconclusive [07106].

**Influence of oral conceptives on tendons and ligament**

Sex differences exist with regards to ligament and tendon injuries. Lower collagen synthesis has been observed in exercising women vs. men, and in users of oral contraceptives (OC) vs. nonusers, but it is unknown if OC will influence tendon biomechanics of women undergoing regular training. Thirty female athletes (handball players, 18-30 years) were recruited: 15 long-term users of OC (7.0 ± 0.6 years) and 15 nonusers (>5 years).
Synchronized values of patellar tendon elongation (obtained by ultrasonography) and tendon force were sampled during ramped isometric knee extensor maximum voluntary contraction to estimate mechanical tendon properties. Furthermore, tendon cross-sectional area and length were measured from MRI images, and tendon biopsies were obtained for analysis of tendon fibril characteristics and collagen cross-linking. Overall, no difference in tendon biomechanical properties, tendon fibril characteristics, or collagen cross-linking was observed between the OC users and nonusers, or between the different phases of the menstrual cycle. In athletes, tendon cross-sectional area in the preferred jumping leg tended to be larger than that in the contralateral leg, and a greater absolute and normalized tendon stiffness, as well as a lower strain, were observed in the jumping leg compared with the contralateral leg. The results indicate that long-term OC use or menstrual phases does not influence structure or mechanical properties of the patellar tendon in female team handball athletes [13283].

Influence of SERMs on steroid metabolism

In a comparable line of investigation, the alteration of steroid profile data by selective estrogen receptor modulators (SERMs, category S4.2) was studied. Although relatively straightforward in detection, the effect of tamoxifen, toremifene, and clomiphene on T, 5alpha-androstane-3α,17β-diol (ADIOL), 5beta-androstane-3alpha,17beta-diol (BDIOL), EpiT, 4-androstenedione, A, and etiocholanolone (E), LH and follicle-stimulating hormone (FSH) were studied. Significant effects were observed for T, EpiT, and 4-androstenedione in males; all other parameters were found unaffected [13012].

Folinic acid supplementation

Although there are numerous benefits to women from athletic participation, a complex combination of endocrine and metabolic factors exaggerates risk for a serious health concern: the female athlete triad. The purpose of one article was to provide updates on new issues related to the triad, specifically the relationship between athletic-associated amenorrhea and endothelial dysfunction-a potential fourth component to the triad that is a concern for future cardiovascular risk, public health issues, and athletic performance. Folic acid should be considered a potential safe and inexpensive therapeutic treatment to restore endothelial-dependent vasodilation [11336].

Laboratory techniques

Estrogens were prohibited in the food producing animals by European Union (96/22/EC directive) and added to the Report on Carcinogens in United States since 2002. Due to very low concentration in serum or urine (pg/mL), the method of control its abuse had not been fully developed. The endogenous estrogens were separated from urines of 18 adult men and women. The exogenous estrogens were chemical reference standards and over the counter preparations. Two patients of dysfunctional uterine bleeding (DUB) administered exogenous estradiol and the urines were collected for 72h. The urinary estrogens were separated by high-performance liquid chromatography (HPLC) and confirmed. The exogenous and exogenous estrogens were analyzed by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) to determine the $^{13}$C/$^{12}$C ratio (delta$^{13}$C‰). The delta$^{13}$C‰ values of reference standard of E1, E2, and E3 have been defined. Two DUB patients’ urinary estradiol delta$^{13}$C‰ values was depleted to -28 after the administration. The
progesterone, 17α-hydroxyprogesterone, pregnanediol, as well as desogestrel and ethinylestradiol from contraceptives were also determined. Stable carbon isotope analysis can distinguish the endogenous and exogenous urinary estrogen in human [12197].

Selective estrogen receptor modulator (SERM)

It was discussed the capabilities of liquid chromatography coupled to mass spectrometry with a time-of-flight system with accurate mass measurement for the detection and characterisation of drug metabolites in biological samples for anti-doping purpose. Urinary excretion samples of three selective oestrogen receptor modulators (SERMs) with a common triphenylethylene structure: clomiphene, toremifene, and tamoxifen, obtained after oral administration of a single dose of each drug, were analysed using a time-of-flight system, after automatic tuning and calibration of the equipment, in positive full scan mode using an electrospray ionisation source. Following this approach we detected most of all significant metabolites reported by others and postulated new metabolites, especially for toremifene, have been characterised: N-demethyl-3-hydroxy-4-methoxy-toremifene and 3-hydroxy-4-methoxy-toremifene; in additiona to this, in the urinary excretion samples of toremifene some metabolites, without the characteristic chlorine isotope pattern, discarded in previous studies, that are also metabolites of tamoxifen, were identified. The lack of certified reference materials does not allow an accurate determination of the limit of detection (LODs) of all metabolites; however an estimation taking into account the response factor of similar compounds allows to estimate that all metabolites are clearly detectable in a range of concentration comprised between 10 ng/mL and 30 ng/mL [08207].

One study was performed to investigate the influence of the intake of selective oestrogen receptor modulators on the urinary endogenous steroids profile. For this purpose the circadian variability of luteinizing hormone, follicle-stimulating hormone, testosterone, 5alpha-androstan-3alpha,17beta-diol, 5beta-androstan-3alpha,17beta-diol, epitestosterone, 4-androstenedione, androsterone and etiocholanolone were measured on eight subjects (four males and four females) by gas chromatography-mass spectrometry and chemiluminescent immunometric assay techniques before and after oral administration of multiple doses of either tamoxifen (80 mg for 2 days) or toremifene (120 mg for 2 days) or clomiphene (100 mg for 2 days). The individual baseline variability of the steroids studied was set up by collecting the urine samples every 3 h, for 3 days prior to the treatment; whereas the evaluation of the effects of the oral administration of multiple doses of selective oestrogen receptor modulators on the steroid urinary profile was assessed by collecting urine samples every three hours for at least five days from the first administration. The results of the measurements showed that, only in male subjects, the relative urinary concentrations of testosterone, epitestosterone and 4-androstenedione were significantly altered generally after the second day of drug administration. While no significant effects were recorded in both sexes on the luteinizing hormone, follicle-stimulating hormone, androsterone, etiocholanolone, 5alpha-androstan-3alpha,17beta-diol and 5beta-androstan-3alpha,17beta-diol urinary levels and on testosterone/epitestosterone, 5alpha-androstan-3alpha,17beta-diol/5beta-androstan-3alpha,17beta-diol and androsterone/etiocholanolone ratios [11113].

The alteration of steroid profile data by selective estrogen receptor modulators (SERMs) was studied. Although relatively straightforward in detection, the effect of tamoxifen, toremifene, and clomiphene on testosterone, 5alpha-androstan-3alpha,17beta-diol, 5beta-androstan-3alpha,17beta-diol, epitestosterone, 4-androstenedione, and etiocholanolone, LH and follicle-stimulating hormone (FSH) were studied. Significant effects were observed for testosterone, epitestosterone, and 4-androstenedione in males; all other parameters were found
unaffected [12017].

**Toremiphene**

Toremifene is a selective estrogen receptor modulator included in the list of prohibited substances in sport by the World Anti-doping Agency. The aim of one study was to investigate toremifene metabolism in humans in order to elucidate the structures of the most abundant urinary metabolites and to define the best marker to detect toremifene administration through the analysis of urine samples. Toremifene (Fareston®) was administered to healthy volunteers and the urine samples were subjected to different preparation methods to detect free metabolites as well as metabolites conjugated with glucuronic acid or sulphate. Urinary extracts were analyzed by LC-MS/MS with triple quadrupole analyzer using selected reaction monitoring mode. Transitions for potential metabolites were selected by using the theoretical [M+H]^+ as precursor ion and m/z 72 or m/z 58 as product ions for N,N-dimethyl and N-desmethyl metabolites, respectively. Toremifene and 20 metabolites were detected in excretion study samples, excreted free or conjugated with glucuronic acid or sulphate. Structures for most abundant phase I metabolites were proposed using accurate mass measurements performed by QTOF MS, based on fragmentation pattern observed for those metabolites available as reference standards. Several metabolic pathways including mono- and di-hydroxylation, N-desmethylation, hydroxymethylation, oxidation, dehalogenation and combinations were proposed. All metabolites were detected up to one month after toremifene administration; the most abundant metabolites were detected in the free fraction and they were metabolites resulting from dehalogenation. Several of the metabolites elucidated in this work have not been reported until now in the scientific literature [11114].

In the present study, toremifene urinary excretion studies were evaluated in order to examine main metabolic reactions and to select target metabolites in doping control analysis. Urine samples from three female subjects were collected every 3 h for at least 15 days after the oral administration of a single dose of Fareston® (60 mg). The elemental compositions of the compounds detected were determined by liquid chromatography-mass spectrometry using a time-of-flight system with accurate mass measurement. More detailed structure elucidation was obtained by monitoring the presence or absence of structure-specific ions, using product ion scan and neutral loss acquisition modes, whereas the metabolites urinary profiles were evaluated in selected reaction monitoring acquisition mode. The results showed that the main routes of phase-I modifications involved carboxylation of the chlorinated side chain, N-demethylation and hydroxylation in different positions. Fifteen metabolites were found in all subjects studied, most of them were detected for more than 10 days in the free, glucuronide and sulphate fractions, with a maximum of excretion generally after 9-22 and 34-47 h from drug administration. These metabolites can be divided in two groups: metabolites with the characteristic chlorine isotope pattern and metabolites without the characteristic chlorine isotope pattern. The most abundant and long-term compounds were the carboxylated metabolites followed by the hydroxylated metabolites. Their product ions originating after collision-induced dissociation were observed to occur prevalently in the dimethylaminoethoxy and in the chlorinated side chains. These structure-specific ions were used to design screening and confirmation procedures to positively identify toremifene administration in doping control analysis [11220].

**Raloxiphene**

A selective, sensitive, accurate and precise liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for determination of raloxifene and its three glucuronides:
raloxifene-6-beta-glucuronide (M1), raloxifene-4’-beta-glucuronide (M2), raloxifene-6,4’-diglucuronide (M3) in urine samples is presented in this paper. The developed analytical method is the first fully validated method capable of simultaneous determination of raloxifene and its glucuronides in real urine samples. Moreover, for the first time a method for determination of raloxifene diglucuronide in relevant biological samples was introduced. Metabolites were obtained by a bioconversion process of raloxifene to its glucuronides using the microorganism Streptomyces sp. and were used as standards for validation. Urine samples were introduced to a simple solid phase extraction prior to the analysis by LC-MS/MS. The method was linear in a wide range with high determination coefficient. The limits of quantification achieved were 1.01, 1.95, 2.83 and 4.69nM for raloxifene, M1, M2 and M3, respectively. The recoveries were higher than 93 percent, the accuracy was within 100 ± 9 percent and the precision was better than 12 percent for all compounds. The developed method was successfully applied to the real urine samples and showed to be appropriate for use in further research of still not completely discovered raloxifene pharmacokinetics. Furthermore, the presented method could also serve for a potential application in anti-doping analysis [11115].

Raloxifene is one of the selective estrogen receptor modulators and is often used to prevent and treat osteoporosis in postmenopausal women. Because of the indirect impact on serum testosterone levels and the potential ability for performance enhancement, it is banned by the World Anti-Doping Agency (WADA). This study established a fast, sensitive and selective liquid chromatography-tandem mass spectrometry method to quantify total raloxifene (unchanged and glucuronidated) in human urine for doping analysis. Urines from six healthy volunteers were collected 240 h after taking a single dose of raloxifene. The concentrations of urinary raloxifene were analyzed by the established method after sample preparation, including hydrolysis with β-glucuronidase. The lowest limit of quantification was 0.5 ng/mL. Linearity was observed for raloxifene concentrations ranging from 0.5 to 100 ng/mL, with a correlation coefficient of 0.999. The recoveries were >93 percent. Inaccuracies were below ±5 percent, and precisions varied from 2.18 to 5.37 percent. The results showed that urinary raloxifene was immediately detectable within 4 h after the administration of only a single dose of raloxifene. Such a result indicates a violation of the WADA rules. Furthermore, ingesting raloxifene would be detectable after 6 days in the urine of males or >10 days in the urine of female [13800].

Laboratory techniques
A selective, sensitive, accurate and precise liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for determination of raloxifene and its three glucuronides: raloxifene-6-β-glucuronide (M1), raloxifene-4'-β-glucuronide (M2), raloxifene-6,4'-diglucuronide (M3) in urine samples is presented in one paper. The developed analytical method is the first fully validated method capable of simultaneous determination of raloxifene and its glucuronides in real urine samples. Moreover, for the first time a method for determination of raloxifene diglucuronide in relevant biological samples was introduced. Metabolites were obtained by a bioconversion process of raloxifene to its glucuronides using the microorganism Streptomyces sp. and were used as standards for validation. Urine samples were introduced to a simple solid phase extraction prior to the analysis by LC-MS/MS. The method was linear in a wide range with high determination coefficient. The limits of quantification achieved were 1.01, 1.95, 2.83 and 4.69 nM for raloxifene, M1, M2 and M3, respectively. The recoveries were higher than 93 percent, the accuracy was within 100 ± 9 percent and the precision was better than 12 percent for all compounds. The developed method was successfully applied to the real urine samples and showed to be appropriate for use in further research of still not completely discovered raloxifene pharmacokinetics. Furthermore, the presented method could also serve for a potential application in anti-doping analysis [11337].
AROMATASE INHIBITORS

Androgens can increase muscular mass and strength and remain the most frequently abused and widely available drugs used in sports doping. Banning the administration of natural or synthetic androgens has led to a variety of strategies to circumvent the ban of the most effective ergogenic agents for power sports. Among these, a variety of indirect androgen doping strategies aiming to produce a sustained rise in endogenous testosterone have been utilized. These include oestrogen blockade by drugs that act as oestrogen receptor antagonists (antioestrogen) or aromatase inhibitors [08204].

The detection of metabolites of the anti-estrogenic substance cyclofenil, listed on the World Anti-Doping Agency Prohibited List since 2004 is described. Target substances are hydroxylated metabolites, bearing an aliphatic hydroxyl group either in the 2-, 3- or 4-position of the aliphatic ring, in addition to the phenolic functions on the aromatic rings. Structural identification used NMR as well as high-resolution mass spectrometry after nano-electrospray ionisation (ESI). Unambiguous detection of all three synthesised cyclofenil metabolites M1-M3 was done using gas chromatography for separation and electron ionisation mass spectrometry for detection of the per-silylated compounds in comparison with a reference urine deriving from an excretion study within the WADA 2007 Educational Programme [08205].

Tamoxifene

Different liquid chromatographic/tandem mass spectrometric (LC/MS/MS) scanning techniques were considered for the characterization of tamoxifene metabolites in human urine for anti-doping purpose. Five different LC/MS/MS scanning methods based on precursor ion scan (precursor ion scan of m/z 166, 152 and 129) and neutral loss scan (neutral loss of 72 Da and 58 Da) in positive ion mode were assessed to recognize common ions or common losses of tamoxifene metabolites. The applicability of these methods was checked first by infusion and then by the injection of solution of a mixture of reference standards of four tamoxifene metabolites available in a laboratory. The data obtained by the analyses of the mixture of the reference standards showed that the five methods used exhibited satisfactory results for all tamoxifene metabolites considered at a concentration level of 100 ng/mL, whereas the analysis of blank urine samples spiked with the same tamoxifene metabolites at the same concentration showed that the neutral loss scan of 58 Da lacked sufficient specificity and sensitivity. The limit of detection in urine of the compounds studied was in the concentration range 10-100 ng/mL, depending on the compound structure and on the selected product ion. The suitability of these approaches was checked by the analysis of urine samples collected after the administration of a single dose of 20 mg of tamoxifene. Six metabolites were detected: 4-hydroxytamoxifen, 3,4-dihydroxytamoxifen, 3-hydroxy-4-methoxytamoxifen, N-demethyl-4-hydroxytamoxifen, tamoxifen-N-oxide and N-demethyl-3-hydroxy-4-methoxytamoxifen, which is in conformity to our previous work using a time-of-flight (TOF) mass spectrometer in full scan acquisition mode [10203].

A molecular imprinted polymer (MIP) has been synthesized in order to specifically extract tamoxifen, a nonsteroidal antiestrogen, and its metabolites from urine by solid-phase extraction (SPE) before HPLC-UV analysis. Clomiphene, a chlorinated tamoxifen analogue, was selected as template for MIP synthesis. Polymerisation was achieved by thermal polymerisation of methacrylic acid (MAA) as functional monomer, ethylene glycol
dimethacrylate (EDMA) as cross-linking agent and acetonitrile as porogen. The efficient elimination of the urinary matrix has been obtained by MIP-SPE but the elution recovery of tamoxifen was initially too low (approximately 14 %). This problem has been overcome following two ways. At first, a preliminary HLB-SPE of the urine has enabled to discard endogenous salts and to percolate an organic sample through the MIP cartridge. Extraction recoveries are equal to 56 and 74 percent for tamoxifen and 4-hydroxytamoxifen, respectively. Then, a second MIP has been prepared with styrene and MAA as functional co-monomers. Strong pi-pi interactions occurring between phenyl groups of styrene and tamoxifen promote rebinding of the analyte by the specific sites. The enhanced hydrophobic character of the imprinted polymer has enabled the direct percolation of urine through MIP-SPE and the easy elimination of endogenous salts from urine with only one aqueous washing step. HPLC-UV analysis has confirmed high extraction recoveries (85 %) for tamoxifen and its metabolite with an enrichment factor of 8. This analytical protocol can selectively detect the presence of tamoxifen metabolites in urines and be useful as a proof of doping in competitive sports [08206].

In men, the stimulation of GH and inhibition of LH secretion by testosterone requires aromatization to estradiol. Tamoxifen, a selective estrogen receptor modulator (SERM), possesses central estrogen antagonistic effect but peripheral hepatic agonist effect, lowering IGF-I. Thus, tamoxifen is likely to perturb the neuroendocrine regulation of GH and gonadal axes. Raloxifene, a SERM, is used for therapy of osteoporosis in both sexes. Its neuroendocrine effects in men are poorly understood. It was conducted a randomized, open-label crossover study. Ten healthy men were randomized to 2-wk sequential treatment with tamoxifen (10 and 20 mg/d) and raloxifene (60 and 120 mg/d), with a 2-wk intervening washout period. Tamoxifen, but not raloxifene, significantly reduced IGF-I levels by 25 ± 6 percent and increased SHBG levels by 20 ± 7 percent at the higher therapeutic dose. There was a nonstatistically significant trend toward a reduction in the GH response to arginine with both SERMs. Both drugs significantly increased LH, FSH, and testosterone concentrations. The mean increase in testosterone and LH was significantly greater with tamoxifen than with raloxifene treatment. It was concluded that tamoxifen, but not raloxifene, reduces IGF-I levels. Both SERMs stimulate the gonadal axis, with tamoxifen imparting a greater effect. We conclude that in therapeutic doses, raloxifene perturbs the GH and gonadal axes to a lesser degree than tamoxifen [10463].

One study was performed to investigate the influence of the intake of selective oestrogen receptor modulators on the urinary endogenous steroids profile. For this purpose the circadian variability of luteinizing hormone, follicle-stimulating hormone, testosterone, 5alpha-androstan-3alpha,17beta-diol, 5beta-androstan-3alpha,17beta-diol, epitestosterone, 4-androstenedione, androsterone and etiocholanolone were measured on eight subjects (four males and four females) by gas chromatography-mass spectrometry and chemiluminescent immunometric assay techniques before and after oral administration of multiple doses of either tamoxifen (80 mg for 2 days) or toremifene (120 mg for 2 days) or clomiphene (100 mg for 2 days). The individual baseline variability of the steroids studied was set up by collecting the urine samples every 3 h, for 3 days prior to the treatment; whereas the evaluation of the effects of the oral administration of multiple doses of selective oestrogen receptor modulators on the steroid urinary profile was assessed by collecting urine samples every three hours for at least five days from the first administration. The results of the measurements showed that, only in male subjects, the relative urinary concentrations of testosterone, epitestosterone and 4-androstenedione were significantly altered generally after the second day of drug administration. While no significant effects were recorded in both sexes on the luteinizing hormone, follicle-stimulating hormone, androsterone, etiocholanolone, 5alpha-androstan-3alpha,17alpha-diol and 5beta-androstan-3alpha,17beta-diol urinary levels and on
testosterone/epitestosterone, 5alpha-androstan-3alpha,17beta-diol/5beta-androstan-3alpha, 17beta-diol and androsterone/etiocholanolone ratios [11456].

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The use of selective oestrogen receptor modulators has been prohibited since 2005 by the World Anti-Doping Agency regulations. As they are extensively cleared by hepatic and intestinal metabolism via oxidative and conjugating enzymes, a complete investigation of their biotransformation pathways and kinetics of excretion is essential for the anti-doping laboratories to select the right marker(s) of misuse. This work was designed to characterize the chemical reactions and the metabolizing enzymes involved in the metabolic routes of clomiphene, tamoxifen and toremifene. To determine the biotransformation pathways of the substrates under investigation, urine samples were collected from six subjects (three females and three males) after oral administration of 50 mg of clomiphene citrate or 40 mg of tamoxifen or 60 mg of toremifene, whereas the metabolizing enzymes were characterized in vitro, using expressed cytochrome P450s and uridine diphosphoglucuronosyltransferases. The separation, identification and determination of the compounds formed in the in vivo and in vitro experiments were carried out by liquid chromatography coupled with mass spectrometry techniques using different acquisition modes. Clomiphene, tamoxifen and toremifene were biotransformed to 22, 23 and 18 metabolites respectively, these phase I reactions being catalyzed mainly by CYP3A4 and CYP2D6 isoforms and, to a lesser degree, by CYP3A5, CYP2B6, CYP2C9, CYP2C19 isoforms. The phase I metabolic reactions include hydroxylation in different positions, N-oxidation, dehalogenation, carboxylation, hydrogenation, methoxylation, N-dealkylation and combinations of them. In turn, most of the phase I metabolites underwent conjugation reaction to form the corresponding glucuron conjugated mainly by UGT1A1, UGT1A3, UGT1A4, UGT2B7, UGT2B15 and UGT2B17 isoenzymes [13274].

**Exemestane**

The renamed category S4 (Hormone and metabolic modulators) includes five sub-groups, of which the class of aromatase inhibitors (4.1) lists exemestane that was subjected to human in vivo metabolism studies. Four metabolites formerly not reported were discussed and
structures were assigned and proposed based on LC-MS and LC-MS/MS (with accurate mass) data. The metabolites include bis-hydroxylated exemestane bearing the two hydroxyl functions at C-6 and the C-6-linked methylene unit, as well as 6-hydroxyandrost-1,4-diene-17β-ol-3-one and two isomers of 6-hydroxyandrost-1,4-diene-3,17-dione. Since the proposed compositions are not yet supported by chemical (or enzymatic) synthesis or available reference material, the suggested products have to be considered tentative but nevertheless useful for screening purposes [13012].

Aminoglutethimide

Aminoglutethimide is an anti-steroid drug marketed under the tradename Cytadren by Novartis around the world. It blocks the production of steroids derived from cholesterol and is clinically used in the treatment of Cushing’s syndrome and metastatic breast cancer. It is also used by body builders. Aminoglutethimide has two mechanisms of action: it blocks aromatase in the generation of estrogens from androstenedione and testosterone and it blocks the conversion of cholesterol to pregnenolone by inhibiting the enzyme P450scc and consequently decreases synthesis of all hormonally active steroids. At low doses, aminoglutethimide is only an effective inhibitor of aromatase, but at higher doses, it effectively blocks P450scc as well. Following an alternate analytical approach, the utility of metallic plasmonic nanoparticles for the isolation and detection of aminoglutethimide (S4.1) with surface-enhanced Raman spectroscopy and plasmon resonance was presented. By means of colloidal silver triangular nanoprisms, a LOD of 0.13 ng/mL was accomplished, demonstrating the high sensitivity of the employed approach; however, the limitation to one particular drug and the unknown specificity under routine doping control conditions might not allow to consider the assay fit-for-purpose [13012].

Formestan

Formestane (F, androst-4-en-4-ol-3,17-dione) is an irreversible aromatase inhibitor with the ability to suppress the estrogen production from anabolic steroids. Consequently, F is mentioned on the World Anti-Doping Agency (WADA) prohibited list and because studies have shown that F is produced endogenously in small amounts, a threshold for urinary excreted F of 150 ng/mL was introduced. Lower concentrations could be due to endogenous production and need further investigation to prove the exact origin through determination of the carbon isotope ratio. However, because the current screening methods are a lot more sensitive, F is detected in practically every urine sample. A strict implementation of this WADA rule would imply that almost every urine sample needs additional investigation to verify an exogenous or endogenous origin. The main aim of this study was to propose and introduce a lower concentration limit of 25 ng/mL beneath which the detected F is considered as being endogenous and no further investigation is needed. The data presented in this paper suggests that this threshold provides a good balance between a sufficiently large detection window and not having to perform isotope ratio mass spectrometry (IRMS) analyses on negative urine samples [13275].

The class of hormone and metabolic modulators of the 2013 WADA Prohibited List comprises five categories. Among the explicitly mentioned aromatase inhibitors (S4.1), particularly formestane was subject of research projects within the past 12 months, arguably due to its known natural occurrence and the resulting analytical challenge in doping controls. Sensitive and specific steroid profiling has demonstrated that formestane is ubiquitous to human urine samples and consequently, analytical approaches were assessed for the determination of the formestane origin. The strategies were similar to those employed for the
detection and discrimination of natural/endogenous anabolic agents from synthetic analogs with the desire of reasonable effort. Levels of 100 ng/mL and later 150 ng/mL were suggested to be indicative for formestane administration and trigger follow-up analyses by IRMS [13009].

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BLOOD DOPING

Overviews

As science marches on, athletes and coaches march close behind. Researchers have long been interested in how red cell mass and blood volume affect exercise capacity. Interest in blood doping soared after the 1968 Mexico City Olympics. Studies in the 1970s and 1980s suggested that transfusing red cells could speed endurance performance. Diverse athletes of the time were accused of blood doping. In the late 1980s, recombinant human erythropoietin (EPO) began to supplant transfusion for doping. EPO use is a suspect in nearly 20 deaths in 4 years in European cyclists. In the 1998 Tour de France, a team was ejected for using EPO and six other teams quit the race. Tests for detecting artificial blood also exist, but it seems it will take widespread, year-round, unannounced, out-of-competition testing and stern penalties to deter blood doping [07107].

WADA describes blood doping as the use of drugs or any technique to increase red blood cell mass: this enables a greater O₂ transport to tissues thereby increasing aerobic capacity and endurance. In clinical practice, patients suffering from chemotherapy induced anaemia, with a haemoglobin concentration below 10 g/dL, benefit from erythropoietin (EPO) therapy, which currently provides an alternative to red blood cell transfusion. Autologous blood transfusion is popular among sports people who engage in doping because its detection is also very difficult, in contrast to the homologous type which can be detected by flow cytometry [12011].

Blood doping practices in sports have been around for at least half a century and will likely remain for several years to come. The main reason for the various forms of blood doping to be common is that they are easy to perform, and the effects on exercise performance are gigantic. Yet another reason for blood doping to be a popular illicit practice is that detection is difficult. For autologous blood transfusions, for example, no direct test exists, and the direct testing of misuse with recombinant human erythropoietin (rhEpo) has proven very difficult despite a test exists. Future blood doping practice will likely include the stabilization of the transcription factor hypoxia-inducible factor which leads to an increased endogenous erythropoietin synthesis. It seems unrealistic to develop specific test against such drugs (and the copies hereof originating from illegal laboratories). In an attempt to detect and limit blood doping, the World Anti-Doping Agency (WADA) has launched the Athlete Biological Passport where indirect markers for all types of blood doping are evaluated on an individual level. The approach seemed promising, but a recent publication demonstrates the system to be incapable of detecting even a single subject as "suspicious" while treated with rhEpo for 10-12 weeks. Sad to say, the hope that the 2012 London Olympics should be cleaner in regard to blood doping seems faint. It was proposed that WADA strengthens the quality and capacities of the National Anti-Doping Agencies and that they work more efficiently with the international sports federations in an attempt to limit blood doping [12223].

Among doping practices, blood doping has become an integral part of endurance sport disciplines over the past decade. The term "blood doping" or "blood boosting," earlier known as "induced erythrocythemia," usually refers to methods or substances administered for non-medical reasons to healthy athletes with the aim of increasing maximal aerobic power and thereby improving aerobic performance. The definition of blood doping therefore must include methods or substances administered for non-medical reasons to healthy athletes for improving aerobic performance. It includes all means aimed at producing an increased or more efficient mechanism of oxygen transport and delivery to peripheral tissues and muscles.
The World Anti-Doping Agency (WADA) defines blood doping as “the misuse of certain techniques and/or substances to increase one’s red blood cell mass, which allows the body to transport more $O_2$ to muscles and therefore increase stamina and performance.” Prohibited procedures include the use of synthetic $O_2$ carriers, the transfusion of red blood cells (RBCs), the infusion of hemoglobin (Hb), and the artificial stimulation of erythropoiesis. Several paragraphs of the “2011 Prohibited List” of the WADA are relevant as regards blood doping. First, under “Prohibited Substances” (“S2. Peptide hormones, growth factors and related substances”), several erythropoiesis-stimulating agents (ESAs) are itemized: erythropoietin (Epo), darbepoetin-alfa, hypoxia-inducible factor (HIF) stabilizers, methoxy-polyethylene glycol-epoetin (CERA), and peginesatide (Hematide; Affymax). Second, under “Prohibited Methods” forbidden blood products are specified (“M1. Enhancement of oxygen transfer”). Furthermore, intravenous infusions (unless clinically legitimated) and the sequential withdrawal, manipulation and reinfusion of whole blood are prohibited (“M2. Chemical and physical manipulation”). Finally, genetic interventions with the potential to enhance sport performance are defined (“M3. Gene doping”), including “the transfer of nucleic acids or nucleic acid sequences, the use of normal or genetically modified cells, and the use of agents that directly or indirectly affect functions known to influence performance by altering gene expression” [11116].

The wide-spread assumption that doping with erythropoietin or blood transfusion is only effective by increasing arterial blood $O_2$ content because of rising hematocrit is not self-evident. “Natural blood dopers” (horses, dogs) increase both hematocrit and circulating blood volume during exercise by releasing stored erythrocytes from the spleen. Improvement of aerobic performance by augmenting hemoglobin concentration may be expected until the optimal hematocrit is reached; above this value maximal cardiac output declines due to the steep increase of blood viscosity. Therefore an enlarged blood oxygen content might only be useful if the normal hematocrit of man during exercise is suboptimal. However, recent studies suggest that cardiac power rises after erythropoietin allowing an unchanged cardiac output in spite of increased viscosity. Other factors underlying improved performance after blood doping might be: augmented diffusion capacity for oxygen in lungs and tissues, increased percentage of young red cells with good functional properties (after erythropoietin), increased buffer capacity, increase of blood volume, vasoconstriction, reduced damage by radicals, mood improvement by cerebral effects of erythropoietin. Also the importance of placebo is unknown since double-blind studies are rare. It is suggested that blood doping has multifactorial effects not restricted to the increase in arterial oxygen content [10309].

Many innovative methods for blood doping have been tested and proven efficient in other fields of medicine, for example criminology. Another strategy that has been suggested recently is the use of a hematologic passport, which is based on the sequential evaluation of some hematological and biochemical parameters. An athlete’s hematologic profile should be fairly stable over time. Using proper sequential determinations, individual reference ranges can be defined for both hemoglobin and hematocrit. The International Ski Federation decided to pursue athletes with hemoglobin values above 175 g/l for men and 155 g/l for women, whereas the International Cycling Union disqualified athletes with hematocrit values exceeding 50 percent for men and 47 percent for women when reticulocytes were above 2 percent. Thus far, the hematologic passport appears to be a feasible solution to identify athletes with non-physiological variations within a global strategy to deter blood doping, but it has not been approved, nor is it being incorporated into developing antidoping policies [10353].

Blood and urine samples can be taken in-competition and out-of competition. With respect to
reservations that a venipuncture is a medical intervention and may violate the tenets of
certain religious or cultural groups, the WADA has stated that there is no basis for such
provisos [11428].

Oxygen is essential for life, and the body has developed an exquisite method to collect
oxygen in the lungs and transport it to the tissues. Hemoglobin contained within red blood
cells (RBCs), is the key oxygen-carrying component in blood, and levels of RBCs are tightly
controlled according to demand for oxygen. The availability of oxygen plays a critical role in
athletic performance, and agents that enhance oxygen delivery to tissues increase aerobic
power. Early methods to increase oxygen delivery included training at altitude, and later,
transfusion of packed RBCs. A breakthrough in understanding how RBC formation is
controlled included the discovery of erythropoietin and cloning of the EPO gene. Cloning of
the EPO gene was followed by commercial development of recombinant human Epo
(rHuEpo). Legitimate use of this and other agents that affect oxygen delivery is important in
the treatment of anaemia (low Hb levels) in patients with chronic kidney disease or in cancer
patients with chemotherapy-induced anaemia. However, competitive sports was affected by
illicit use of rHuEpo to enhance performance. Testing methods for these agents resulted in a
cat-and-mouse game, with testing labs attempting to detect the use of a drug or blood
product to improve athletic performance (doping) and certain athletes developing methods to
use the agents without being detected [08208].

Optimum performance in aerobic sports performance requires an efficient delivery to, and
consumption of, oxygen by the exercising muscle. It is probable that maximal oxygen uptake
in the athlete is multifactorial, being shared between cardiac output, blood oxygen content,
muscle blood flow, oxygen diffusion from the blood to the cell and mitochondrial content. Of
these, raising the blood oxygen content by raising the haematocrit is the simplest acute
method to increase oxygen delivery and improve sport performance. Legal means of raising
haematocrit include altitude training and hypoxic tents. Illegal means include blood doping
and the administration of EPO (erythropoietin). The ability to make EPO by genetic means
has resulted in an increase in its availability and use, although it is probable that recent
testing methods may have had some impact. Less widely used illegal methods include the
use of artificial blood oxygen carriers (the so-called "blood substitutes"). In principle these
molecules could enhance aerobic sports performance; however, they would be readily
detectable in urine and blood tests. An alternative to increasing the blood oxygen content is
to increase the amount of oxygen that haemoglobin can deliver. It is possible to do this by
using compounds that right-shift the haemoglobin dissociation curve (e.g. RSR13). There is a
compromise between improving oxygen delivery at the muscle and losing oxygen uptake at
the lung and it is unclear whether these reagents would enhance the performance of elite
athletes. However, given the proven success of blood doping and EPO, attempts to
manipulate these pathways are likely to lead to an ongoing battle between the athlete and
the drug testers [08209].

Owing to the continuous progress of technology, biology, and sport medicine, blood doping is
still a controversial issue that will remain a major challenge for years to come. Blood doping
is unfair and not always detectable by current antidoping strategies; the potential risks for
health outweigh any potential benefit. As long as it is proceed further in the understanding of
the intricate molecular mechanisms that regulate erythropoiesis, innovative and revolutionary
therapeutic resources will become commercially available. As occurred earlier for other
performance-enhancing substances, the side effects of these therapeutic agents when used
by athletes will become apparent in the future. The long history of doping in sports teaches
us how eagerly people will reach for premature technology, with little fear of potential side
effects or complications. Athletes may take advantage of many innovative therapies to
improve athletic performance, and genetic doping is presently the most attractive method.
Staying ahead in the fight against the use of blood doping involves staying ahead in basic and clinical research. There is growing evidence of unsuspected and previously undetected agents being used, and the potential list appears to be endless. Awareness of this emerging problem involves the need for cooperation among manufacturers, sport federations, antidoping agencies, and laboratory professionals to ensure that methods for the detection of putative doping agents are available at the time of product release. Scientists should be aware that the continued integrity of sports competition and athletes’ health both depend on their ability to outpace the efforts of dopers; this calls for on-going development and the application of reliable detection strategies [10353].

The World Anti-Doping Agency (WADA) defines blood doping as “the misuse of certain techniques and/or substances to increase one’s red blood cell mass, which allows the body to transport more $O_2$ to muscles and therefore increase stamina and performance.” Prohibited procedures include the use of synthetic $O_2$ carriers, the transfusion of red blood cells (RBCs), the infusion of hemoglobin (Hb), and the artificial stimulation of erythropoiesis. Several paragraphs of the “2011 Prohibited List” of the WADA are relevant as regards blood doping. First, under “Prohibited Substances” (“S2. Peptide hormones, growth factors and related substances”), several erythropoiesis-stimulating agents (ESAs) are itemized: erythropoietin (Epo), darbepoetin-alfa, hypoxia-inducible factor (HIF) stabilizers, methoxy polyethylene glycol-epoetin (CERA), and peginesatide (Hematide®; Affymax®). Second, under “Prohibited Methods” forbidden blood products are specified (“M1. Enhancement of oxygen transfer”). Furthermore, intravenous infusions (unless clinically legitimated) and the sequential withdrawal, manipulation and reinfusion of whole blood are prohibited (“M2. Chemical and physical manipulation”). Finally, genetic interventions with the potential to enhance sport performance are defined (“M3. Gene doping”), including “the transfer of nucleic acids or nucleic acid sequences, the use of normal or genetically modified cells, and the use of agents that directly or indirectly affect functions known to influence performance by altering gene expression.” Hemoglobin mass is a key factor for maximal exercise capacity. Some athletes apply prohibited techniques and substances with intent to increase hemoglobin mass and physical performance, and this is often difficult to prove directly. Autologous red blood cell transfusion cannot be traced on reinfusion, and also recombinant erythropoietic proteins are detectable only within a certain timeframe. Novel erythropoietic substances, such as mimetics of erythropoietin (Epo) and activators of the Epo gene, may soon enter the sports scene. In addition, Epo gene transfer maneuvers are imaginable. Effective since December 2009, the World Anti-Doping Agency has therefore implemented “Athlete Biologic Passport Operating Guidelines,” which are based on the monitoring of several parameters for mature red blood cells and reticulocytes. Blood doping may be assumed, when these parameters change in a nonphysiologic way. Hematologists should be familiar with blood doping practices as they may play an important role in evaluating blood profiles of athletes with respect to manipulations, as contrasted with the established diagnosis of clinical disorders and genetic variations [11428].

During the last 30 years, the artificial increase of red blood cell volume ("blood doping") has changed the level of performance in all endurance sports. Many doping scandals have shown the extent of the problem. The detection of blood doping relies on two different approaches: the direct detection of exogenous manipulating substances (erythropoietic stimulants) or red cells (homologous transfusion) and the indirect detection, where not the doping substance or technique itself, but its effect on certain biomarkers is measured. Whereas direct detection using standard laboratory procedures such as isoelectric focusing can identify erythropoietic stimulants, homologous blood transfusion is identified through mismatches in minor blood group antigens by flow cytometry. Indirect methods such as the athlete biological passport are the only means to detect autologous transfusion and may also be used for the detection of erythropoietic stimulants or homologous transfusion. New
techniques to unmask blood doping include the use of high-throughput “omics” technologies (proteomics/metabolomics) and the combination of different biomarkers with the help of mathematical approaches. Future strategies should aim at improving the use of the available data and resources by applying pattern recognition algorithms to recognize suspicious athletes and, on the basis of these findings, use the appropriate testing method. Different types of information should be combined in the quest for a forensic approach to anti-doping.

Blood doping in sports has been a hot topic of present. Longitudinal follow up of hematological parameters in different endurance sports, during the 1990s and early 2000s, has provided considerable suspicions about extensive blood manipulation, with performance enhancing effects. Recent doping revelations in the media also prove that blood doping is not an anticipated myth but it is, in fact, real. Erythropoiesis stimulating agents and autologous blood transfusions are used in synergy with substantial effect on the maximum oxygen uptake and delivery to muscles. Whilst both methods of blood manipulation represent a potential health hazard, in the context of an elevated hematocrit, nevertheless despite a number of suspicious deaths amongst athletes, this has not yet been fully documented. A reliable test for detection of recombinant human erythropoietin was implemented in 2000, but this is probably circumvented by microdose regimens. The Athlete's Biological Passport represents the progeny of the idea of an indirect approach based on long term monitoring of hematological parameters, thus making it possible to detect autologous blood doping and erythropoietin use after the substance is excreted. Nevertheless with advances in anti-doping measures it is possible that the levels of excretion of substances used can be masked. Clearly more sensitive and specific diagnostic tools and research/development in these areas of major concern are warranted, which, combined with changes in the athlete's attitude, will help in reaching the vision of fair play.

Blood doping is based on the use and administration of any illicit substance or procedure aimed at increasing and optimising oxygen delivery to the exercising muscles and, therefore, includes blood transfusions, administration of erythropoiesis-stimulating substances (namely hormones and growth factors), blood substitutes and artificial altitude facilities, possibly mixed and/or combined. The use of blood transfusions, either autologous or homologous, is an effective strategy for increasing oxygen delivery to the muscles and can be used immediately before a single competition or even during long-lasting sporting events to maintain a stable haemoglobin (Hb) concentration, especially when a natural decrease is expected as a result of physiological adaptation to endurance exercise. The procedure is not new, since it became popular nearly 40 years ago, but then suddenly declined due to its inclusion among the list of prohibited methods issued by the International Olympic Committee (IOC). Another reason that contributed greatly to the sudden decay of popularity of blood transfusions among elite athletes was the cloning of the erythropoietin gene and subsequent development of recombinant erythropoietin (rHuEpo) and its introduction among the armamentarium of illicit ergogenic aids. The great success of rHuEpo and analogues (erythropoiesis-stimulating agents, ESA) was mainly the consequence of the remarkable biological and technical advantages that these substances had over traditional means of increasing the red cell mass, such as blood transfusions. These advantages include easier supply (from healthcare facilities as well as from the “black market”), more comfortable administration (small subcutaneous doses) and the sharp and long-lasting effects on erythropoiesis (i.e. “blood boosting”). Once the concentration of Hb has been raised through ESA administration, the high concentration can be maintained by weekly administered microdoses of ESA which have a detection window of only 12-18 hours, for rHuEpo, compared to 3 days for regular doses. The subsequent development and implementation of (more or less) reliable electrophoretic techniques to screen for rHuEpo misuse caused a sudden resurgence of blood transfusions, which also took advantage of new procedures for
collecting and storing the erythrocytes (e.g. freezing), allowing their use over a very long period of time as well as their harvesting during rest periods, thus avoiding a decline of Hb and aerobic sport performance during the competitive season [13290].

The oxygen carrying capacity of the organism has been addressed as performance limiting in most endurance sport disciplines. From basic physiology, it is known that the oxygen transport of the body is mediated through cardiac output and hemoglobin, both entities being key factors in the Fick principle, which defines oxygen uptake. VO$_{2\text{max}}$, the maximum oxygen uptake, and total hemoglobin (tHb) are both well-established determinants for aerobic exercise capacity. Possible ways to manipulate VO$_{2\text{max}}$ can be examined by Fick’s principle: 

\[ \text{VO}_{2\text{max}} = \text{CO} \left( \text{CaO}_2 - \text{CvO}_2 \right) \]

where CO is cardiac output, CaO$_2$ is arterial oxygen content and CvO$_2$ is venous oxygen content. Systematic endurance training increases cardiac output through increased plasma volume (PV) and eccentric hypertrophy of the heart. Induced hypervolemia with dextran or other plasma volume expanders increases CO and VO$_{2\text{max}}$ in untrained individuals, but the VO$_{2\text{max}}$ remains unchanged in athletes, with only a small increase in CO. This is due to a lower diastolic reserve capacity, which does not compensate for the reduction in CaO$_2$. Arteriovenous difference is already high in the working muscle. Oxygen extraction has been measured to 85 percent in a maximum intensity setting with a one legged knee extensor exercise with supramaximal blood flow, hence reducing the capillary transit time further than with two legged exercises. CaO$_2$ on the other hand can easily be modified by elevating the hemoglobin level either by ESA or blood transfusions, thus increasing VO$_{2\text{max}}$ [13005].

One of the prime training targets for endurance athletes is therefore to increase their oxygen uptake and thereby increase their cardiac output and the oxygen carrying capacity of the blood. It is well known that regular endurance training leads to enlargement of the cardiac cavities and thus cardiac output and, on the other hand, induces an increase in red cell volume, which will improve the oxygen transport. However, these adaptations are slow, require dedicated training over many years, and there is a high interindividual difference in the ability to adapt to training. Thus, athletes and coaches have searched for possibilities to bypass these natural adaptations. It is well known that hypoxia will accelerate red cell production and thus increase red cell volume so that altitude training and different other types of hypoxic interventions are nowadays standard training strategies for most elite athletes competing in endurance events. In light of the inventive spirit of human nature, it was clear that sooner or later athletes would seek artificial means to increase their oxygen transport capacity without having to go through the cumbersome and time-consuming process of hypoxic training. From a scientific point of view, the experiments laying the base for a beneficial effect of increased red cell mass stem from military investigations, which demonstrated that the infusion of red cells had the same physiological effects as the adaptation of the erythropoietic system to the hypoxia of altitude and that the “tolerance” of hypoxia (measured through heart rate response to exercise) was improved in the transfused subjects. In the following decades, this topic was further developed with additional investigations that showed a beneficial effect of increased red cell volume on endurance performance. The positive effect of increased red cell volume depended on the amount of transfused red cells with increases in VO$_{2\text{max}}$ of up to 17 percent [13006].

Based on testimonies and confiscated diaries, it has been revealed a doping regimen where a cyclist “mobilizes” with EPO during the low season and “harvests” blood after one month. The blood is retransfused just before completion or even during longer stage races and then withdrawn after the finish. In research early investigators of autologous blood transfusions could not support a performance enhancing effect. When whole blood is stored at 4 °C the red blood cell count is reduced by 33 percent after 4 weeks. Eventually, when red blood cells were stored with cryoprotective agents like glycerol, a number of studies have proven a
correlation between blood transfusions and improved VO$_{2\text{max}}$ or work capacity. The increase in maximal oxygen uptake is typically around 5-10 percent with a dose dependent pattern. 10,000 m time for "highly trained" male runners has been reduced by one minute after retransfusion of 400 ml red blood cells. The increase in VO$_{2\text{max}}$ is correlated to the increase in hemoglobin. Thus, the result is more dependent on dosage than method. The blood volume is not affected by either method and the performance enhancing effect is solely due to elevated Hb [13005].

During the last 30 years, the artificial increase of red blood cell volume ("blood doping") has changed the level of performance in all endurance sports. Many doping scandals have shown the extent of the problem. The detection of blood doping relies on two different approaches: the direct detection of exogenous manipulating substances (erythropoietic stimulants) or red cells (homologous transfusion) and the indirect detection, where not the doping substance or technique itself, but its effect on certain biomarkers is measured. Whereas direct detection using standard laboratory procedures such as isoelectric focusing can identify erythropoietic stimulants, homologous blood transfusion is identified through mismatches in minor blood group antigens by flow cytometry. Indirect methods such as the athlete biological passport are the only means to detect autologous transfusion and may also be used for the detection of erythropoietic stimulants or homologous transfusion. New techniques to unmask blood doping include the use of high-throughput "omics" technologies (proteomics/metabolomics) and the combination of different biomarkers with the help of mathematical approaches. Future strategies should aim at improving the use of the available data and resources by applying pattern recognition algorithms to recognize suspicious athletes and, on the basis of these findings, use the appropriate testing method. Different types of information should be combined in the quest for a forensic approach to anti-doping [13006].

Blood doping in sports has been a hot topic of present. Longitudinal follow up of hematological parameters in different endurance sports, during the 1990s and early 2000s, has provided considerable suspicions about extensive blood manipulation, with performance enhancing effects. Recent doping revelations in the media also prove that blood doping is not an anticipated myth but it is, in fact, real. Erythropoiesis stimulating agents and autologous blood transfusions are used in synergy with substantial effect on the maximum oxygen uptake and delivery to muscles. Whilst both methods of blood manipulation represent a potential health hazard, in the context of an elevated hematocrit, nevertheless despite a number of suspicious deaths amongst athletes, this has not yet been fully documented. A reliable test for detection of recombinant human erythropoietin was implemented in 2000, but this is probably circumvented by microdose regimens. The Athlete's Biological Passport represents the progeny of the idea of an indirect approach based on long term monitoring of hematological parameters, thus making it possible to detect autologous blood doping and erythropoietin use after the substance is excreted. Nevertheless with advances in anti-doping measures it is possible that the levels of excretion of substances used can be masked. Clearly more sensitive and specific diagnostic tools and research/development in these areas of major concern are warranted, which, combined with changes in the athlete's attitude, will help in reaching the vision of fair play [13005].

Relevant for doping controls is the hemoglobin mass (Hb$_{\text{mass}}$). It represents an attractive complement to established markers for the detection of autologous blood doping due to its independence from plasma volume; however, factors such as its within-subject variability (e.g. resulting from reduced training or altitude exposure) as well as technical aspects necessitated in-depth investigations. In one study, a total of 130 athletes (rowing, swimming, running, cycling, kayaking, and football) were tested on approximately 6 occasions within one year using the standard CO-rebreathing method. The within-subject coefficient of variation
was found as high as 4 percent, which was considered to be of limited applicability in sports drug testing if used as single parameter only. In combination with other markers, potential utility was nevertheless recognized. Reduced training volumes showed substantial influence on Hb_{mass} as explicitly demonstrated in a follow-up study with nine triathletes. Within a period of 30 days of detraining, a significant decrease of Hb_{mass} accounting for 3.1 percent was measured, corroborating the need to consider numerous individual factors when applying Hb_{mass} as an anti-doping measure. It was also drawn comparable conclusions from a study with 21 individuals mimicking a 42 weeks cycling season. The athletes underwent ten CO-rebreathing tests for Hb_{mass} determination during the period of the investigation. At the 99 percent specificity level, 10 out of 11 "doped" persons returned positive test results; however, one false positive outcome was recorded as well. Increasing the specificity level to 99.9 percent eliminated the false positive finding but reduced the sensitivity to 73 percent, enabling the detection of 8 out of 11 "doped" volunteers. In order to minimize the impact of technical issues on the variability of Hb_{mass} measurements, the influence of different spectrophotometers on the analytical result as well as the impact of quality controls were assessed, identifying the hemoximeters as a major contributor to inter-laboratory variations, which was minimized with adequate corrections via standardized calibrator samples [13012].

**Prevalence of blood doping**

By using similar models as in the biological passport, but with lower specificity it is possible to estimate the prevalence of doping. Analyses of blood values from the contestants of the 2001 Nordic World Ski Championships revealed that 50 percent of the medal winners and 33 percent of 4th to 10th positions had highly abnormal blood profiles. In comparison only 3 percent of the competitors who finished 41-50 had a highly abnormal blood profile. When comparing individuals, hematocrit and thereby Hb is significantly correlated to VO_{2max} in the untrained. It has also been researched the development of hemoglobin concentrations in Finnish and Swedish cross country skiers from 1987 to 1999. In the beginning the athletes were below the average for the general population in accordance with the compensatory plasma volume expansion of training. In the 1990s the average hemoglobin concentration increased with over 1.0 g/100 mL. The highest individual values were almost 20.0 g/100 mL for both sexes, higher than what is found in permanent mountain dwellers. In that time period, cross country skiers refined the use of altitude training. However, this cannot explain the rise in Hb alone. Still, there is only a handful of studies documenting an effect of altitude training because there is a large interindividual variation of response. When Fédération Internationale de Ski (FIS) issued a regulation for maximum Hb values in 1996 (18.5 g/100 mL for males and 16.5 g/100 ml for females), the maximum values were reduced by 1.5 and 4.2 g/100 mL in males and females. This regulation exists today in different sports federations, but the upper limits are reduced to a Hct or Hb of 0.50 and 17.0 g/100 mL for males and 0.47 and 16.0 g/ 100 mL for females [13005].

It was also compared blood samples from the late 1990s to 2007 in international cross country skiers. The average Hb value fell initially from about 16.2 to a nadir in 2002–2003 of 14.8 g/100 ml. During this time period the EPO test was introduced and FIS announced a strengthening of the anti-doping program. From 2001 to 2007 the average Hb reached the highest values in the Olympic seasons 2001-2002 and 2005-2006. In 2006 the hemoglobin values increased significantly (1.0 g/100 mL in females) in just one week before the Olympics. Samples collected at 1200 m above sea level were in general only 0.15 g/100 mL higher than samples collected below 600 m over sea level in females. In cycling, one can identify some of the trends from cross country skiing like the significant reduction in number of reticulocytes after 2002. In autologous blood doping, reticulocytes increase after phlebotomy and decrease to below pre value after reinfusion. With EPO the opposite is true.
There should be no between season variations since samples are drawn throughout the season for all consecutive years. The current Hb limit of 17.0 g/100 mL is arbitrary. In one study 3.9 percent of the blood donors and 10.4 percent of male elite rowers were found to exceed the limit. This retrospective study confirms violation of the Hb and Hct regulation does not automatically mean doping. However, it does not prove that more athletes have a natural elevated hemoglobin concentration than the normal population because the subjects were not controlled for blood doping. The blood doping prevalence is probably skewed between nations. In the early 2000s the prevalence of blood doping in track and field endurance athletes estimated with the ABPS model ranged from 1 to 48 percent between different nations The average prevalence was 18 percent [13005].

**Biochemical markers of hypoxia**

Although tissues may exist regardless of reduced oxygen pressure, this requires glycolytic ATP generation, which is very expensive from the energetic viewpoint. Hypoxia is defined as the condition in which oxygen pressure is reduced at the level of bodily tissues. There are many clinical situations during which decreased tissue oxygenation may occur. It may be transient or chronic, as well as systemic or local. An emergent need exists for monitoring and diagnosis with respect to numerous possible clinical circumstances leading to hypoxia and its life-threatening consequences. The assessment of global oxygen homeostasis relies on blood gas analysis and lactate concentration, but such an approach does not fully reflect the local oxygenation of tissues. Oxygen needle microelectrode measurements reveal great differences in tissue pO$_2$ levels. Local pO$_2$ levels depend on many factors, among which the most important are: the distance to the nearest capillary, the extracellular and intracellular fluid diffusion rates and intracellular measurements of the number and activity levels of mitochondria. Thus, nowadays, it is impossible to establish an accurate normal value ranges for local tissue pO$_2$. Oxygen deficiency is an important gene regulator. A sequence-specific DNA-binding factor, the hypoxia induced factor (HIF), is the fundamental hypoxia response protein. 70 genes identified so far have been found to be HIF-dependent. They are responsible for increased oxygen delivery, i.e. by boosting angiogenesis due to vascular endothelial growth factor (VEGF) release and the enhancement of red blood cell production by erythropoietin (EPO). VEGF-induced angiogenesis is one of several key hypoxia adaptations. An enhanced vascular bed in response to hypoxia affects almost every bodily tissue and organ. This was observed particularly in skeletal muscles as well as in the brain. The expression of a few hypoxia markers does not require HIF activation. An especially interesting member of this group is osteopontin (OPN), whose synthesis increases during hypoxia. OPN was originally linked to bone remodeling, but currently it seems to posses an important role in immunity, inflammation and tumor pathogenesis. Quantification of hypoxia is clinically essential both for therapy and prognosis. Taking account of the fact that the concept of oxygen pressure at the tissue level is not quantitative (norms do not exist, results are incomparable), biochemical markers are preferable. Particularly significant in this context are hypoxia-induced proteins such as HIF, EPO, VEGF or potentially OPN [12224].

**Theoretical aspects on energy transfer in the body**

The time course of energy metabolism during moderate exercise involves primarily the phosphagen system (for the first 10 to 15 s), followed by anaerobic glycolysis for the next 1 to 2 min and aerobic metabolism for physical activities lasting more than 2 min. ATP molecules normally present within muscle cells can be promptly used to sustain muscle
contraction; phosphocreatine is an additional reserve of energy that can be used to rapidly synthesize ATP. Both systems provide energy at a very rapid rate, but when a muscle fiber is undergoing a sustained contraction, these energy reserves are quickly exhausted. When muscle fibers are actively contracting, each thick filament breaks down roughly 2500 ATP molecules/s. Because even a small skeletal muscle contains thousands of muscle fibers, the ATP demands are enormous and, therefore, skeletal muscles must rely on alternative mechanisms to supply energy. Most cells generate ATP through aerobic metabolism in mitochondria and glycolysis in the cytoplasm. Aerobic metabolism normally provides up to 95 percent of the energy demand of a resting cell. In this process, mitochondria absorb oxygen, adenosine diphosphate (ADP), phosphate ions, and organic substrates that enter the tricarboxylic acid cycle (also known as the citric acid cycle or the Krebs cycle). While carbon atoms are released as carbon dioxide, hydrogen is shuttled to respiratory enzymes in the inner mitochondrial membrane where their electrons are removed. After a series of intermediate steps, protons and electrons are combined with oxygen to form water. By this process, a large amount of energy is efficiently produced, as each organic molecule fed to the tricarboxylic acid cycle generates 17 ATP molecules. During extensive physical exercise, the demand for energy, along with mitochondrial ATP production, progressively increase to a maximum rate that is determined by the availability of oxygen, which cannot diffuse into the muscle fiber fast enough to enable the mitochondria to fulfill the on-going energy expenditure. At peak levels of exertion, mitochondrial activity can provide only about one-third of the ATP required. Therefore, oxygen becomes progressively depleted, and muscles cannot get sufficient amounts to perform at their optimal potential, in terms of both power and resistance. Such a relative gap between oxygen demand and availability is conventionally called “oxygen debt.” Owing to the relative depletion of oxygen to produce ATP via traditional mechanisms, muscle tissue is compelled to shift to the anaerobic pathway, culminating in ATP production through conversion of pyruvic acid, provided by the enzymatic pathway of glycolysis, to lactic acid. Therefore, the process of anaerobic glycolysis enables the generation of additional energy when mitochondria are unable to fulfill the current energy demand. However, anaerobic energy production has its drawbacks. Although nearby 80% of the lactate produced diffuses from the muscles and is transported to the liver for conversion to glucose or glycogen, in conditions of extensive training, it cannot be completely cleared and lactate gradually accumulates at both the site of synthesis and in the blood. As the relative concentration of intracellular lactate can become extremely elevated in muscles, this process can persist for several minutes after the end of the exercise. As the progressive accumulation of lactate lowers the intracellular pH and alters the functional characteristics of key enzymes, the overall efficiency of the muscular contraction finally declines, producing the characteristic symptoms of fatigue, pain, and muscle soreness that may develop several hours or even days after particularly strenuous or unaccustomed exercise. Lactic acidosis typically occurs when the concentration of lactate in blood exceeds 4 mmol/l. Highly trained athletes have maximal oxygen uptakes that may be double those of sedentary people and that permits greater muscular activity coupled to a reduced production and accumulation of lactic acid. Oxygen is carried to peripheral tissues and muscles by two efficient delivery systems: 3 percent is carried in solution (plasma), whereas the remaining 97 percent is bound to hemoglobin, the main protein in red blood cells. Practices that are aimed at producing an increase in hemoglobin in blood or a more efficient mechanism of oxygen transport and delivery are associated with improved energy production, allowing the muscles to become more fatigue resistant and to perform better [10353].

Carotid body arterial chemoreceptors are essential for a normal hypoxic ventilatory response (HVR) and ventilatory acclimatization to hypoxia (VAH). However, recent results show that O2-sensing in the brain is involved in these responses also. O2-sensing in the rostral ventrolateral medulla, the posterior hypothalamus, the pre-Bötzinger complex and the nucleus tractus solitarius contribute to the acute HVR. Chronic hypoxia causes plasticity in
the brain that contributes to VAH and represents another time domain of central O$_2$-sensing. The cellular and molecular mechanisms of acute O$_2$-sensing in the brain remain to be determined but they appear to involve O$_2$-sensitive ion channels and heme oxygenase-2, which acts by a different mechanism than has been described for the carotid body. It is not known if plasticity in such mechanisms of acute central O$_2$-sensitivity contributes to VAH. However, O$_2$-sensitive changes in gene expression in the brain do contribute to VAH and demonstrate another mechanism of O$_2$-sensing that is important for ventilatory control. This time domain of O$_2$-sensing in the brain involves gene expression under the control of hypoxia inducible factor-1$\pm$ (HIF-1$\pm$ and potentially several HIF-1$\pm$ targets, such as erythropoietin, endothelin-1, heme oxygenase and tyrosine hydroxylase [09147].

Human muscle operates along a continuum of power output, which is set through bioenergetic and anatomical principles. In turn, environmental and intrinsic factors during contractile work exert pronounced control over muscle performance by instructing muscle remodelling. This phenotypic control is specifically indicated with intense exercise at altitude, when extra strain is put on energy supply and the temperature-dependent mechanical efficiency of contraction. While it is classically thought that chronic exposure to hypoxia is maladaptive, repeated short episodes of reduced oxygenation alone or in combination with intense endurance work is now understood to preserve exercise performance when atmospheric oxygen levels are low. Endurance training at moderate altitude exploits the temperature-dependent malleability of energy supply that may maximize metabolic flux at altitude. The contribution of genomic mechanisms is important to the plasticity of metabolic pathways in exercised muscle. This is highlighted by the association of distinct gene polymorphisms in master governors of mitochondrial and vascular growth with exercise phenotypes. Feedforward control of human locomotor muscle by exercise involves the transient upregulation of transcript expression for metabolic processes. The response of the mitochondrial transcriptome to intense exercise is graded with respect to mitochondrial content and deoxygenation during muscle work and reflects exercise-induced mitochondrial biogenesis. This supports the notion that genome-mediated muscle malleability is under feedback control by design constraints of the pathway of oxygen. Thus, activity-dependent and genetic mechanisms contribute to the interindividual difference in the metabolic bottlenecks in athletes performing in exceptional environmental conditions [09148].

In a comparative study on erythrocytes (RBCs) drawn from mountaineers before and after a high-altitude stay, it was observed that upon returning to sea level, their RBCs displayed a senescent-like phenotype as indicated by their density and the partial loss of membrane proteins which are shed by ageing RBCs. The aim of one study was therefore to investigate possible changes in the membrane skeleton of these RBCs and to compare them with pathological RBCs. It was analysed the proteins of RBC ghosts obtained from the subjects before and after returning to sea level by two-dimensional electrophoresis and mass spectrometry. It was observed lower expression and fragmentation of beta-actin after exposure to hypoxia. This suggested an alteration in membrane skeleton structure, which was confirmed by beta-actin release in cell lysates during ghost preparation. It was observed a similar actin fragmentation and release in RBC lysates from beta-thalassaemic patients. In conclusion, these results indicate that after exposure to hypoxia, RBCs display a modification of their actin and cytoskeleton instability [09149].

**Hemoglobin mass and physical performance**

In aerobic sport disciplines, such as long-distance running, cycling, or cross-country skiing, the main factors determining performance are a high delivery of O$_2$ to the exercising skeletal muscles and its use. The rate of maximal O$_2$ uptake ($O_2$ max) is dependent on a high cardiac output (Q) and a wide difference for arterialvenous O$_2$ (a-vO$_2$), that is, the Fick equation.
Because $Q_{\text{max}}$ is difficult (if not impossible) to manipulate to higher values during competitions, the distribution of $Q$ during maximal exercise to the working skeletal muscles is close to 80 percent, and arterial $O_2$ extraction is already in the range of approximately 90 percent at maximal exercise, the only variable that remains open for manipulations in regards to increasing exercise performance is the arterial $O_2$ content. Accordingly, in a given person, changes in Hb concentration by either RBC transfusion or hemodilution will increase or decrease $O_{2\text{max}}$, respectively. On the group basis, however, concentration of hemoglobin is not predictive of $O_{2\text{max}}$, whereas the total mass of Hb ($H_{\text{mass}}$) correlates very well with $O_{2\text{max}}$. Indeed, a somewhat reduced hemoglobin concentration is sometimes, but not always, observed among athletes, whereas $H_{\text{mass}}$ is usually increased compared with normal healthy persons. The first experiments with blood transfusions and exercise performed demonstrated that the transfusion of 450 mL of whole blood on 4 consecutive days decreases submaximal exercise heart rate (in hypoxia) for several weeks, and hence predicted that exercise performance would be increased. Accordingly, it was calculated in a recent review that a change of 1 g in $H_{\text{mass}}$ will produce a change in $O_{2\text{max}}$ of 4 mL/min whereas the effects on submaximal exercise performance are probably variable according to competition distance. It should also be noted here that volume loading (i.e. plasma volume expansion) in itself does not lead to an improved exercise performance in elite athletes, again highlighting the role of $H_{\text{mass}}$. If, however, a plasma volume expander is administrated simultaneously with increments in $H_{\text{mass}}$, then performance will probably increase more than when just augmenting the total red cell volume [11428].

**Erythropoiesis**

Erythropoiesis is part of the large process of haematopoiesis, which involves the production of mature cells found in the blood and lymphoid organs. Haematopoiesis is continuously required because of the normal turnover in the cell populations in the blood and lymphoid organs. In the normal adult human, the daily turnover of erythrocytes exceeds $10^{11}$ cells. During periods of increased erythrocyte loss, due to haemolysis or haemorrhage, the production of erythrocytes increases rapidly and markedly. However, overproduction of erythrocytes does not occur, even after the most severe loss of erythrocytes. In haematopoiesis, a few pluripotent haematopoietic stem cells in the bone marrow proliferate and differentiate to give rise to all the cellular components of the blood and the lymphoid system. During this process, an individual haematopoietic cell undergoes an apparently random process called commitment. When a cell undergoes commitment, its potential to proliferate becomes limited and its potential to develop into multiple types of mature cell is also restricted. Thus, these haematopoietic cells are termed committed, lineage specific progenitor cells. The major stages of differentiation in mammalian erythropoiesis are as follows. The most immature stage of committed erythroid progenitors is the burst forming unit-erythroid (BFU-E). The next major stage of erythroid progenitor cell development is the colony forming unit-erythroid (CFU-E). A continuum of erythroid progenitor stages exists between the BFU-E and CFU-E, with decreasing proliferative potential as the progenitors approach the CFU-E stage. The descendant cells of the CFU-E are termed erythroid precursor cells. These erythroid precursors are proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts, and orthochromatic erythroblasts. The orthochromatic erythroblasts do not divide, but they enucleate, forming the nascent erythrocyte called the reticulocyte [06145].

*Effects of marathon*
In marathon runners changes in red blood cell count, haematocrit and haemoglobin in relation to haemodilution have been reported. Moreover, it has been hypothesized that strenuous exercise induces oxidant stress through several different mechanisms. One study investigated the haematological variables, iron status and oxidative indices before, immediately and 48 h after a race in 8 healthy trained males aged 33-44 years running a 21-km marathon in 79 ± 3 min. The haematological parameters were determined by standard procedures. Erythropoietin and soluble-transferrin receptor were evaluated immunoenzymatically. Nontransferrin-bound iron (NTBI) was assayed by high-performance liquid chromatography after nitrilotriacetic acid chelation. Malonyldialdehyde (MDA) concentration was assayed colorimetrically. The total number of reticulocytes rose significantly after the run with a significant increase in the high-RNA-content fraction. Erythropoietin rose by 26 percent and by 25 percent immediately and 48 h after the race, respectively. Serum iron and serum ferritin remained unchanged but NTBI and serum MDA increased significantly immediately after running. Significant positive correlations at any time between MDA and polymorphonuclear neutrophils, MDA and NTBI, polymorphonuclear neutrophils and NTBI, and between lactate dehydrogenase and NTBI were observed. It was concluded that the erythropoietic changes observed in marathon runners are the results of several interacting mechanisms that involve either the haemopoietic system per se or erythrocyte haemolysis and oxidative stress [06146].

Effects of training in juniors

During the world junior speed skating championship 2002 all athletes (60 males, 56 females) were subjected to hematologic blood testing one day before the competition as requested by International Skating Union (ISU). This study aimed to obtain hematologic reference values for junior athletes, whilst the influence of endurance training on hematologic variables of young athletes was studied. Hematologic results of athletes were compared to results of non-athletes matched by age and gender (14 males, 17 females). The blood analysis was done on an ADVIA 120. To compare measurement of ferritin, erythropoietin, and soluble transferrin receptor in serum as well as in EDTA-plasma, serum and EDTA-blood was obtained from the control group. In hemoglobin and hematocrit it was found no significant difference between the two groups, whereas the number of erythrocytes was lower in athletes. The mean corpuscular volume was higher in athletes, whilst the corpuscular hemoglobin content was only marginally higher in athletes than in non-athletes. Consequently corpuscular hemoglobin concentration mean was lower in athletes than in non-athletes. There was no difference of erythropoietin and soluble transferrin receptor, whilst in ferritin we found a difference between the groups. Endurance training does not change the values of hemoglobin and hematocrit. Increased mean corpuscular volume and decreased corpuscular hemoglobin concentration mean could be a result of changed properties of red blood cell-membrane caused by acidosis and higher osmolality during the training. In junior athletes we did not find an iron overload as described in some adult athletes [06147].

Red blood cells

Red blood cells (RBCs) during microcirculation, aging and storage, lose N-acetylenuraminic acid (NANA) and other biomaterials thereby altering cell structures, some properties and functions. Such cell damage very likely underlies the serious adverse effects of blood transfusion. However, a controversy has remained since 1961-1977 as to whether with aging, the RBCs, suffering loss of NANA, do have a decreased charge density. Any correlation between the changes in the cell properties with cell aging is also not clear. Therefore, to remove the ambiguity and uncertainty, it was carried out multiparametric
studies on Percoll fractions of blood of 38 volunteers (lightest-young-Y-RBCs, densest-old-O-RBCs, two middle fractions). It was found that there were striking differences between the properties of Y-RBCs and O-RBCs. The potential of Y-RBCs decreased gradually with aging. Studies in parallel on RBC fractions incubated with both positively charged quantum dots and SNA-FITC along with their potentials provide for the first time direct visual evidence about the lesser amount of charge density and NANA on O-RBCs, and a collinear decrease in their respective potentials. Close correlation was found between the surface charge on an aging RBC and its structure and functions, from the cell morphology, the membrane deformability to the intracellular Hb structure and oxidation ability. This quantitative approach not only clarifies the picture but also has implications in biology and medicine [11120].

Hematological parameters are used for monitoring health status of athletes, to prevent sports’ anemia and also for suspecting blood doping. It is important to monitor professional athletes during the training and competitive season, because some modifications of the hematological parameters are induced from long-lasting and continuous exercise, although these variations are not usually clinically relevant and fall into reference intervals described for general population. Moreover, hematological parameters are integrated in formulae used in the anti-doping context for screening the athletes. The reference intervals applied to athletes are usually those found in the general population. The erythrocyte (RBC) mass is often considered the most important parameter for evaluating the training effectiveness. The RBC mass should be related to whole body mass, inducing also gender differences evidenced by different reference intervals usually proposed for males and females. However, in athletes the gender difference on VO$_{2max}$ is principally related to the cardiac size, measured as left ventricular mass, accounting for 68 percent of the difference. The lack of correlation between BMI and Hb in the whole group of athletes indicates that in a population with consistent number of subjects the body characteristics do not apparently influence the Hb concentration. Athletes are thought to be physically normal and healthy by definition, but high training workload and psychophysical stress from competitions can modify their homeostasis, inducing apparently pathological biochemical and hematological results. In general, similar reference intervals for hematological parameters are described in athletes’ population and general population, but some differences exist. Regardless of exercise and possible related effects, the reference intervals in athletes should take into account also the anthropometrical characteristics of the subjects that can markedly differ among sports disciplines. It was pointed out the need of considering body mass index (BMI) in evaluating and interpreting serum creatinine data in athletes, when a correlation between this parameter and BMI. The relation between mass and hematological parameters has been not extensively studied. Therefore, it was investigated the relation between hematological parameters and BMI in 126 top-level male athletes: 32 rugby players of Italian National team, nine triathletes of Italian National team, 27 football players of a Italian Second Division team, 24 cyclists of a ProTour team, 21 sailors of a America’s Cup yacht, 11 basketball players of a Italian First Division team, and two ice dancers of Italian National team. The age range was 17-35 years. The BMI was calculated as weight/(height)$^2$ and supplied by teams’ physicians. The blood drawings were performed at rest in the morning before the start of the training and competitive season. An informed consent for blood drawings has been obtained. The correlation between BMI and hematological parameters in the whole group of athletes gave significant result for erythrocytes (RBC), but not for hemoglobin (Hb) and reticulocytes (Ret). The correlation between mean values of the considered parameters in groups of athletes competing in different sports and BMI, gave non-significant data for RBC and Ret, and a weak correlation for Hb. The lack of correlation between BMI and Hb and between BMI and Ret represents an important finding for the use of the parameters in antidoping context. The relative stability of the parameters in athletes, together with the absence of relation with BMI, reinforce the integration of Hb and Ret in formulae applied in antidoping context. In conclusion, the type of sport discipline and body mass characteristics can influence in part
Hb and RBC, but not Ret. Thus, the interpretation of Ret values is not influenced by anthropometrical characteristics and could be identical in different sports disciplines [07108].

The analysis of the cytosolic red blood cell (RBC) proteome is negatively affected by the high intracellular amount of hemoglobin complicating the detection of low-abundant cytosolic proteins. In this study, an alternative approach for the preparation of hemoglobin-depleted RBC lysates is presented, which was established in combination with downstream 2D PAGE analysis and Orbitrap MS. Hemoglobin removal was accomplished by using HemoVoid® depletion reagent, which enabled a very efficient enrichment of low-abundant proteins by simultaneously reducing the hemoglobin concentration of the sample. After defining selected sample preparation protocol characteristics including specificity/selectivity, precision and linearity, a 2D reference map (pH 4-7) of the cytosolic RBC proteome was generated and a total of 189 different proteins were identified. Thus, the presented approach proved to be highly suitable to prepare reproducible high-resolution 2D protein maps of the RBC cytosol and provides a helpful tool for future studies investigating disease- or storage-induced changes of the cytosolic RBC proteome [12225].

In recent years, multivariate statistical models have been validated as reliable approaches to detect recombinant human erythropoietin (r-huEPO) during current use (ON-model), as well as after recent use (OFF-model). Later research has focused only on the OFF-model, which is based on Hb concentrations and Ret%, because it has been claimed able to specifically detect recent blood doping practices, that are more likely to be discovered in unannounced anti-doping or intra-competition testing. Because of the variations in Hb and Ret%, the calculated OFF-score showed a consistent decrease from the resting value to T2 and a slight increase towards T3, however, still lower than the baseline value. From a medical point of view, despite the statistical significance of some variations, not all of them necessarily play a physiologically relevant role. Indeed, it is unlikely that the significant variations observed in WBC (12-14 %), MCV (0.2-1.0 %), MCH (0.5-1.0 %), MCHC (1.0-3.0%), and platelets (3.9-8.1 %) have any physiological importance. On the other hand, the variation in RBC, Hb, Ht, and Ret count indicated noticeable adaptive modifications in the bone marrow microenvironment in response to the elevated metabolic demand [13296].

**Effects of iron on performance**

It was investigated the iron-related haematological parameters in both male and female athletes participating in different sporting disciplines necessitating different metabolic energy demands. A total of 873 athletes (514 males and 359 females) were divided according to gender and to the predominant energy system required for participation in sport (aerobic, anaerobic or mixed) and haematological and iron-related parameters were measured. For both male and female athletes, significant differences related to the predominant energy system were found at a general level. According to the ferritin cutoff value of 35 microg/L, whole body iron and sTfr significantly differed in all three groups of male and female athletes. The percentage of hypochromic erythrocytes in male athletes was significantly higher only in those who required an anaerobic energy source, whilst in the females hypochromic erythrocytes and hemoglobin were significantly different only in anaerobic and mixed energy source athletes. According to the ferritin cutoff value of 22 microg/L, in females, whole body iron, sTfr and hypochromic erythrocytes were significantly higher in all three groups of athletes than those below the aforementioned cutoff value. It was concluded that the predominant energy system required for participation in sport affects hematological parameters. sTfr and body iron proved to be reliable parameters for monitoring the dynamics of iron metabolism and could contribute to successful iron-deficiency prevention [11121].
Testing of blood

Full blood counts are now used as evidence that athletes have used banned blood doping. This has led to legal scrutiny of the efficacy of preanalytical procedures such as specimen homogenisation. To characterise the impact of different mixing strategies on whole-blood homogeneity manual inversion, mechanical mixing and automatic mixing performed by the Sysmex XT-2000i were evaluated. Automated mixing by the instrument, or 1 min of mechanical mixing, thoroughly homogenised specimens even for tubes that had been refrigerated and left undisturbed for 36 h. Manual inversions were almost as effective, provided that specimens were first allowed to equilibrate to ambient temperatures. It was concluded that current sport guidelines that mandate at least 15 min of mechanical mixing are excessive. Except where specimens are presented to the instrument manually, mechanical mixing is redundant in the context of full blood counts [12233].

Freezing red blood cells

Red blood cells undergo structural and biochemical aging-related changes during storage, and therefore, the conventional storage of blood at 4°C is allowed for only 5 to 7 weeks depending on storage solution. Because the recovery of reinfused RBCs in the circulation is inversely proportional to their storage period, the period of storage at 4°C becomes a compromise between the recovery of reinfused RBCs after reinfusion and the in vivo resynthesis of RBCs during the storage period to compensate for the donated RBCs. Adding glycerol to RBCs minimizes the ice crystal formation in the RBCs during freezing. This allows the RBC storage for up to 10 years at −20°C or −80°C. However, a drawback with freezing blood is the additional loss of RBCs during the freeze-thaw-wash process. Different storage procedures yield different amounts of transfused hemoglobin and hence influence the sensitivity of any new test based on blood markers. It is well recognized that the reinfusion of RBCs results in considerable posttransfusion hemolysis of up to 25% within the first 24 hours after reinfusion. Therefore, a sensitivity of 35 percent would apply only during the first minutes to hoursafter reinfusion and thereafter decreases as in vivo hemolysis would occur. Using the frozen storage procedure would reduce the amount of reinfused hemoglobin and, thereby, the sensitivity [12009].

Effect of endurance training on erythrocyte deformability

Higher erythrocyte deformability may reduce the risk of circulatory diseases by enhancing oxygen delivery and reducing the load on the cardiovascular system. The effect of endurance training on erythrocyte deformability is not clear. One study explored the impact of endurance training on erythrocyte deformation and shape and investigated the underlying mechanisms of hemorheological alterations. Forty male Wistar rats were randomly divided into two groups: sedentary (S; n=21) and exercised (E; n=19). Hematological indices and erythrocyte shape were measured at the end of the 11th week. The gene expression of erythropoietin (Epo) and the Epo receptor (EpoR) was quantified using reverse transcription-PCR (RT-PCR), and Epo protein expression was analyzed using Western blotting. Endurance training significantly decreased the abnormality ratio of erythrocyte shape. The deformability indicator (DI) of red blood cells was lower in the E group than in the S group. Eleven weeks of endurance training increased Epo mRNA and protein expression in the kidney, EpoR mRNA expression in the bone marrow, and relative circulating Epo compared to the sedentary group. The decrease in the erythrocyte morphological index and the maximum of deformability indicator were associated with an increase in relative circulating Epo. In conclusion, 11 weeks of endurance training increased erythrocyte deformability. Epo and
EpoR may contribute to the decreased morphological index and deformability indicator in erythrocytes during endurance training in rats [13297].

**Hemoglobin and hematocrit**

There is limited information regarding the optimal hemoglobin level for physical activity and most studies followed relatively few participants. The object of this study was to assess iron storage levels in a population of healthy young males and their impact on physical fitness. Blood samples were drawn from 358 consenting infantry recruits for hemoglobin, iron, ferritin, transferrin, folic acid, and B₁₂ levels. A detailed medical and nutritional history was noted. Recruits performed a field fitness test including a 2,000-m run. Mean hemoglobin was 13.8 ± 1.0 g/dL. Level of hemoglobin lower than 14 and 12 g/dL were found in 54 percent and 5 percent of the recruits, respectively. Mean ferritin was 57 ± 34 ng/ml, with 15 percent of the recruits under 25 ng/ml. On multivariate analysis, after adjusting for pre-induction sports activity, intermediate pre-induction hemoglobin level (12-14 g/dL) was associated with significantly faster 2,000-m running time (than both the lower hemoglobin group (n=16) and the higher hemoglobin group (n=166). The subjects in this study were non-athletic healthy young men. The high rate of abnormally low hemoglobin and ferritin values probably indicates a nutritional deficit in this population. The slower running results in the group with hemoglobin below 12 g/dL are in line with previous work, indicating the need for iron supplementation. The decrease in running ability with increased hemoglobin above 14 g/dL is surprising and will need further evaluation [07109].

Hemoglobin (Hb) and reticulocytes (Ret) are measured as indirect markers of doping in athletes. It was studied the diurnal variation, the impact of exercise, fluid intake and ambient temperature in athletes on these parameters. Hourly venous blood samples were obtained from 36 male athletes of different disciplines (endurance (END) and non-endurance (NON-END)) over 12 h during a typical training day. Seven inactive subjects served as controls (CON). Hb and Ret were determined. A mixed model procedure was used to analyse the data. At baseline, Hb was similar for all groups, END showed lower Ret than NON-END and CON. Exercise showed a significant impact on Hb (+0.46 g/dL), the effect disappeared approximately 2 h after exercise. Hb decreased over the day by approximately 0.55 g/dL. There was no relevant effect on Ret. Fluid intake and ambient temperature had no significant effect. Hb shows significant diurnal- and exercise related variations. In an anti-doping context, most of these variations are in favour of the athlete. Blood samples taken after
exercise might therefore provide reliable results and thus be used for the longitudinal monitoring of athletes if a timeframe for the re-equilibration of vascular volumes is respected [10101].

**Regulation of red blood cell mass**

One article reviewed the regulation of production of RBCs at several levels. It was focused on the regulated expansion of burst-forming unit-erythroid erythroid progenitors by glucocorticoids and other factors that occur during chronic anemia, inflammation, and other conditions of stress. It was also highlighted the rapid production of RBCs by the coordinated regulation of terminal proliferation and differentiation of committed erythroid colony-forming unit-erythroid progenitors by external signals, such as erythropoietin and adhesion to a fibronectin matrix. It was discussed the complex intracellular networks of coordinated gene regulation by transcription factors, chromatin modifiers, and miRNAs that regulate the different stages of erythropoiesis [11458].

**Circadian rhythm**

In the fight against doping in sports, indirect detection methods using Hb and Hct are starting to play a more and more important role. The goal of this investigation was to quantify the changes in plasma volume (PV) during a 2 week study of endurance exercise simulating a cycling stage race and to test for PV changes relative to the circadian rhythm of the athlete. Fifteen endurance trained triathletes and cyclists performed a standardised 3 day taper (no exercise), followed by a 9 day cycling stage race simulation. Hb showed a mean increase of 4 percent during the 3 day taper period which was caused by a reduction in PV. During the exercise phase Hb dropped by 1.5 g/dL or 11 percent, which relates to an average expansion of PV by 0.6 l or 16 percent. The fluctuations in morning PV showed a wide inter-subject variability. The smallest change was 493 ml (10 %), which was accompanied with a drop in Hb from 14.8 g/dl to 13.7 g/dl. The largest increase was 1277 mL (or 25 %). The corresponding Hb values for this subject dropped from 15.8 g/dL to 13.1 g/dL. The Hb concentration of the morning and evening samples showed a mean difference of approximately 0.6 g/dL or 3.7 percent, which was observed both during the taper (without exercise) and stage race phases. This study showed that exercise induced changes of PV in healthy trained endurance athletes can be up to 1277 mL or 25 percent when tested at the same time of the day. This fact needs to be considered in the interpretation of blood profiles as this value might even be higher if the test was to be performed at different times of the day. In the study it was able to demonstrate that the circadian rhythm of Hb concentration remains stable even under maximal PV expansion and reaction to exercise. This finding could be implemented in the athlete biological passport model to help reduce the variation of biological markers [11459].

**Seasonal variations of hemoglobin**

The influence of training and competition workloads is crucial for evaluation of longitudinal hematological data in athletes. There are only a few papers on the variation of aematological parameters during long-lasting periods and, especially, during an entire competitive season. It was summarized that some hematological parameters can be influenced by long-term training and competition periods. Hemoglobin (Hb) and hematocrit (Ht) are decreased during the more intense periods of training, throughout the season. In different sport disciplines, the decline of Hb ranges from 3 to 8 percent during the competition season, while the range of reticulocytes (Ret%) varies from 5 to 21 percent. Reticulocytes are also decreased after long periods of training and competitions, but their variation is not necessarily associated with that
of Hb. The qualitative variations (trend of modifications) of haematological parameters are roughly independent of the sport discipline, but quantitatively (amount of modifications) dependent on sport discipline. The modifications are more evident in cycling, running, swimming than they are in football and rugby. The variations of haematological parameters within the same sport discipline are qualitatively concordant and quantitatively different among separate but consecutive competitive seasons. These findings are described in aerobic and team sports sportsmen. The definition of reliable reference ranges in sportsmen would only be possible by following the best laboratory practices. For antidoping purposes more studies investigating hematological modifications during the season are advisable [11117].

**Stability of hemoglobin under testing conditions**

With the setting up of the newly Athlete's Biological Passport antidoping programme, novel guidelines have been introduced to guarantee results beyond reproach. It was investigated in this context, the effect of storage time on the variables commonly measured for the haematological passport. It was also wanted to assess for these variables, the within and between analyzer variations. Blood samples were obtained from top level male professional cyclists (27 samples for the first part of the study and 102 for the second part) taking part to major stage races. After collection, they were transported under refrigerated conditions (2 °C <T<12 °C), delivered to the antidoping laboratory, analysed and then stored at approximately 4 °C to conduct analysis at different time points up to 72 h after delivery. A mixed-model procedure was used to determine the stability of the different variables. As expected haemoglobin concentration was not affected by storage and showed stability for at least 72 h. Under the conditions of the investigation, the reticulocytes percentage showed a much better stability than previous published data (> 48 h) and the technical comparison of the haematology analyzer demonstrated excellent results. In conclusion, the data clearly demonstrate that as long as the World Anti-Doping Agency's guidelines are followed rigorously, all blood results reach the quality level required in the antidoping context [11118].

Hematologic parameters are commonly utilized in sports medicine and antidoping testing. However, there are no universally accepted methodologies for comparing the performance of automated blood analyzer systems. To address this problem, it was selected and examined 19 studies from a review of literature published from 2000 to 2010. Meaningful discrepancies were found between measurements obtained with different analytical systems. Because harmonization and clear standardization of methods are lacking, the analytical variability often largely exceeds intra- and inter-individual biological differences, producing equivocal test results unreliable for clinical and antidoping testing. A central criticality to applying the Bayesian approach is analytical variability, but the use of different analytical technologies precludes the comparison of inter-methods for determining the robustness of blood variables and their clinical significance. Therefore, future multicenter studies are needed to compare analytical methodologies and blood analyzer systems, and to establish worldwide-accepted standards and quality control protocols [11119].

**Statistical models for blood cell survival**

The aim of one work is to compare different labelling methods that are commonly used to estimate the lifespan of red blood cells (RBCs), e.g. in anaemia of renal failure, where the effect of treatment with erythropoietin depends on the lifespan of RBCs. A previously developed model for the survival time of RBCs that accounts for plausible physiological processes of RBC destruction was used to simulate ideal random and cohort labelling methods for RBCs, as well as the flaws associated with these methods (e.g. reuse of label...
and loss of the label from the surviving RBCs). Random labelling with radioactive chromium and cohort labelling using heavy nitrogen were considered. Blood sampling times were determined for RBC survival studies using both labelling methods by applying the theory of optimal design. It was assessed whether the underlying parameter values of the model are estimable from these studies, and the precision of the parameter estimates were calculated. In theory, parameter estimation would be possible for both types of ideal labelling methods without flaws. However, flaws associated with random labelling are significant and not all parameters controlling RBC survival in the model can be estimated with good precision. In contrast, cohort labelling shows good precision in the parameter estimates even in the presence of reuse and prolonged incorporation of the label. A model based analysis of RBC survival studies is recommended in future to account for limitations in methodology as well as likely causes of RBC destruction [11460].

Preanalytical mixing of whole-blood specimens

Full blood counts are now used as evidence that athletes have used banned blood doping. This has led to legal scrutiny of the efficacy of preanalytical procedures such as specimen homogenisation. To characterise the impact of different mixing strategies on whole-blood homogeneity manual inversion, mechanical mixing and automatic mixing performed by the Sysmex XT-2000i were evaluated. Automated mixing by the instrument, or 1 min of mechanical mixing, thoroughly homogenised specimens even for tubes that had been refrigerated and left undisturbed for 36 h. Manual inversions were almost as effective, provided that specimens were first allowed to equilibrate to ambient temperatures. Current sport guidelines that mandate at least 15 min of mechanical mixing are excessive. Except where specimens are presented to the instrument manually, mechanical mixing is redundant in the context of full blood counts [11583].

Blood parameters during endurance exercise

Hematological assessment is crucial for evaluating athletes healthy status. Professional athletes experience physiological modifications during competitions and over a season: the risk of sports anaemia is high. Few descriptions of hematological parameters behaviour during a 3-weeks cycling stage race have been published. It was studied nine professional cyclists engaged in the 2011 Giro d'Italia stage race. Pre-analytical and analytical phases tightly followed academic and anti-doping authorities’ recommendations. Hematological and iron metabolism parameters were measured days -1 (pre-race), 12 and 22 during the race. Hemoglobin, red blood cells and hematocrit decreased during the race with a stabilisation in the second half, but final values were lower than baseline. Reticulocytes did not modify, whilst the immature reticulocyte fraction increased. No differences were found in red blood cells volume and corpuscular haemoglobin content, neither in iron metabolism markers. The acute phase proteins, haptoglobin and C-reactive protein, both increased over the race, while haemoglobin and haptoglobin were inversely related. These data are important for improving the knowledge of physiological modifications in hematological and iron metabolism parameters of professional athletes during highly demanding competitions [12232].

During flights

WADA's biological passport uses Hb and Ret% as markers for detection of blood doping. Athletes often have to travel long distances when they go for competition or training camps. Thus it is a legitimate discussion if the influence of travelling has a significant impact on these markers. For that reason the aim of this study was to investigate Hb and Ret% before and after a day of travelling including a flight of 8 h. Fifteen male endurance athletes
performed a 14 h journey including an 8 h flight. Hydration status, bodyweight and fluid intake was controlled. Blood was sampled in the morning and evening of day 1 and day 2 for interday comparisons and to control if intraday fluctuations are a result of travelling or circadian rhythm. Day 1. The subject’s bodyweight did not show a significant difference between the two timepoints of day 1, with 72.9 ± 5.9 kg in the morning and 72.5 ± 5.8 kg in the evening. The subjects showed a positive fluid balance of 982 ± 780 ml. Hb showed a significant decrease from 14.5 ± 0.6 g/dL to 14.0 ± 0.6 g/dL while Ret% remained unchanged (1.09 ± 0.25 % vs 1.03 ± 0.29 %). Day 2. The bodyweight of the subjects was 71.9 ± 5.8 kg in the morning and 72.9 ± 5.7 kg in the evening. The fluid balance was 407 ± 908 mL. The high SD is a sign that some of the subjects had a negative fluid balance, which was the case for four subjects. Hb showed a significant decrease from 14.9 ± 0.6 g/dL to 14.2 ± 0.5 g/dL while Ret% remained unchanged (1.03 ± 0.30 % vs 1.06 ± 0.32 %). The homeostasis of the human body regarding its fluid balance and hydration remains stable during changing environmental conditions which occur at a typical travel day including an 8 h flight. It was concluded that an 8 h flight does not change the haematological variables used in the biological passport in a bigger range than by circadian fluctuations. Thus blood samples after flights up to 8 h can be used for the interpretation in antidoping [11461].

**Total hemoglobin mass**

Training and hypoxia-associated changes in maximal oxygen uptake are mediated by different blood adaptations. Training increases blood volume because of plasma and red cell volume expansion, resulting in increased cardiac output, whereas hypoxia increases only red cell volume, leading to increased hemoglobin concentration and oxygen transport capacity. Blood doping mimics the altitude effects, however, by far exceeding its magnitude [10102].

Determination of total haemoglobin mass (Hb\text{mass}) via carbon monoxide (CO) depends critically on repeatable measurement of percent carboxyhaemoglobin (%HbCO) in blood with a hemoximeter. The main aim of one study was to determine, for an OSM3 hemoximeter, the number of replicate measures as well as the theoretical change in percent carboxyhaemoglobin required to yield a random error of analysis (Analyser Error) of ≤1 percent. Before and after inhalation of CO, nine participants provided a total of 576 blood samples that were each analysed five times for percent carboxyhaemoglobin on one of three OSM3 hemoximeters; with approximately one-third of blood samples analysed on each OSM3. The error of analysis was calculated for the first two (duplicate), first three (triplicate) and first four (quadruplicate) measures on each OSM3, as well as for all five measures (quintuplicates). Two methods of CO-rebreathing, a 2-min and 10-min procedure, were evaluated for error of analysis. For duplicate analyses of blood, the Analyser Error for the 2-min method was 3.7, 4.0 and 5.0 percent for the three OSM3s when the percent carboxyhaemoglobin increased by two above resting values. With quintuplicate analyses of blood, the corresponding errors reduced to .8, .9 and 1.0 percent for the 2-min method when the percent carboxyhaemoglobin increased by 5.5 above resting values. In summary, to minimise the error of analysis to about 1 percent or less on an OSM3 hemoximeter, researchers should make ≥5 replicates of percent carboxyhaemoglobin and the volume of CO administered should be sufficient increase percent carboxyhaemoglobin by ≥5.5 above baseline levels [10356].

The purpose of one study was to determine the reliability of total hemoglobin mass (Hb\text{mass}) measurement in the field and to establish the variability of Hb\text{mass} during a cycling race, to assess its viability as an additional antidoping detection parameter in a control-matched longitudinal study in a International Cycling Union’s (UCI) ProTour stage race in 6
professional cyclists and 5 recreationally active controls. Seventy-two Hb\text{mass} tests using the optimized carbon monoxide rebreathing method were performed over 7 consecutive days, before and throughout the tour. Fasted venous blood was obtained for measurement of hematocrit (Hct) and hemoglobin concentration [Hb] in the morning before stages 1, 3, and 6 (D1, D3, and D6). Reliability of Hb\text{mass} measurement was established using typical error calculated from 2 baseline measures. Individual change scores and coefficients of variation were used to assess stability during racing. Typical error for Hb\text{mass} was 1.3 percent (95 % confidence limits 0.9 to 2.5 %). Calculated 95 percent and 99.99 percent confidence interval for percent change in Hb\text{mass} were ± 3.6 percent and ± 7.2 percent, respectively. Mean Hb\text{mass} remained within ± 1.9 percent of baseline in cyclists and ± 0.5 percent in controls. In all cases, individual change scores for both cyclists and controls fell within the 95 percent confidence interval. There was a decrease in Hct (8.1 % ± 2.8 %) and [Hb] (9.7 % ± 3.2 %) throughout the tour in cyclists but not in controls. It was demonstrated that Hb\text{mass} can be measured reliably via CO-rebreathing during a cycling tour. Unlike concentration of hemoglobin and hematocrit, total hemoglobin mass remains stable over 6 days of racing in professional cyclists and may have potential in an antidoping context [10103].

The purpose of one study was to reveal erythropoietin doping. It was recently suggested that the assessment of total haemoglobin mass (tHb) by means of the carbon monoxide rebreathing technique should be implemented in anti-doping work. Since erythropoietin may increase the hemoglobin concentration [Hb] simply by reducing plasma volume it was injected eight human subjects with epo for 15 weeks and directly tested the feasibility hereof. Erythropoietin treatment increased [Hb] in all subjects at all time points (range 3.8-18.8 %). In approximately half the subjects this was mainly the consequence of an increased tHb, but in the remaining subjects the change was the result of a decrease in the plasma volume. After the initial epo "boosting" period the assessment of tHb could not detect epo injections in 50 percent of the subjects in the remaining "maintenance" period. In the authors’ opinion the variability observed over time when assessing tHb is not justifiable in an anti-doping setting [10104].

The aims of one study were to evaluate total hemoglobin mass (tHb\text{mass}) in international field hockey players, to examine the correlation between tHb\text{mass} and maximum oxygen uptake (VO\text{2max}); and to assess influences of iron status on tHb\text{mass} and on VO\text{2max}. The players of the German women's (n=17, aged 21-31 years) and men's (n=17, aged 19-32 years) national field hockey team were investigated. tHb\text{mass} was measured by an optimized carbon monoxide rebreathing method. The following parameters were measured in venous blood: Hemoglobin concentration (Hb\text{conc}), hematocrit (Hct), number and percentage of reticulocytes, reticulocyte hemoglobin content, serum iron, serum ferritin, serum transferrin, unsaturated iron-binding capacity, and serum soluble transferrin receptor concentration. VO\text{2max} was determined in a treadmill test. tHb\text{mass} correlated to VO\text{2max} in women and in men, whereas Hb\text{conc} and Hct did not. The investigated parameters of iron status showed no association to tHb\text{mass} or to VO\text{2max}. In conclusion, tHb\text{mass} can be used as an indicator for endurance capacity in elite field hockey players, whereas Hb\text{conc} may not. tHb\text{mass} or VO\text{2max} were not influenced by the actual iron status of the investigated athletes [10105].

To evaluate total hemoglobin mass (tHb\text{mass}) in international field hockey players; to examine the correlation between tHb\text{mass} and maximum oxygen uptake (O\text{2max}); and to assess influences of iron status on tHb\text{mass} and on O\text{2max}. The players of the German women's (n=17) and men's (n=17) national field hockey team were investigated. tHb\text{mass} was measured by an optimized carbon monoxide rebreathing method. Total hemoglobin mass correlated to O\text{2max} in women and in men, whereas hemoglobin concentration and hematocrit did not. The investigated number and percentage of reticulocytes, reticulocyte hemoglobin content, serum iron, serum ferritin, serum transferrin, unsaturated iron-binding
capacity, and serum soluble transferrin receptor concentration showed no association to tHbmass or to $O_{2\text{max}}$. In conclusion, total hemoglobin mass can be used as an indicator for endurance capacity in elite field hockey players, whereas hemoglobin concentration may not. Total hemoglobin mass or $O_{2\text{max}}$ were not influenced by the actual iron status of the investigated athletes [09150].

Total haemoglobin mass (tHb) as a direct parameter of the blood system and ultimate target of all blood transfusions has not been evaluated for its post-transfusion survival and stability. Therefore, the purpose of one study was to investigate the latter which may also be relevant from an anti-doping perspective as autologous blood transfusions remain impossible to detect. The tHb was determined by the CO rebreathing method prior to and after donation of 1 unit of whole blood, as well as prior to and after reinfusion (weekly up to 56 days) of the erythrocyte concentrate in 10 men. The mean tHb content of the derived erythrocyte concentrate was $60 \pm 3$ g, while the net tHb increases after transfusion of $51$ g ($95 \%$ confidence intervals $33-69$ g) permitted proof of an elevated tHb for at least 56 days after transfusion. It was concluded that the results show that an elevated tHb induced by autologous transfusion allowed continuous identification although, as expected, a slow decrease of tHb has been revealed in the observation period. In reference to anti-doping, CO rebreathing permits proof of a supraphysiologically elevated tHb but possibly only if a stable baseline value is known [09151].

The assessment of total hemoglobin mass (tHb-mass) with the optimized carbon monoxide-rebreathing procedure (oCOR) is discussed as a promising method to detect blood doping. The method requires repeated measurements of the carboxyhemoglobin fraction (%HbCO) using spectrophotometers (CO oximeters). In order to determine whether %HbCO measurements with different spectrophotometers yield similar tHb-masses, the results of 57 tHb-mass calculations from simultaneous %HbCO measurements with two different spectrophotometers were analyzed. For the comparison of longitudinal tHb-mass alterations (deltaHb-mass), 3 tHb-mass measurements were obtained at 6-month intervals (33-37 subjects). Apparently, the analytical variation in tHb-mass determination with oCOR increases considerably with the use of different spectrophotometers. Therefore, agreement on the use of one spectrophotometer that accurately measures low %HbCO values is needed if oCOR should be used in an anti-doping setting [11122].

Determination of total haemoglobin mass (Hbmass) via carbon monoxide (CO) depends critically on repeatable measurement of percent carboxyhaemoglobin (%HbCO) in blood with a hemoximeter. The main aim of one study was to determine, for an OSM3 hemoximeter, the number of replicate measures as well as the theoretical change in percent carboxyhaemoglobin required to yield a random error of analysis (Analyser Error) of $\leq 1$ percent. Before and after inhalation of CO, nine participants provided a total of 576 blood samples that were each analysed five times for percent carboxyhaemoglobin on one of three OSM3 hemoximeters; with approximately one-third of blood samples analysed on each OSM3. The Analyser Error was calculated for the first two (duplicate), first three (triplicate) and first four (quadruplicate) measures on each OSM3, as well as for all five measures (quintuplicates). Two methods of CO-rebreathing, a 2-min and 10-min procedure, were evaluated for Analyser Error. For duplicate analyses of blood, the Analyser Error for the 2-min method was 3.7, 4.0 and 5.0 percent for the three OSM3s when the percent carboxyhaemoglobin increased by two above resting values. With quintuplicate analyses of blood, the corresponding errors reduced to .8, .9 and 1.0% for the 2-min method when the percent carboxyhaemoglobin increased by 5.5 above resting values. In summary, to minimise the analyser error to $\leq 1\%$ on an OSM3 hemoximeter, researchers should make $\geq 5$ replicates of percent carboxyhaemoglobin and the volume of CO administered should be sufficient increase percent carboxyhaemoglobin by $\geq 5.5$ above baseline levels [11123].
An increase of hemoglobin (Hb) mass is the key target of blood doping practices to enhance performance as it is a main determinant of maximal oxygen uptake. Although detection methods exist for doping with recombinant EPO and homologous blood transfusions, autologous transfusions remain virtually undetectable. In this context, the most sensitive parameter would be a determination of Hb mass itself. The purpose of one study therefore was to establish whether Hb mass measurements by the optimized CO-rebreathing method allow screening for the withdrawal and reinfusion of autologous red blood cells. The optimized CO-rebreathing method was used for evaluation of Hb mass in two groups at three time points (duplicate measurements: baseline, after donation, and after reinfusion). Group I (n=6) was to donate and receive 1 unit of packed red cells (PRC) in contrast to two PRC in group II (n=4). The time span between withdrawal and reinfusion was 2 days. The mean Hb content of the blood units was 59 ± 4 g (group I) and 108 ± 1 g (group II). Hb mass decreased significantly after blood and increased significantly after reinfusion (group I: 70 ± 16 g; group II: 90 ± 9 g) but was still lower than at baseline. The total error of measurements for the duplicate measures ranged between 0.8 and 3.1 percent (Hb mass: 6-22 g). It was concluded that Hb mass determination with the optimized CO-rebreathing method has sufficient precision to detect the absolute differences in Hb mass induced by blood withdrawal and autologous reinfusion. Thus, it may be suited to screen for artificially induced alterations in Hb mass [07110].

Hemoglobin mass is a main determinant of maximal oxygen uptake. Blood doping aims at increasing this variable. Limits for hematocrit and hemoglobin concentration are used as indicators of blood doping. However, these variables are measures of concentration, do not represent total hemoglobin mass and are altered by vascular volumes shifts. Direct estimation of hemoglobin mass could improve blood tests. It is unknown if physical exercise alters hemoglobin mass. The purpose of one study was to investigate the reaction of hemoglobin mass and other vascular compartments to heavy exercise in athletes. Hemoglobin mass and vascular compartments were evaluated using the optimised CO rebreathing method in 7 elite cyclists during a stage race. Simultaneously, hemoglobin concentration and hematocrit were analysed. Hemoglobin mass (pre-race 958 ± 123 g, end race 948 ± 106 g) and red cell volume did not change significantly over the study period, while plasma volume and blood volume tended to increase. Hematocrit (pre-race 44.1 ± 2.5 %, end race 40.9 ± 1.59 %) and hemoglobin concentration (pre-race 15.8 ± 0.9 g/dL, end race 14.7 ± 0.7 g/dL) decreased. During the study, a plasma volume expansion as adaptation to prolonged exercise occurred. Hemoglobin concentration and aematocrit decreased accordingly, whereas hemoglobin mass remained stable. Hemoglobin mass might therefore be a suitable screening tool for blood manipulations [07111].

A high hemoglobin mass (Hbmass) is associated with a high maximum aerobic power (VO2max), however, the extent to which Hb(mass) is influenced by training is currently unclear. Accordingly, this study monitored changes in Hbmass and VO2max in 12 previously untrained adults (aged 18-25 years) following 40 days of regular physical activity. Hbmass and VO2max were assessed at the start and end of a 40-day physical activity program, which comprised of approximately 40 min of daily, moderate-intensity physical activity. Relative VO2max increased by 11 percent, yet there was no significant change in relative Hbmass (1.7 %) and body mass (0.2 %) during the 40-day period. There was a significant correlation between Hbmass and VO2max at the start of the study but not between the change in relative VO2max and the change in relative Hbmass. The results support the concept of relative stability in Hbmass with approximately 1 month of moderate-intensity physical activity suggesting that Hbmass may be used for talent identification and possibly for anti-doping purposes [12226].
Hemoglobin mass (Hbmass) is another parameter independent of plasma volume shifts. Hbmass is the total amount of circulating hemoglobin level in the blood. Because of its lower variability compared with Hb, it has been suggested as a potential candidate for the hematologic module of the Athlete Biological Passport. The hematologic module of the Athlete Biological Passport consists of longitudinal monitoring of Hb and OFF-hr and other RBC indices of blood manipulation that aims to identify suspicious values and profiles. Hbmass has been measured for more than a century by the use of carbon monoxide (CO) by the indicator dilution principle. Hemoglobin level mass would be an advantageous parameter in detecting ABT especially during longer periods of strenuous exercise, that is, during the Tour de France, where Hbmass is stable but Hb decreases because of plasma volume expansion. In a noncompetition setting, relatively stable Hb values would be considered normal, but when observed during such strenuous events such as the Tour de France, they must be considered indicative of blood transfusions. The WADA recommends in the Operating Guidelines for the Biological Passport that a value or sequence exceeding the 99.9 percent specificity level should be reviewed by a panel of experts. This implies 1 false positive for every thousand analyses. Nevertheless, individual antidoping organizations are allowed to use lower specificity levels before further scrutinization is performed [12009].

It was quantified the changes of hemoglobin mass (Hbmass) and maximum oxygen consumption (VO2max) after 22 days training at 1300-1800 m combined with nightly exposure to 3000-m simulated altitude. It was hypothesized that with simulated 3000-m altitude, an adequate beneficial dose could be as little as 10 h/24 h. Fourteen male collegiate runners were equally divided into 2 groups: altitude (ALT) and control (CON). Both groups spent 22 days at 1300-1800 m. ALT spent 10 h/night for 21 nights in simulated altitude (3000 m), and CON stayed at 1300 m. VO2max and Hbmass were measured twice before and once after the intervention. Blood was collected for assessment of percent reticulocytes (%retics), serum erythropoietin (EPO), ferritin, and soluble transferrin receptor (sTfR) concentrations. Compared with CON there was an almost certain increase in absolute VO2max (8.6 %, 90 % confidence interval 4.8 to 12.6 %) and a likely increase in absolute Hbmass (3.5 %; 90 % confidence interval 0.9 to 6.2%) at postintervention. The %retics were at least very likely higher in ALT than in CON throughout the 21 nights, and sTfR was also very likely higher in the ALT group until day 17. EPO of ALT was likely higher than that of CON on days 1 and 5 at altitude, whereas serum ferritin was likely lower in ALT than CON for most of the intervention. Thus, together the combination of the natural and simulated altitude was a sufficient total dose of hypoxia to increase both Hbmass and VO2max [13292].

One study sought to quantify the effects of reduced training, surgery and changes in body mass on haemoglobin mass (Hbmass) in athletes. Hbmass of 15 athletes (6 males, 9 females) was measured 9 ± 6 times over 162 ± 198 days, during reduced training following injury or illness. Additionally, body mass (n=15 athletes) and episodes of altitude training (n=2), iron supplementation (n=5), or surgery (n=3) were documented. Training was recorded and compared with pre-injury levels. Analysis used linear mixed models for ln(Hbmass), with Sex, Altitude, Surgery, Iron, Training and log(Body Mass) as fixed effects, and Athlete as a fixed and random effect. Reduced training and surgery led to 2.3 and 2.7 percent decreases in Hbmass, respectively. Altitude and iron increased Hbmass by 2.4) and 4.2 percent, respectively. The effect of changes in body mass on Hbmass was not statistically significant. The estimates for the effects of surgery and altitude on Hbmass should be confirmed by future research using a larger sample of athletes. These estimates could be used to inform the judgements of experts examining athlete biological passports, improving their interpretation of Hbmass perturbations, which athletes claim are related to injury, thereby protecting innocent athletes from unfair sanctioning [13293].
Within-subject variation in hemoglobin mass

Illicit autologous blood transfusion to improve performance in elite sport is currently undetectable, but the stability of longitudinal profiles of an athlete’s hemoglobin mass ($H_{\text{b mass}}$) might be used to detect such practices. The aim of one study was to quantify within-subject variation of $H_{\text{b mass}}$ in elite athletes, and the effects of potentially confounding factors such as reduced training or altitude exposure. A total of 130 athletes (43 females and 87 males) were measured for $H_{\text{b mass}}$ an average of six times during a period of approximately 1 year using carbon monoxide rebreathing. Linear mixed models were used to quantify within-subject variation of $H_{\text{b mass}}$ and its associated analytical and biological components for males and females, as well as the effects of reduced training and moderate altitude exposure in certain athletes. The maximum within-subject coefficient of variation (CV) for $H_{\text{b mass}}$ was 3.4 percent for males and 4.0 percent for females. The analytical CV was about 2.0 percent for both males and females, and the long-term biological CV, after allowing for analytical variation, was 2.8 percent for males and 3.5 percent for females. On average, self-reported reduced training resulted in a 2.8 percent decrease in $H_{\text{b mass}}$ and altitude exposure increased $H_{\text{b mass}}$ by 1.5% to 2.9%, depending on the duration and type of exposure. It was concluded that the within-subject CV for $H_{\text{b mass}}$ of about 4 percent indicates that athletes may experience changes up to about 20 percent with a 1-in-1000 probability. Changes of this magnitude for measures taken a few months apart suggest that $H_{\text{b mass}}$ has a limited capacity to detect autologous blood doping. However, changes in $H_{\text{b mass}}$ may be a useful indicator when combined with other measures of blood manipulation [12227].

Influence of exercise

It was investigated whether 12 months of chronic endurance training would affect hematology, CD4(+) lymphocyte transferrin receptor (CD71) expression, CD4(+) intracellular iron and the incidence of upper respiratory tract illnesses (URTI) in Ironman triathletes compared with untrained men. Resting venous blood samples were taken from 15 Ironman triathletes (TR) and 12 untrained men (UT) every 4 weeks for 12 months. Erythrocyte, leukocyte and platelet concentration, haematocrit, haemoglobin (Hb) and mean corpuscular haemoglobin (MCHC) were measured with a full blood count. CD4(+) lymphocytes were analysed for changes in transferrin receptor (CD71) expression (CD4(+)CD71(+)), and intracellular iron (Fe$^{3+}$), by flow cytometry. The TR group had significantly lower Hb, MCHC, and platelets for 10, 9 and 11 months, respectively; lower CD4(+)CD71(+) (3 months) and Fe(3+) (1 month), respectively; higher CD4(+)CD71(+) (1 month); a higher lymphocyte count for 4 months. There were no between-group differences in other variables. In both groups haematology and lymphocytes increased during spring, early summer and winter and decreased during late summer/late winter, with an inverse relationship between CD4(+)CD71(+) and Fe$^{3+}$. The TR group reported significantly fewer URTI than the UT. Low Hb and MCHC suggest an iron deficiency which may affect triathlete performance. Monthly changes in lymphocytes, CD4(+)CD71(+) and Fe(3+) suggested that spring, summer and late autumn are associated with CD4(+) proliferation. There may be seasonal relationships between haematology and lymphocyte function, independent of endurance training, possibly affecting performance but not the incidence of URTI [10357].

Exercise in humans augments the mobilization of circulating hematopoietic progenitor cells (CD34(+)) from the bone marrow. It was investigated the effect of inflammation on erythroid marrow activity by mobilization of erythroid progenitor cells (EPIs) along with soluble markers of erythropoiesis. Ten healthy athletes who participated in an ultradistance foot race participated in the study. Peripheral blood mononuclear cells were isolated, before (phase I),
at the end (phase II), and at 48 h post-race (phase III). EPs were detected as burst colony forming units (BFU-e) and colonies were scored at day 14. Markers of inflammation (C-reactive protein, serum amyloid-A, interleukin-6, ferritin and S100B) and bone marrow activity (erythropoietin, soluble transferrin receptor and lipocalin-2) were assessed. An approximately three-fold decrease in BFU-e number was observed at phase II. sTfR concentrations were also decreased at phase II and remained decreased at phase III. However, EPO and lipocalin-2 concentrations reached a maximum value at phase II, with a tendency to decrease at phase III. These findings indicate that exercise-induced inflammation modulates bone marrow homeostasis leading to an increase in leukocyte turnover and a decrease in erythroid compartment. It appears that lipocalin-2 is the main factor that regulates the production and mobilization of EPs [10106].

Erythropoietin (Epo) treatment increases hematocrit (Htc) and, consequently, arterial O₂ content. This in turn improves exercise performance. However, because elevated blood viscosity associated with increasing Htc levels may limit cardiac performance, it was suggested that the highest attainable Htc may not necessarily be associated with the highest attainable exercise capacity. To test the proposed hypothesis that an optimal Htc in acute and chronic Epo-treated mice exists – i.e. the hematocrit that facilitates the greatest O₂ flux during maximal exercise – hematocrit levels of wild-type mice were acutely elevated by administering novel erythropoiesis-stimulating protein (NESP; wtNESP). Furthermore, in the transgenic mouse line tg6 that reaches Htc levels of up to 0.9 because of constitutive overexpression of human Epo, the Htc was gradually reduced by application of the hemolysis-inducing compound phenylhydrazine (PHZ; tg6PHZ). Maximal cardiovascular performance was measured by using telemetry in all exercising mice. Highest maximal O₂ uptake (VO₂max) and maximal time to exhaustion at submaximal exercise intensities were reached at Htc values of 0.58 and 0.57 for wtNESP, and 0.68 and 0.66 for tg6PHZ, respectively. Rate pressure product, and thus also maximal working capacity of the heart, increased with elevated Htc values. Blood viscosity correlated with VO₂max. Apart from the confirmation of the Htc hypothesis, we conclude that tg6PHZ adapted better to varying Htc values than wtNESP because of the higher optimal Htc of tg6PHZ compared to wtNESP. Of note, blood viscosity plays a critical role in limiting exercise capacity [10107].

**Influence of injury and training on hemoglobin mass**

One study sought to quantify the effects of reduced training, surgery and changes in body mass on haemoglobin mass (Hbmass) in athletes. Hbmass of 15 athletes (6 males, 9 females) was measured 9 ± 6 (mean ± SD) times over 162 ± 198 days, during reduced training following injury or illness. Additionally, body mass (n=15 athletes) and episodes of altitude training (n=2), iron supplementation (n=5), or surgery (n=3) were documented. Training was recorded and compared with pre-injury levels. Analysis used linear mixed models for ln(Hbmass), with gender, altitude, surgery, iron, training and log(Body Mass) as fixed effects, and athlete as a fixed and random effect. Reduced training and surgery led to 2.3 and 2.7 percent decreases in Hbmass, respectively. Altitude and iron increased Hbmass by 2.4 and 4.2 percent, respectively. The effect of changes in body mass on Hbmass was not statistically significant. The estimates for the effects of surgery and altitude on Hbmass should be confirmed by future research using a larger sample of athletes. These estimates could be used to inform the judgements of experts examining athlete biological passports, improving their interpretation of Hbmass perturbations, which athletes claim are related to injury, thereby protecting innocent athletes from unfair sanctioning [13295].

**Total hemoglobin mass in endurance-trained**

To evaluate differences in total haemoglobin mass (tHb mass) and in red blood cell profile between elite endurance-trained (END) and non-endurance-trained (nEND) male and female adolescent athletes, tHb mass (CO rebreathing) and specific variables of red blood cell
profile (haemoglobin concentration, haematocrit, erythrocyte indices) were determined in 59 elite junior athletes (29 END, 30 nEND). It was hypothesized that at the age of 15-17 years, regular endurance training might induce a significant increase in tHb mass and changes in red blood cell profile. Therefore, all parameters were again determined after 6, 12 and 18 months in a subset of 27 subjects (17 END, 10 nEND). In END, tHb mass related to body weight was about 15 percent greater than in nEND whereas no significant differences were observed for the red blood cell profile. In both groups, tHb mass related to body weight and the variables of red blood cell profile had not changed significantly after 6, 12 and 18 months of regular training. In conclusion, in elite junior athletes, differences in tHb mass between END and nEND were similar, however, smaller compared with previously in adult athletes reported values. At the age of 15-17 years, 18 months of regular training did not induce significant changes in tHb mass beyond alterations explained by physical growth and also variables of red blood cell profile did not change significantly [11462].

**Influence of acute hard training**

Sensitivity of the Athlete Blood Passport for blood doping could be improved by including total haemoglobin mass (Hb_mass), but this measure may be unreliable immediately following strenuous exercise. It was examined the stability of Hb_mass following ultra-endurance triathlon (3.8 km swim, 180 km bike, 42.2 km run). Twenty-six male sub-elite triathletes, 18 racers and 8 controls, were tested for Hb_mass using CO re-breathing, twice 1-5 days apart. Racers were measured before and 1-3 h after the triathlon. Controls did no vigorous exercise on either test day. Serum haptoglobin concentration and urine haemoglobin concentration were measured to assess intravascular haemolysis. There was a 3.2 percent increase in Racers' Hb_mass from pre-race (976 g ± 14.6 %) to post-race (1 007 g ± 13.8 %), as opposed to a -0.5 percent decrease in controls (pre-race 900 g ± 13.9 %, post-race 896 g ± 12.4 %). Haptoglobin was -67 percent reduced in racers (pre-race 0.48 g/L ± 150 %, post-race 0.16 g/L ± 432 %), compared to -6 percent reduced in controls (pre-race 1.08 g/L ± 37 %, post-race 1.02 g/L ± 37 %). Decreased serum haptoglobin concentration in racers, which is suggestive of mild intravascular blood loss, was contrary to the apparent Hb_mass increase post-race. Ultra-endurance triathlon racing may confound the accuracy of post-exercise Hb_mass measures, possibly due to splenic contraction or an increased rate of CO diffusion to intramuscular myoglobin [12228].

**In detraining**

Haemoglobin mass (Hb_mass) determination using CO rebreathing may assist to detect illegal blood doping practices, however variations in Hbmass with periods of intensive training and detraining must be quantified. This study aimed to determine the effect of a 30-day period of detraining on Hb_mass in ultra-endurance triathletes. Nine male recreational triathletes (29-44 years) participated in the study. Hb_mass was assessed using CO rebreathing 30 days and 10 days before an ultra-endurance triathlon and after about 10, 20 and 30 days of detraining following the race. VO2max was assessed 10 days before the race and also after the 30-day detraining period, which consisted of an 87 percent reduction in training hours. After 30-days of detraining there was a 3.1 percent decrease in mean Hb_mass from 868 ± 99 to 840 ± 94 g, and a 4.7 percent decrease in mean VO2max from 4.83 ± 0.29 to 4.61 ± 0.41 L/min as well as a 2.8 percent increase of body mass from 75.1 ± 6.4 to 77.1 ± 6.1 kg and a 28 percent increase in skinfold total from 43.9 ± 14.2 to 55.1 ± 14.0 mm. Individual decreases in Hb_mass following detraining would need to be considered if using Hb_mass for anti-doping purposes [12229].

**A stable parameter**

A high hemoglobin mass (Hb_mass) is associated with a high maximum aerobic power (VO2max),
however, the extent to which Hb mass is influenced by training is currently unclear. Accordingly, one study monitored changes in Hb mass and VO2max in 12 previously untrained adults (aged 18-25 years) following 40 days of regular physical activity. Hb mass and VO2max were assessed at the start and end of a 40-day physical activity program, which comprised of approximately 40 min of daily, moderate-intensity physical activity. Relative VO2max increased by 11 percent, yet there was no significant change in relative Hb mass (1.7 %) and body mass (0.2 %) during the 40-day period. There was a significant correlation between Hb mass and VO2max at the start of the study, but not between the change in relative VO2max and the change in relative Hb mass. The results support the concept of relative stability in Hb mass with approximately 1 month of moderate-intensity physical activity suggesting that Hb mass may be used for talent identification and possibly for anti-doping purposes [11125].

Influence of travelling

Dehydration, fluid shifts or changes in coagulation occurring during air travel can trigger distinct reactions in the haematological system. Athletes are concerned that these effects might impair sporting performance, increase the risk of thrombosis or cause abnormalities in blood values that might be mistaken for doping in the 'Athlete's biological passport' (ABP) a longitudinal monitoring of haematological variables in antidoping. The aim of one study was to investigate key variables of the ABP before and after a long-haul flight in athletes. Fifteen endurance athletes were submitted to ABP blood samples in the morning before and after arrival of an 8 h flight. Two additional samples were obtained in the morning and the evening 3 days after the travel. Twelve nontravelling subjects served as controls. Hemoglobin concentration was higher before than after travel in athletes (+0.5 g/dL), a similar pattern was observed 3 days after the travel. No difference was observed in the control group. Reticulocyte percentage did not show any significant changes in neither of the groups. It was concluded that the observed changes are in line with normal diurnal variations. There is no indication that travel will affect haematological variables in way that might be mistaken for blood doping [12230].

Influence of erythropoietin

In healthy subjects, administration of recombinant human erythropoietin (rHuEpo) increases haemoglobin concentration not only by the well known increase in red blood cell mass but also by a decrease in plasma volume. The decrease in plasma volume, which precedes the increase in red cell mass, appears to be a rapid responding mechanism regulated by the renin-angiotensin-aldosterone system to control haematocrit. Theoretically, normal human red blood cells can persist in the circulation for approximately 17 weeks. However, neocytolysis, the selective hemolysis of young circulating red blood cells which contributes to the regulation of red cell mass, seems to appear during specific conditions that cause a rapid decrease in erythropoietin levels such as spaceflight, high altitude exposure or blood doping. Little is known about the time course of these mechanisms post rHuEpo administrations. Recombinant human erythropoietin (rHuEpo) increases haemoglobin mass (Hb(mass)) and maximal oxygen uptake (VO2max). One study defined the time course of changes in Hb(mass), VO2max as well as running time trial performance following 4 weeks of rHuEpo administration to determine whether the laboratory observations would translate into actual improvements in running performance in the field. Nineteen trained men received rHuEpo injections of 50 IU/kg body mass every two days for 4 weeks. Hb(mass) was determined weekly using the optimized carbon monoxide rebreathing method until 4 weeks after administration. VO2max and 3,000 m time trial performance were measured pre, post administration and at the end of the study. Relative to baseline, running performance significantly improved by 6 percent after administration and remained significantly enhanced
by 3 percent 4 weeks after administration, while VO₂_max was also significantly increased post administration and remained significantly increased 4 weeks after rHuEpo. Hb(mass) was significantly increased at the end of administration compared to baseline. The rate of decrease in Hb(mass) toward baseline values post rHuEpo was similar to that of the increase during administration but Hb(mass) was still significantly elevated 4 weeks after administration compared to baseline. It was concluded that running performance was improved following 4 weeks of rHuEpo and remained elevated 4 weeks after administration compared to baseline. These field performance effects coincided with rHuEpo-induced elevated VO₂_max and Hb(mass) [13294].

**Laboratory technique**

The assessment of total hemoglobin mass (tHb-mass) with the optimized carbon monoxide-rebreathing procedure (oCOR) is discussed as a promising method to detect blood doping. The method requires repeated measurements of the carboxyhemoglobin fraction (%HbCO) using spectrophotometers (CO oximeters). In order to determine whether %HbCO measurements with different spectrophotometers yield similar tHb-masses, the results of 57 tHb-mass calculations from simultaneous %HbCO measurements with two different spectrophotometers (RapidLab, OSM3) were analyzed. For the comparison of longitudinal tHb-mass alterations (delta tHb-mass), 3 tHb-mass measurements were obtained at 6-month intervals (33-37 subjects). Because of significant differences in %HbCO measurements, the limits of agreement for tHb-mass (OSM3) and tHb-mass(RapidLab) were 11 percent (95 % reference range -6.8 to +15.6 %) and the correlation of deltahHb-masses as determined with the two spectrophotometers over two time intervals was weak. In only about 70 percent of all deltahHb-mass estimations did deltahHb-mass(OSM3) and deltahHb-mass(RapidLab) show the same direction of change. Apparently, the analytical variation in tHb-mass determination with oCOR increases considerably with the use of different spectrophotometers. Therefore, agreement on the use of one spectrophotometer that accurately measures low %HbCO values is needed if oCOR should be used in an anti-doping setting [12231].

The sensitivity of the athlete blood passport to detect blood doping may be improved by the inclusion of total hemoglobin mass (Hb_mass), but the comparability of Hb_mass from different laboratories is unknown. To optimize detection sensitivity, the analytical variability associated with Hb_mass measurement must be minimized. The aim of one study was to investigate the efficacy of using quality controls to minimize the variation in Hb_mass between laboratories. Three simulated laboratories were set up in one location. Nine participants completed three carbon monoxide (CO) re-breathing tests in each laboratory. One participant completed two CO re-breathing tests in each laboratory. Simultaneously, quality controls containing Low (1-3 %) and High (8-11 %) concentrations of percent carboxyhemoglobin (%HbCO) were measured to compare hemoximeters in each laboratory. Linear mixed modeling was used to estimate the within-subject variation in Hb_mass, expressed as the coefficient of variation, and to estimate the effect of different laboratories. The analytic variation of Hb_mass was 2.4 percent when tests were conducted in different laboratories, which reduced to 1.6 percent when the model accounted for between-laboratory differences. Adjustment of Hb_mass values using quality controls achieved a comparable analytic variation of 1.7 percent. The majority of between-laboratory variation in Hb_mass originated from the difference between hemoximeters, which could be eliminated using appropriate quality controls [11124].

**Reticulocytes**

Reticulocytes (Ret) are a key variable in the emerging concept of the athlete's biological
The ratio between the amount of hemoglobin in the mature erythrocyte population and the passport and the longitudinal monitoring of biological parameters in the field of anti-doping. In this context, knowledge on the variability of Ret in athletes and the influence of exercise is necessary. The aim of one study was to evaluate longitudinal variation in Ret and the influence of short- and long-term exercise. Ret% in 793 samples of 238 athletes were determined and analyzed in different study parts for inter- and intra-individual variation and the impact of long- (competitive season) and short-term (all out) exercise. Median Ret% was 0.9 (99.5 % confidence interval 0.4 to 2.7). Intra-individual variation for Ret% was 0.0118; inter-individual variation 0.0124. During periods of intensive exercise Ret% was slightly but significantly lower (mean - 0.1 %). After a short, all-out exercise bout, Ret% was increased (+0.5 %). Athletes mostly display similar Ret% than the normal population; however, intra-individual variation in athletes is higher. During the competitive season of endurance athletes, Ret% is slightly decreased. After short bouts of intense exercise Ret% is increased [10466].

Reticulocytes are the transitional cells from erythroblasts to mature erythrocytes. Reticulocytes are present in blood for a period of 1-4 days and can be recognized by staining with supravital dyes, such as new methylene blue, or fluorescent markers, which couple residual nucleic acid molecules, a hallmark of the immature forms of erythrocytes. Although reticulocytes could be counted through a microscope (there is a standard of International Committee for Standardisation in Haematology for manual counting), this method is reported to be time consuming, inaccurate and imprecise. The integration of the reticulocyte count in automated hematology systems allowed the widespread use of these parameters, although the lack of calibration material and different markers, technologies and software used in automated systems could engender discrepancies among data obtained from different analytical systems. The importance of reticulocytes in sports medicine derives from their sensitivity, the highest among haematology parameters, in identifying the bone marrow stimulation, especially when recombinant human erythropoietin is fraudulently used. Automated systems are also able to supply information on volume, density and the haemoglobin content of reticulocytes. Some of the related parameters are also used in algorithms for identifying abnormal stimulation of bone marrow as reticulocytes haematocrit. The pre-analytical variability of reticulocytes (transportation, storage, biological variability) should be taken into account in sports medicine also. Reticulocytes remain stable for almost 24 hours at 4 degrees C from blood drawing, they are affected by transportation, and biological variability is not high in general. It could be remarked, however, that the intra-individual variability is high when compared with other haematological parameters such as haemoglobin and haematocrit. The intervals of data reported in athletes are very similar to reference intervals characterizing the general population. The reticulocyte count shows some modifications after training and during the competition season. The variability induced by exercise cannot be overlooked since the so-called haematological passport, a personal athlete's document in which haemoglobin and other parameters are registered, may be introduced by sports federations. Exposure to naturally high altitude and "living high – training low" programmes determined contentious results on reticulocytes. Simulated high altitude induced by intermittent hypobaric hypoxia does not modify reticulocytes, despite an increase in erythropoietin serum concentration. The variability among athletes competing in different sport disciplines is apparently limited. The knowledge of the behaviour of reticulocytes in training and competitions is crucial for defining their role in an antidoping control context. It is important for sport physicians and clinical pathologists to know the reticulocyte variability in the general population and in athletes, the pre-analytical warnings, the different methodologies for counting reticulocytes and the derived parameters automatically available, and, finally, the possible influence of training, competitions, type of sport and altitude [08210].

The ratio between the amount of hemoglobin in the mature erythrocyte population and the
reticulocytes (RBCHb:RetHb ratio) has previously been suggested as a marker to screen for erythropoietin-abuse. It was speculated that the reinfusion of blood would lead to a marked increase in this ratio, making it a valuable parameter in the screening for autologous blood doping. Three bags of blood (approximately 201 ± 11 g of Hb) were withdrawn from 16 males and stored at either -80 degrees C (n=8) or +4 degrees C (n=8) and reinfused 10 weeks or 4 weeks later, respectively. Seven subjects served as controls. Different erythrocyte parameters were measured on a hematological analyzer serially throughout and during a 4 week wash-out period. By using RBCHb:RetHb ratio cut-off limits of 146 (1:100) ("suspicious") and 183 (1:1000) ("positive"), 35 percent (-80 T) and 20 percent (+4 T) of all samples obtained during a 4 week wash-out period were identified as "suspicious", and 19 percent (-80 T) and 4 percent (+4 T) as "positive". In total, 7 out of 16 (44 %) subjects had at least one sample exceeding 183. Compared to the currently used indirect parameters, the RBCHb:RetHb ratio is the best indicator of autologous blood doping after reinfusion, and the parameter could be used in a testing setting, once stability validation has been performed [09153].

Automated haematological analysers still represent the gold standard for the study of reticulocyte maturation even if this technique is based on structural properties and staining affinity rather than on functional aspects. On the contrary, flow cytometry allows the simultaneous analysis of multiple cellular characteristics including functional features. The aim of one study was now to investigate whether simultaneous analysis of different reticulocyte parameters using flow cytometry may add functional information when considering their pattern of maturation. Thirty-nine healthy donors and 31 haemodialysed patients on treatment with rHuEpo were analysed. TO/CD71 scattergraph reticulocyte analysis designed a peculiar distribution which was similar among the same group of subjects (H or HDT), but different between healthy donors and haemodialysed patients. Distribution of the percentage of reticulocytes in low, medium and high boxes did not show any difference between healthy donors and haemodialysed patients groups, while the analysis using flow cytometry pointed out statistically significant differences between healthy donors and haemodialysed patients groups in the three boxes where the TO(+)/CD71(+) reticulocytes were localized. The present study suggests that TO/CD71 analysis was reproducible and could detect different pattern of maturation of a particular clinical setting [09154].

The role of reticulocytes (Ret) in sports medicine became clear when the count of immature erythrocytes was introduced in protocols used for anti-doping purposes. Because specific research regarding seasonal variations in Ret is lacking, it was assessed Ret (and [Hb]) in top-level male and female skiers during four consecutive competitive seasons. A significant difference between males and females was found for [Hb] and Ret values: [Hb] was lower and Ret was higher in females. The difference was maintained across all four competitive seasons. Marked within-subject differences in [Hb], Ret and immature reticulocyte fraction values were noted; the within-subject variability was greater than the between-subject variability in both genders. For instance, a difference for Ret was consistently shown between first and second blood drawings, i.e. between basal value, before the start of training and competition, and the value at middle of season, when training workload was at highest level. Unlike Ret%, the analysis of variance showed significant changes in [Hb] values across competitive seasons for both genders. Comparison between consecutive seasons (e.g. 2005-2006 vs 2006-2007) showed significant differences for both parameters. The behaviour of [Hb] and Ret during the various seasons was parallel in females, whereas a discrepancy existed in males. In general, inter-individual variability is quite high, thus, Ret and [Hb] modifications should be referred only to the single athlete. It was thus confirm the validity of the use of Ret counts for anti-doping purposes [10111].
Hemoglobin concentration and percent reticulocytes were analyzed in blood samples taken pre-competition, post-competition, and during out of competition testing in elite speed skaters. Percent reticulocytes during screening was not different from the values obtained post-race, and no significant gender difference was found. Mean hemoglobin concentration both in males and females was slightly but significantly higher at 1425 m altitude compared to <750 m altitude (0.23 g/dl increase in males and 0.48 g/dl increase in females). Mean percent reticulocytes at 1425 m altitude is significantly higher (0.24 % in males and 0.27 % in females, respectively) compared to blood sampled <750 m altitude. The distribution of percent reticulocytes shows 11 out of 11 500 samples with percent reticulocytes below 0.4 percent. From the 171 samples with a values >2.4 percent in 52 skaters at least two consecutive samples yielded a percent reticulocytes above 2.4 percent. In 50 individuals with generally normal values but at least in two consecutive samples values above 2.4 percent the pattern required additional testing. In conclusion, percent reticulocytes are a robust hematological parameter, including acute exercise [10112].

Automated haematological analysers still represent the gold standard for the study of reticulocyte maturation even if this technique is based on structural properties and staining affinity rather than on functional aspects. On the contrary, flow cytometry allows the simultaneous analysis of multiple cellular characteristics including functional features. Aim was to investigate whether simultaneous analysis of different reticulocyte parameters using flow cytometry may add functional information when considering their pattern of maturation. Thirty-nine healthy donors (H) and 31 haemodialysed patients on treatment with rhHuEpo (HDT) were analysed. Reticulocyte counts and their stages of maturation were studied both with ADVIA 2120 and by flow cytometry. TO/CD71 scattergraph reticulocyte analysis designed a peculiar distribution which was similar among the same group of subjects (H or HDT), but different between H and HDT. Distribution of the percentage of reticulocytes in low, medium and high boxes calculated by ADVIA 2120 did not show any difference between H and HDT groups, while the analysis using flow cytometry pointed out statistically significant differences between H and HDT groups in the three boxes where the TO+/CD71+ reticulocytes were localized. The present study suggests that TO/CD71 analysis was reproducible and could detect different pattern of maturation of a particular clinical setting [10113].

Reticulocytes (Ret) are a key variable in the emerging concept of the athlete's biological passport and the longitudinal monitoring of biological parameters in the field of anti-doping. In this context, knowledge on the variability of Ret in athletes and the influence of exercise is necessary. The aim of one study was to evaluate longitudinal variation in Ret and the influence of short- and long-term exercise. Reticulocytes in 793 samples of 238 athletes were determined and analyzed in different study parts for inter- and intra-individual variation and the impact of long- (competitive season) and short-term (all out) exercise. Median percentage of reticulocytes was 0.9 (95 % confidence interval 0.4 to 2.7). Intra-individual variation for Ret% was 0.0118; inter-individual variation 0.0124. During periods of intensive exercise Ret% was slightly lower (mean - 0.1 %). After a short, all-out exercise bout, Ret% was increased (+0.5 %). Athletes mostly display similar Ret% than the normal population; however, intra-individual variation in athletes is higher. During the competitive season of endurance athletes, Ret% is slightly decreased. After short bouts of intense exercise Ret% is increased. These data can be used for the interpretation of blood profiles in athletes [10355].

The Sysmex R-500 (R-500) Hematology Analyzer is a bench-top system appropriate for the analysis of limited batches of blood samples. The R-500 provides percentage proportional (RET%), absolute reticulocyte (RET#), and absolute red blood cell (RBC#) counts. The system was validated, according to the International Committee for Standardization in Hematology, International Standards Organization (ISO/IEC) 17025, and World Antidoping
Agency (WADA) specifications. The instrument calibration was performed according to the manufacturer and validation parameters comprised linearity, precision, uncertainty (intermediate and long-term precision), comparability, effect of drift, carryover, stability, and accuracy. The linearity and the comparability studies for RET#, RET%, and RBC# were expressed in regression factors (R2) and coefficients of correlation, respectively. For the precision studies, the coefficients of variation for RET#, RET%, and RBC# were 9.5 percent, 9.8 percent, and <1.5 percent, respectively. For the intermediate precision studies, the coefficients of variation for RET#, RET%, and RBC# were 3.1 percent, 3.6 percent, and 0.6 percent, respectively. Carryover was found to be negligible. Sample stability was demonstrated at both room temperature and at 4 degrees C over a 24-hour period. Comparability studies for the R-500 were performed using a Sysmex SE-9500. The total evaluation led to the conclusion that the R-500 is an accurate and precise analyzer and because of to its relatively limited size, it can be considered a portable instrument, capable to be used in sports competition and training sites, where doping control and health tests are conducted. The analytical methodology of RET% measurement by the R-500 has been incorporated into the Doping Control Laboratory of Athens' Scope of Accreditation according to the ISO/IEC 17025 and WADA specifications [07112].

The role of reticulocytes (Ret) in sports medicine became important when the count of immature erythrocytes has been introduced in protocols used and officially approved for anti-doping purposes. The use of modern automated analysers, which allow the easy count and the description of characteristics of reticulocytes, increased the possible use of these parameters in sports medicine. It was studied the behavior of Ret and immature reticulocyte fraction (IRF) in top-level athletes practising rugby, ski, soccer and cycling, throughout a competitive season. It was aimed at increasing the knowledge of physiological characteristics of these sportsmen and supplying valuable suggestions to trainers and sports physicians. It was observed a stability of Ret counts, also during training and competitions, although some modifications, namely decrease during competitions periods in cyclists, and in rugby and soccer players, occurred. No significant correlation was found between Ret count and Hb in each sport discipline. IRF values tend to be high in athletes owing to continuous bone marrow stimulation linked to haemolysis, typical of sports activities. It was confirmed the validity of the use of Ret counts for antidoping purposes and also for evaluating health status and iron metabolism of sportsmen [07113].

The percentage of reticulocytes has been investigated such as the injection of the granulocyte colony-stimulating factor (G-CSF), for which anecdotal evidence existed as to its abuse in elite sport. In a controlled study, the repeated administration of therapeutic dosages of G-CSF (10 microg/kg/day) over a period of 5 days resulted in a statistically significant increase of %reticulocytes while all volume-dependent parameters such as red blood cell count, haematocrit and haemoglobin were found decreased [13009].

Reticulocytes (Ret), immature precursors of erythrocytes, contain nucleic acid (RNA) residues, are larger in volume than mature red blood cells (RBCs) but have a lower hemoglobin (Hb) concentration. All enucleated RBCs with two blue-stained subparticles are counted as Ret and related to 1000 erythrocytes in the same field. Automatic counting methods rely on staining the particles with classical dyes (new methylene blue, brilliant cresyl blue, or similar) or with fluorophores (acridine orange) which bind the RNA residues, making them recognizable and numerable. Automation combines simultaneous enumeration of stained particles and recording of particle size with the advantage of high precision, since a high number of events are collected for each count and better throughput [13009].

Produced by thered bone marrow from mature erythroblasts that undergo enucleation, they still retain RNAresidues. After a brief period in the bone marrow, they are released into the
blood stream where they remain for up to 4 days until they mature into erythrocytes. Their lifetime is much shorter than that of mature RBCs, which have a mean lifetime of 120 days. While circulating in the bloodstream, Ret undergo several rapid changes as they mature: membrane remodeling, volume change, and extrusion of organelles. Reticulocytes develop from erythroblasts and circulate in the bloodstream for about 1-4 days before maturing into erythrocytes. With the introduction of reticulocyte count in equations and statistical models for detecting suspected blood doping, its application to sports medicine has attracted growing interest in reticulocyte behavior during training and competition seasons in athletes and experimental blood doping treatment in healthy volunteers. An update on recent publications is therefore needed to improve the interpretation of reticulocyte analysis and its variability in sportsmen. Reticulocyte count constitutes a robust parameter during the preanalytical phase, but cell stability can be assured only if blood samples are kept at constantly cold temperatures (4 degrees C) and test results will differ depending on the blood analyzer system used. Marked intraindividual variability is the principal finding to be evaluated when exercise-induced changes are observed or illicit procedures suspected. Furthermore, reticulocyte variability is greater than that of other hematological parameters such as hemoglobin or hematocrit. Ideally, any variation should be interpreted against long-term time series for the individual athlete: values obtained from large athlete cohorts ought to be used only for extrapolating outliers that deserve further examination. Reticulocyte distribution in athletes is similar to that found in the general population, and a gender effect in some sports disciplines or selected athlete groups may be seen. Reticulocyte variability is strongly influenced by seasonal factors linked to training and competition schedules and by the type of sports discipline. Published experimental data have confirmed the high sensitivity of reticulocyte analysis in identifying abnormal bone marrow stimulation by either erythropoietin administration or blood withdrawal and reinfusion [13298].

Extended intervals between sample collection and analyses render athlete's whole-blood specimens collected in the field for antidoping purposes susceptible to storage degradation. The aim of this study was to characterize the stability of key blood variables under different storage durations and temperatures. It was evaluated stability of full blood count indices (plus reticulocytes) in individual tubes left undisturbed during 36, 48, 72, 96, 120, 144 and 168 h of storage at approximately 4, 6 and 12°C. Samples were measured on a Sysmex XT-2000i instrument. The two key variables in the context of antidoping (haemoglobin concentration, reticulocytes) were stable for at least 168 h, except under 12°C (stable 48 h only). Volume-dependent variables changed in a predictable manner that enabled a nomogram to be generated to predict original values provided storage duration and temperature were known. It was concluded that key blood results can be relied upon for at least 7 days if storage temperature is kept at 4-6°C [13299].

Sports anemia

Reticulocyte evaluation in sportsmen is important because of its usefulness in monitoring athletes’ health, as, for example, in detecting sports anemia. Professional athletes are particularly prone to developing this condition in which Hb loss is accompanied by an increase in Ret count. Sport-induced anemia is associated with various causes, but erythrocyte destruction due to intravascular hemolysis is the major one responsible for continuous stimulation of bone marrow which can be monitored by Ret count. Sports anemia is evidenced by marked changes in blood cell count and iron-associated parameters during periods of intense training or, even more accentuated, at the start of the training season, and it is frequently encountered in endurance athletes. It is a transient condition, with only 8 percent of elite athletes presenting frank anemia (Hb concentration below reference limits: 135 g/L in males and 120 g/L in females). Training induces an increase in plasma volume
which, through hormonal (e.g., Epo) and osmotic responses, stimulates erythropoiesis, inducing augmentation of the red cell mass, including RBC and immature erythroid forms (e.g., reticulocytes). While the plasma volume may rise by over 20 percent, the increase in red cell mass will range between 10 and 18 percent, with a final relative decrease in hematocrit. The advantages of these changes are that plasma expansion decreases blood viscosity and improves blood flow in large vessels, while the greater deformability of newly formed erythrocytes increases capillary flow. It is unlikely, however, that such exercise-induced hematological variations will be exclusively due to hemodilution. For instance, microcytosis in endurance athletes is secondary to reactive reticulocytosis due to intravascular hemolysis. Hemolysis may result from the breakdown of erythrocytes in exercising muscles (swimmers, weightlifters, rowers) or from the impact with the ground (runners). Within this context, it is useful for sports physicians and clinical pathologists to understand the effect various conditions (type of sport and training, competition schedules, altitude) can have on Ret values. Ret measurement in sports medicine attracted attention after Ret count began being applied for antidoping purposes [13298].

**Influence of erythropoietin**

It has been reported the effect of rhEpo administration in healthy subjects on the Ret values to highlight the physiological response to hormone stimulation. In detail, bolus rhEpo injections (150 U or 300 U/kg body weight) increase the immature reticulocyte fraction (IRF) starting 36 h after a single dose of rhEpo, peak after 3-4 days, and normalize within 7 days. rhEpo transiently increases the lifespan of circulating Ret from a baseline value of 1.7-3.4 days and increases Ret values twofold by inducing increased Ret release from the bone marrow and prolonging the maturation time of circulating Ret. After frequent weekly injections for 14 days and a concomitant doubling in reticulocyte percentage (Ret%), Ret% returned to basal levels even with weekly rhEpo injections and continuously high, suggesting a decreased sensitivity to prolonged rhEpo treatment [13298].

**Blood drawing**

Accuracy and rigorous standardization of blood collection techniques are needed to avoid spurious changes in hematological parameters and of Ret in particular. According to a study on 36 athletes, the use of different sampling techniques (cannula or syringe) had no significant impact on Ret% measurement when carried out over a day, during different days of a year with equal timing of sampling days to preclude the effect of climatic extremes, nor did the environmental temperature modify the Ret% values [13298].

**Measurement**

Reticulocytes are the most sensitive index available to authorities who seek to sanction athletes for blood doping based on deviations beyond individual reference ranges. Because such data comprise longitudinal results that are generated by different laboratories, the comparability of reticulocyte counts from different instruments is of crucial importance. To enhance between-instrument comparability of reticulocyte counts reported by the Sysmex XT-2000i automated haematology analyser it was optimised recalibration of instruments towards assigned values of control material (e-CHECK) in tandem with fresh blood verification. In terms of reticulocyte counts reported as a percentage of all cells in a fresh blood sample, it was possible to recalibrate all three test instruments so that the mean of 10 samples was within 0.1 percent of the comparative instrument’s mean value. This approach provides a straightforward means of reducing between-instrument differences in reticulocyte counts generated by the Sysmex XT-2000i [13300].
**Diurnal variation**

Reticulocytes count shows a physiological circadian rhythm with an acrophase occurring at 01:00 a.m. (95% confidence interval between 07:48 p.m. and 04:28 a.m.), reflecting the diurnal variations in serum erythropoietin that peaks at 01:00 a.m. These variations in circulating Ret constitute 37 percent of the total variability encountered over a day. The intraindividual daily range is 130 percent, with the highest value expressed as a percent of the lowest. The sleep/wake cycle is responsible for synchronizing the body’s biological clock, and resetting it can take up to 42 days. After intercontinental flights across time zones, athletes involved in competitions worldwide often experience circadian rhythm disruption [13298].

**Biological variability**

Ashenden reported three different values for analytical variation with regard to reticulocytes: as 9.1, 7.6, and 5.9 percent for low, medium, and high Ret%. Intraindividual variability was found to be greater than interindividual variability in both genders among top-level ski athletes. The within-subject variance for Ret in 793 samples obtained from 238 athletes was 0.0118, the between-subject variance was 0.0124. The intraindividual CV calculated on 500 samples from 43 subjects was 8.37 ± 1.64 percent in endurance athletes, 8.08 ± 2.27 percent in nonendurance athletes, and 7.4 ± 1.12 percent in nonathletes. These values suggest an enhanced stimulation of bone marrow in the athletes, although the differences among the groups were not significant. By comparison, the coefficient of variation of Hb in the same individuals was 2.65 ± 0.67, 3.0 ± 0.79, and 2.36 ± 0.42 percent, respectively. High intraindividual variability among elite athletes was reported in a large group of speed skaters, especially on precompetition screening when very high values for some athletes (>3.0 %) were measured. Thus Ret intraindividual variability is higher in athletes than in nonathletes. Moreover, Ret variations should be interpreted against long-term time series in the individual athlete; values obtained from large athlete cohorts should only be used for extrapolating outliers which deserve further examination [13298].

**Kinetics of reticulocytes production in humans**

RBCs are produced in the bone marrow; their formation involves a vast variety and number of cells at different stages of maturation, starting with the first stem cell progeny committed to erythroid differentiation and ending with the mature circulating RBC. Under normal conditions, the rate of RBC production is such that the red cell mass in the body is regulated and constant. It takes approximately from 12 to 15 days for a cell at the burst-forming unit-erythroid stage to mature into an erythroblast. Within 6-8 days, a cell in this stage proliferates and differentiates into a colony-forming unit-erythroid, which needs another 5-7 days to proliferate and develop into basophilic erythroblasts after the nucleus has been extruded, the cell is referred to as a Ret, an immature RBC larger in volume than erythrocytes by about 24 percent, with a lower Hb concentration (about 17 %) and a similar Hb content. Ret lifetime is much shorter than that of erythrocytes (1-4 vs 120 days) [13298].

**Reported reticulocyte values in athletes**

Recent studies have confirmed that reticulocytes are not normally distributed. The data were recorded in athletes before the competition season. The differences in Ret values could have been due to different training workloads. It should be remarked that the results are similar for different groups of athletes (endurance and anaerobic athletes), when presented as median
and percentiles (25th–75th). Conversely, different results were recorded in athletes practicing the same discipline (triathlon) probably due to different workloads. Elite speed skaters showed values clearly higher than those measured in athletes practicing other disciplines: a specific cluster could be recognized, but the particular methodology could also be cited to explain this difference. Ret values are not influenced by body mass index (BMI) of athletes. In a study on a heterogeneous group of male professional athletes (n=126) practicing different sports and characterized by a wide range in BMI (22-28 kg/m²), no correlation was found. BMI partially influenced Hb and erythrocyte values, reinforcing the robustness of Ret as a parameter for evaluating the hematological status in athletes. From an analysis of studies investigating seasonal variability of hematological parameters in athletes, it appears that variations in Ret and Hb are unrelated. However, there are no apparent differences between athletes and sedentary people when athletes are at rest. Differences eventually emerge for small groups of athletes during the competitive season, and controls as endurance athletes could have higher or lower values than the general population, depending on the phase of training [13298].

**Gender effects on reticulocytes**

Gender could be a source of variation in Ret distribution. Higher Ret values were described for female Alpine ski athletes during four consecutive seasons. The differences between genders were confirmed in all seasons; however, the trends of Ret changes within each season and between consecutive seasons ran parallel in both genders. Differences in Ret% between genders and a high between-subject variability were observed. Within gender analysis revealed that although the values remained stable in the males (no period or season-related effect), differences between periods within seasons were noted in the females. In contrast, no gender-related influence on reticulocytes distribution was observed in endurance athletes. In a large group (n=873) of athletes practicing different sports disciplines, the Ret values were higher for the females. Thus a gender effect may be observed in some sports disciplines or selected groups of athletes [13298].

**Stability of reticulocytes**

Instability is defined as an absolute difference, quotient, or percentage deviation from results obtained from measurement at time 0 and after a given period of time. It has been found that reticulocytes are less stable than Hb, but stability depends on the counting method applied. Storage at cold temperatures (ideally 4°C) is essential to guarantee the stability of Ret values [13298].

**Effects of exercise on reticulocytes**

Ret% increased after short-term exhaustive exercise as demonstrated in 23 Caucasian endurance athletes (19 males, 4 females; age range, 18-56 years). Blood samples were drawn immediately before and within 10 min after the end of a standardized incremental test until exhaustion on a treadmill or cycling ergometer (duration, 30-45 min). The increase in Ret was low (mean, 0.05 %) but evident in nearly all subjects. Thus, depending on its intensity, acute exercise can modify Ret count. However, exercise intensity is a key factor for determining Ret changes: no increase was observed by the same authors over a day in 36 male athletes, 20 endurance (cycling, triathlon), and 16 nonendurance (ball disciplines) mainly training at moderate intensity. In professional triathletes out-of-competition observed over a period of 63 days, a significant decrease in Ret% was found only at the end of the study (between day 49 and days 56 and 63), while no change in MCV was noted. A decrease in Ret after long periods of training and competition was observed, but its variation
was not necessarily associated with that of Hb. In a review article, the authors also compared the differences in Ret-related parameters between athletes practicing different sports at different levels and controls as measured on different automated systems. Generally, no variations in all Ret-related parameters (Chr, RetHb, and MCVr) were found between the athletes and the controls. Thus, training and competitions influence Ret values during a season; Ret decrease can be seen during the more intense phase of the season, but this effect is not always evident. The differences between consecutive seasons are greater than within season differences for Ret in the same group of athletes; the differences are gender specific. Average Ret values decrease in the past decade in elite athletes. Ret changes during a season are not always parallel to changes in Hb. Qualitative variations in Ret are mainly independent of the sports discipline, but quantitatively dependent on the sports discipline. Ret is influenced by exposure to altitude, level of altitude, and especially the duration of exposure to hypoxia are crucial for determining Ret changes [13298].

Reticulocytes in doping

Ret is a crucial parameter for suspecting and discovering the illicit use of substances and/or procedures which can enhance oxygen transfer to muscles. The use of Ret for detecting suspected recombinant erythropoietin (rHuEpo) abuse has been widely described. A study on the use of different rHuEpo treatment schemes involved 24 healthy male volunteers divided into three groups of eight subjects each. Two groups received rHuEpo injections (65 U/kg) for a 4-week period (2 weeks “boosting” phase plus 2 weeks “maintenance”). During the boosting phase, the subjects were injected with the hormone every second day (4 injections/week) and during the maintenance period, they had one injection per week. The injection period was followed by a 3-week washout period. The third group received rHuEpo (60 U/kg) for 10 weeks, with 3 weeks of boosting (the frequency of injections was the same as for the other two groups), followed by 7 weeks of maintenance, with one injection per week and 1 week of washout. Ret increased in all treated subjects during the boosting period; the increase was more pronounced in the two groups treated with 2 weeks of boosting and 2 weeks of maintenance. Ret values were higher than the highest cutoff (2.4%) in 8 out of 24 subjects during the boosting period, 4 from the groups treated for 2 weeks, and 4 from the group treated for 3 weeks. During the maintenance period, only one subject, treated for 2 weeks, had Ret >2.4 percent. No subjects had Ret <0.2 percent during the entire observation period. Ret was the most sensitive parameter for discovering the rHuEpo treatment. Blood manipulation can also be performed by expanding and diluting plasma to mask increased enhancement of Hb and erythrocytes. In a study the testing of desmopressin-induced hemodilution in eight physically active males was studied. After desmopressin treatment, a significant decrease in Hkr, Hb, and OFFhr was found. The mean Ret value before and after desmopressin treatment was 0.8 ± 0.2 and 0.6 ± 0.2 percent, respectively. Desmopressin abuse is an effective method for diluting blood. Ret behavior during blood manipulation by withdrawal and reinfusion (autotransfusion) has been recently investigated in studies involving adults or athletes to define variations in hematological parameters when high quantities of blood are drawn, stored, and then reinfused in healthy subjects. In a study involving seven male triathletes for a 63-day period, from day 1 to day 21, the training workload was progressively increased up to a regular weekly training of 5 h. No significant differences between consecutive measurements of Ret and MRV were found; the final values at 5 and 12 days after reinfusion differed significantly from the value measured after blood withdrawal. The authors stated that some absolute variations in hematological parameters in a period of 15 days could be used for detecting suspected blood manipulation: 6 percent for Hkr%, 4 percent for Hb, and 20 percent for OFFhr. These values, however, are lower than possible changes induced by training and competitions. Ret remains constant during blood withdrawal. Its decrease during recovery and after reinfusion is more informative. However, if the amount of blood withdrawn is high, that is, three blood bags over
a period of 2 weeks or two blood bags in 1 week, Ret will increase due to the natural stimulation of bone marrow to release new erythrocytes. This increase is followed by a decrease, with a normalization of values during the recovery period, and then an additional decrease after reinfusion which inhibits bone marrow activity. In conclusion, Ret is a valid parameter for detecting suspected blood doping as Ret is a specific and sensitive parameter for detecting suspected rhHuEpo abuse. Ret is also a sensitive parameter for detecting suspected blood manipulation by transfusion during the recovery phase after withdrawal and after reinfusion. Moreover, Ret changes during blood manipulation by transfusion depend on the quantity of blood drawn and reinfused and the time of testing [13298].

**Neocytolyis**

Neocytolyis, the selective hemolysis of young circulating red blood cells (RBCs), contributes to the physiologic control of red cell mass and to pathophysiologic phenomena such as anemia of renal disease, anemia after spaceflight, and blood doping by athletes. Progress in understanding the process is hampered by the lack of established markers to distinguish young from older RBC. Twelve potentially informative RBC surface markers were assayed by flow cytometry in normal blood samples, and 4 were preferentially expressed in young RBC. To create a model of neocytolyis, 3 normal volunteers had recombinant human erythropoietin (rhEpo) administered until mild erythrocytosis occurred, then were studied upon rhEpo withdrawal. Neocytolyis ensued that most evident from a rapid rise in serum ferritin as the iron from young RBC was transferred back to stores. Five additional volunteers had surface markers monitored during and after rhEpo administration. Three subjects with marginal baseline iron stores had blunted response to rhEpo, no significant neocytolyis, and no change in RBC surface marker expression. Two subjects with adequate baseline iron stores developed erythrocytosis followed by neocytolyis. Decreased expression of CD44 (homing-associated cell adhesion molecule) and CD71 (transferrin receptor) seemed to correlate best with neocytolyis; CD35 (complement receptor) less so. Of note, further studies are needed to determine if these changes are causative of red cell destruction. Thus, this study begins to establish a human model of neocytolyis, to establish markers differentiating young and old RBC, and to establish a basis for better definition of the process. Although the study is preliminary, the results support the possibility that flow could be useful to detect blood doping because neocytolyis should predictably occur in athletes who surreptitiously blood dope [09152].

The phenomenon of neocytolyis regulates sudden changes in erythropoietin concentrations in the body, inducing apoptosis of young RBCs. The reintroduction of old, but still efficient RBCs, cannot substantially influence the downregulation of RBC mass when it is excessive. Hence, neocytolyis can explain some of the potential pitfalls of autologous transfusion when the hemoglobin increase is lower than expected and serum bilirubin concomitantly increases. This may be due not to improper storage of the blood, but rather to excessive introduction of RBCs, stimulating a downregulation of erythrocyte release [06005].

**Polycytemia**

Polycytemia is defined by the increase of hematocrit and haemoglobin respectively. Possible causes might be neoplastic diseases like polycythemia vera with proliferation of a cell clone. More often one will find reactive forms resulting from chronic hypoxemia. A physiologic form of polycythemia can be found in highlanders and athletes training at high altitude. With increasing frequency erythropoietin and it's analoga are being used as doping substances to induce Polycytemia. Red cell proliferation induced by chronic hypoxemia is
the most common form in patients. In this instance the lung itself can be the cause (hypoxemia with hypocapnea in blood gas analysis) or hypoventilation caused by an insufficient respiratory pump (hypercapnea with hypoxemia in blood gas analysis) induces a compensatory Polycythemia. The former form can be treated with long term oxygen therapy and the latter by non-invasive ventilation, either approach corrects hypoxemia and reduces Polycythemia within some weeks [10470].

**Blood conservation and transfusions**

In 1628 the English physician William Harvey described the blood circulatory system. Shortly afterward, the first reported blood transfusion was attempted. Since then, substantial improvements have been made in the techniques employed for blood transfusion. Blood transfusions were originally used to support critically ill patients with severe forms of acute and chronic anemia. However, recent advances in biotechnology have allowed the separation of whole blood in its components. Because patients seldom require all of the components of whole blood, it makes sense to transfuse only that portion needed for a specific condition or disease. This treatment is conventionally referred to as “blood component therapy.” Typically, up to four components may be derived from 1 unit of blood: red blood cells (RBCs), platelets, plasma, and cryoprecipitated antihemophilic factor (AHF). RBCs may be stored under refrigeration for a maximum of 42 days, or they may be frozen for up to 10 years. Platelets must be stored at room temperature and may be kept for a maximum of five days. Fresh frozen plasma, mainly used for the therapy of acquired and congenital bleeding disorders, is stored frozen for usually up to one year. Cryoprecipitated AHF, which contains one or more specific clotting factors, is made from fresh frozen plasma and may be stored frozen for up to 1 year. Additional products manufactured from whole blood include albumin, immune globulin, specific immune globulins, and clotting factor concentrates. Blood transfusions can be traditionally classified as autologous, where the blood donor and transfusion recipient are the same, or as allogeneic, where the blood is transfused into someone other than the donor. The most common autologous donation is the preoperative donation of blood for possible re-transfusion up to six weeks before or following elective surgery. As a significant amount of iron is removed by each autologous donation, an adequate time for recovery of not less than 72 h from the last donation, and appropriate iron supplements, are usually required for patients undergoing autologous donations. An important step in ensuring the safety of allogeneic transfusions is the screening of donated blood for infectious diseases. Today, nine tests for infectious diseases are traditionally performed. Hepatitis B (HBV) and syphilis tests were in place before 1985. Since then, tests for human immunodeficiency virus (HIV-1 and HIV-2), human T-lymphotropic virus (HTLV-I and -II), and the hepatitis C virus (HCV) have been introduced. Current evidence suggests that blood transfusions are unlikely to be beneficial in the absence of active blood loss when the hemoglobin concentration exceeds 100 g/L (hematocrit > 30 %). The benefits arising from blood transfusions may exceed the risks when the hemoglobin concentration falls to 70 g/L (hematocrit < 21 %). Therefore, the majority of existing guidelines conclude that transfusion is rarely indicated when the hemoglobin concentration is greater than 100 g/l, and it is almost always indicated when it falls below a threshold of 60 g/L in healthy, stable patients or likely a higher threshold in older or sicker patients [10353].

Blood transfusion is an effective and unmediated means of increasing the number of red blood cells in the circulation in order to enhance athletic performance. Blood transfusion became popular in the 1970s among elite endurance athletes and declined at the end of the 1980s with the introduction of recombinant erythropoietin. The successive implementation in 2001 of a direct test to detect exogenous erythropoietin and in 2004 of a test to detect
allogeneic blood transfusion forced cheating athletes to reinfuse fully immunologically compatible blood. The implementation of indirect markers of blood doping stored in an Athlete's Biological Passport provides a powerful means to deter any form of blood transfusion [10109].

It was observed earlier that, in endurance sports such as cycling, triathlon, cross-country skiing, and marathon running, ways of boosting the blood's oxygen-carrying capacity can enhance performance by over 20 percent. The first clear evidence of blood doping through blood transfusion came from a controlled experiment carried out in 1947. Since then, transfusions have long been used for this purpose, as they are an extremely straightforward, simple, and effective method of increasing the oxygen carrying capacity of blood. There are two methods of doping through blood transfusions: autologous and allogeneic; for convenience and safety, the former is reportedly much more used. The traditional procedure of autologous blood transfusion begins by the withdrawal of 1 to 4 units of blood (1 unit = 450 ml of blood) several weeks before competition. The blood is centrifuged, the plasma components are immediately reinfused, and the corpuscular elements, principally RBCs, are stored refrigerated at 4°C or frozen at −80°C. As blood stored by refrigeration displays a steady decline in the number of RBCs, a substantial percentage, up to 40 percent, of the stored RBCs may not be viable. The freezing process, conversely, limits the aging of the cells, allowing the storage of the blood for up to 10 years with a 10-15 percent loss of RBCs. Stored RBCs are then reinfused, usually 1 to 7 days before a high-endurance event. When properly performed, this process increases hemoglobin and hematocrit levels by up to 20 percent, but it is not completely safe and free from side effects. A large infusion of RBCs may be associated with hyperviscosity syndrome, which is characterized by increased blood viscosity and decreased cardiac output and blood flow velocity and results in reduction of peripheral oxygen delivery. Additional complications may be phlebitis, septicemia, bacterial infection, and air/clot embolism. In addition, allogeneic transfusions may trigger transfusion reactions characterized by fever, urticaria, and anaphylactic shock and are significantly associated with transmission of blood-borne infectious diseases including hepatitis, acquired immunodeficiency syndrome, malaria, cytomegalovirus, and Creutzfeldt-Jacob disease. There are anecdotal reports of athletes (the 1984 United States Olympic cycling team and the Spanish cyclist Manzano among others) experiencing severe side effects from blood transfusions, and some of them nearly died after being injected with poorly stored blood. Earlier studies have shown that a greater than 5% increase in circulating hemoglobin is necessary to improve performance, suggesting that athletes would need to infuse at least 1 unit of blood to obtain a surreptitious athletic advantage [10353].

For the last decade, the only way to test for blood doping by allogeneic transfusion has been to assess the hematocrit or hemoglobin threshold; several sport federations banned athletes if random tests gave values over an arbitrary limit. Recently, a more reliable approach to the detection of blood transfusion in athletes has been proposed. The method is based on the quantification of antigenically distinct donor and recipient RBCs by flow cytometry, through the use of standard blood bank antisera in combination with a fluorescent-labeled secondary antibody directed against human immunoglobulin. This strategy allows detection of even a single unit of blood transfused, provided that there is at least one antigen mismatch between donor and recipient. As individual RBCs display an almost unique antigen pattern, which is under strict genetic regulation, the possibility of two blood samples matched for ABO and Rh factor being identical for the panel of 12 blood group antigens tested is less than 1:500. The test currently involves RBC phenotyping for the antigens C, c, E, e, K, k, Fya, Fyb, Jka, Jkb, S, and s. Therefore, flow cytometry appears to be the ideal technique for detecting allogeneic transfusion with a high degree of analytical efficiency. However, this test has some limitations. First, it does not detect autologous transfusion with blood donated and stored beforehand, as the RBCs surface antigens of the donor and recipient are identical. An
additional problem is the lack of an easy confirmatory test, such as DNA analysis. A final concern arises from persons who are blood group chimeras, as the identification of a second RBC population may indicate either a transient (allogeneic RBC transfusion) or static (life-long chimera) phenomenon. However, serial testing will enable discrimination between these two situations [10353].

Blood doping consists of any illicit means used to increase and optimize oxygen delivery to the muscles and includes blood transfusions, administration of erythropoiesis-stimulating substances, blood substitutes, natural or artificial altitude facilities, and innovative gene therapies. The use of blood transfusion, an extremely straightforward, practical and effective means of increasing an athlete’s red blood-cell supply in advance of competition, became rather popular in the 1970s, but it has suddenly declined following the widespread use of recombinant human erythropoietin among elite endurance athletes. Most recently, following implementation of reliable tests to screen for erythropoiesis-stimulating substances, blood transfusions have made a strong resurgence, as attested by several positive doping tests. Doping by blood transfusion can be classified as homologous, where the blood is infused into someone other than the donor, and autologous, where the blood donor and transfusion recipient are the same. The former case produces more clinically relevant side effects, but is easily detectable using current antidoping protocols based on erythrocyte phenotyping by flow cytometry and, eventually, erythrocyte genotyping by DNA testing. Since the donor and recipient blood are identical in autologous blood doping, this is less risky, though much more challenging to detect. Indirect strategies, relying on significant deviations from individual hematological profiles following autologous blood donation and reinfusion, are currently being investigated. For the time being, the storage of athletes' blood samples to allow testing and sanctioning of guilty athletes once a definitive test has been introduced may represent a reliable deterrent policy [06005].

Since the early 1960s it has been recognized that any means to increase and optimize oxygen delivery to the muscles would profoundly enhance an individual's athletic performance, especially for demanding physical exercise. Basically, this process, also known as induced erythrocythemia, blood boosting or, more recently, blood doping, consists of techniques administered to the athlete to “increase one’s red blood cell mass, which allows the body to transport more oxygen to muscles and therefore increase stamina and performance.”. Erythrocythemia induced by the infusion of 400–920 mL of packed red blood cells (RBCs) effectively enhances performance capacity in endurance track races, probably due to an increase in oxygen delivery to the working muscles. Reinfusion of 750 mL of RBCs substantially increases maximum oxygen consumption, although any performance improvement is not apparently associated with observed changes in the hematological profile. It is noteworthy that the maximum oxygen increase per gram change in hemoglobin is fairly comparable following either reinfusion of blood or recombinant human erythropoietin injections and persists for several weeks post-reinfusion, regardless of the return to baseline of the main hematological values. Increased performance for 3 h to 14 days following reinfusion of 1350 mL of autologous blood is also evident in cross-country skiers, based on improved race time over a specified distance. Significant improvements in treadmill endurance tests have also been reported following induced erythrocythemia . Apparently, blood transfusions also work for anaerobic sports, as the extra proton-buffering capacity of infused hemoglobin raises the lactate threshold and contributes to lower lactate generation during submaximal exercise. Hence, the great potential to improve athletic performance by blood transfusion is well-established [06005].

Consistent evidence shows that a greater than 5 percent increase in circulating hemoglobin is necessary to improve athletic performance, suggesting that athletes would need to infuse at least one unit of blood to obtain a surreptitious athletic advantage. The greater the amount
of blood transfused, the greater is the expected performance improvement; hence, larger infusions of either homologous or autologous RBCs may be associated with the hyperviscosity syndrome, characterized by increased blood viscosity, decreased cardiac output and blood flow velocity, finally resulting in a reduction in peripheral oxygen delivery [06005].

**Blood transfusions in general**

The widespread availability and relatively low cost of blood for transfusions are paramount needs for modern healthcare systems. Blood requirements for surgical and medical treatment are increasing steadily while the availability is stable or even decreasing, so that the demand is already exceeding the supply. Current technology and increasing knowledge on tissue and cell engineering, especially those based on the use of haematological stem cells to obtain mature and efficient blood particles, is opening appealing strategies and approaches for obtaining safe and disposable blood to be used for clinical purposes. RBC have been widely investigated in the past, and their metabolic pathways and mechanical properties have been exhaustively described and also reproduced in vitro. The production of biomaterial particles with similar (or even identical) chemical and physical properties as those of mature, functional RBC has been reported, so we have already alerted the scientific community as well as anti-doping authorities on the possible, surreptitious use of these bioengineering techniques for unfair and illicit purposes in sports [12234].

Blood doping is based on the use and administration of any illicit substance or procedure aimed at increasing and optimising oxygen delivery to the exercising muscles and, therefore, includes blood transfusions, administration of erythropoiesis-stimulating substances (namely hormones and growth factors), blood substitutes and artificial altitude facilities, possibly mixed and/or combined. The use of blood transfusions, either autologous or homologous, is an effective strategy for increasing oxygen delivery to the muscles and can be used immediately before a single competition or even during long-lasting sporting events to maintain a stable haemoglobin (Hb) concentration, especially when a natural decrease is expected as a result of physiological adaptation to endurance exercise. The procedure is not new, since it became popular nearly 40 years ago, but then suddenly declined due to its inclusion among the list of prohibited methods issued by the International Olympic Committee (IOC). Another reason that contributed greatly to the sudden decay of popularity of blood transfusions among elite athletes was the cloning of the erythropoietin gene and subsequent development of recombinant erythropoietin (rHuEpo) and its introduction among the armamentarium of illicit ergogenic aids. The great success of rHuEpo and analogues (erythropoiesis-stimulating agents, ESA) was mainly the consequence of the remarkable biological and technical advantages that these substances had over traditional means of increasing the red cell mass, such as blood transfusions. These advantages include easier supply (from healthcare facilities as well as from the "black market"), more comfortable administration (small subcutaneous doses) and the sharp and long-lasting effects on erythropoiesis (i.e. "blood boosting"). Once the concentration of Hb has been raised through ESA administration, the high concentration can be maintained by weekly administered microdoses of ESA which have a detection window of only 12-18 hours, for rHuEpo, compared to 3 days for regular doses. The subsequent development and implementation of (more or less) reliable electrophoretic techniques to screen for rHuEpo misuse caused a sudden resurgence of blood transfusions, which also took advantage of new procedures for collecting and storing the erythrocytes (e.g. freezing), allowing their use over a very long period of time as well as their harvesting during rest periods, thus avoiding a decline of Hb and aerobic sport performance during the competitive season. The predominant form of blood doping by transfusion is autologous blood transfusion, i.e. the blood donor and the recipient are the same. Homologous transfusions, in which the blood is drawn from a donor who is not the recipient, can also be
used, but the potential side effects and the easier identification by traditional red blood cell (RBC) phenotyping and flow cytometry substantially limit the use of this practice [12234].

**Autologous transfusion**

Autologous blood transfusions (ABTs) have been used by athletes for approximately four decades to enhance their performance. Although the method was prohibited by the International Olympic Committee in the mid 1980s, no direct detection method has yet been developed and implemented by the World Anti-Doping Agency (WADA). Several indirect methods have been proposed with the majority relying on changes in erythropoiesis-sensitive blood markers. Compared with the first methods developed in 1987, the sensitivity of subsequent tests has not improved the detection of blood doping. Nevertheless, the use of sophisticated statistical algorithms has assured a higher level of specificity in subsequent detection models, which is a crucial aspect of antidoping testing particularly to avoid “false positives.” Today, the testing markers with the best sensitivity/specificity ratio are the Hbmr model (an algorithm based on the total amount of circulating hemoglobin level [hemoglobin level mass] and percentage of reticulocytes) and the OFF-hr model (algorithm based on hemoglobin level concentration and percentage of reticulocytes). Only the OFF-hr model is currently approved by WADA. Recently, alternative indirect strategies for detecting blood doping have been proposed. One method is based upon a transfusion-induced immune-response resulting in specific changes in gene expression related to leukocytes such as T lymphocytes. Another method relies on detecting increased plasticizer metabolite levels in the urine caused by the leakage of plasticizers from the blood bags used during the blood storage. These methods need further development and validation across different types of transfusion regimes before they can be implemented. In addition, several research projects have been funded by WADA in recent years and are now under development. Although strategies to detect autologous blood transfusion have improved, a highly sensitive test to detect small volumes of transfused autologous blood has not yet been implemented [12009].

The use of blood doping is forbidden by the World Anti-Doping Agency. Several practices, such as blood transfusions are used to increase oxygen delivery to muscles and all of them are highly pursued. In this regard, the development of accurate methodologies for detecting these prohibited practices is one of the current aims of the anti-doping control laboratories. Flow cytometry methods are able to detect allogeneic blood transfusions but there is no official methodology available to detect autologous blood transfusions. One paper reviewed protocols, including the Athlete Biological Passport, that use indirect markers to detect misuse of blood transfusions, especially autologous blood transfusions. The methods of total haemoglobin mass measurements and the detection of metabolites of blood bags plasticizers in urine are reviewed. The latter seems to be an important step forward because it is a fast screening method and it is based on urine, a fluid widely available for doping control. Other innovative approaches to blood transfusion detection are also mentioned. A combination of the reported methodologies and the implementation of the Athlete Biological Passport is becoming a promising approach [12235].

Blood doping represents one main trend in doping strategies. Blood doping refers to the practice of boosting the number of red blood cells (RBCs) in the bloodstream in order to enhance athletic performance, by means of blood transfusions, administration of erythropoiesis-stimulating substances, blood substitutes, natural or artificial altitude facilities, and innovative gene therapies. While detection of recombinant EPO and homologous transfusion is already feasible through electrophoretic, mass spectrometry or flow cytometry-based approaches, no method is currently available to tackle doping strategies relying on autologous transfusions. It was exploited an in vitro model of autologous transfusion through a 1:10 dilution of concentrated RBCs after 30 days of storage upon appropriate dilution in
freshly withdrawn RBCs from the same donor. Western blot towards membrane Prdx2 and Percoll density gradients were exploited to assess their suitability as biomarkers of transfusion. Membrane Prdx2 was visible in day 30 samples albeit not in day 0, while it was still visible in the 1:10 dilution of day 30 in day 0 RBCs. Cell gradients also highlighted changes in the profile of the RBC subpopulations upon dilution of stored RBCs in the fresh ones. From this preliminary in vitro investigation it emerges that Prdx2 and RBC populations might be further tested as candidate biomarkers of blood doping through autologous transfusion, though it is yet to be assessed whether the kinetics in vivo of Prdx2 exposure in the membrane of transfused RBCs will endow a sufficient time-window to allow reliable anti-doping testing [12236].

There are two main strategies for doping by blood transfusion: autologous, whereby the blood donor and transfusion recipient are the same person, and homologous (allogeneic), whereby blood is transfused into someone other than the donor. The traditional procedure for autologous blood transfusion involves the withdrawal of 1 to 4 units of blood (1 units 450 mL of blood, corresponding to 225 mL of concentrated RBCs at a packed cell volume of 0.75) several weeks before competition to allow re-establishment of the RBC mass to the baseline level. The blood is immediately centrifuged, the plasma components are reinfused and the corpuscular elements (principally RBCs) are refrigerated at 4°C or frozen at -80°C. Stored erythrocytes are then traditionally reinfused into the athlete 1-7 days before a major endurance event [06005].

The drawing of blood for appropriate storage induces natural stimulation of the bone marrow by a complex mechanism that involves the hypoxia inducible factor (HIF) pathway. At the same time, administration of recombinant human erythropoietin at low, subtherapeutic doses increases bone marrow production. Recombinant human erythropoietin can be subcutaneously administered several weeks before competition or during resting periods, allowing the collection of up to 3-5 units of blood in a month, with minimal changes in hemoglobin concentrations, as clearly demonstrated by preoperative medicine. Blood reinfusion then increases the hemoglobin concentration, which is already raised by the exogenous hormone. At the time of competition, athletes finally show up with increased hemoglobin, but virtually undetectable erythropoietin in urine using the official electrofocusing technique. The success of the old technique of blood transfusion in hemodoping is also linked to the disappointing results of new technologies for inducing bone marrow to release RBC cohorts and increase hemoglobin [06005].

As if not complex enough for the anti-doping fight, biotechnology is offering new solutions for intensive care patients requiring blood transfusions, which in turn opens new flood gates for potential abuse. Examples of these new yet not approved/launched products are erythrocytes prepared ex vivo from hematopoietic stem cells as well as erythrocyte-mimicking synthetic biomaterial particles that might require complementary or extended doping control analytical strategies in the future. However, with the increasing knowledge and in-depth investigation of ex vivo stored erythrocytes and concomitantly occurring alterations at the proteome and metabolome levels, alternative (and possibly comprehensive) strategies might arise enabling the detection of different kinds of blood doping. Pilot studies were conducted concerning membrane proteins of red blood cells, using commonly accepted approaches such as 2D gel electrophoresis and iTRAQ labeling with LC-MS/MS quantitation. These studies yielded significant alterations of cytoskeletal-derived proteins such as spectrin beta, ankyrin-1, tropomodulin-1, beta adducin, and tropomyosin as well as transmembrane proteins including glycophorin C and aquaporin-1 with a minimum increase or decrease of at least a factor of 1.5. Focusing on cytosolic proteins and their changes under transfusion medicine-based ex vivo storage conditions, Walpurgis et al. employed 2D difference gel electrophoresis (2D DIGE) to accurately quantify alterations of protein amounts by respective
varying spot volumes, which are all individually corrected by their corresponding internal standard. Following identification of 14 protein candidates, their characterization was conducted by LC-MS/MS revealing transglutaminase 2, beta actin, and copper chaperone as potential marker proteins for storage-induced lesions of blood products and, thus, possibly for doping control purposes. The validity of the characterized proteins was cross-checked by western blot analysis. Despite the significant alterations of membrane and cytosolic proteins as determined in these two aforementioned studies, both approaches necessitate further evaluation since the dilution of these arguably indicative modifications might not remain detectable once the blood is re-infused [13009].

The predominant form of blood doping by transfusion is autologous blood transfusion, i.e. the blood donor and the recipient are the same. Homologous transfusions, in which the blood is drawn from a donor who is not the recipient, can also be used, but the potential side effects and the easier identification by traditional red blood cell (RBC) phenotyping and flow cytometry substantially limit the use of this practice. The detection of autologous blood transfusion is more challenging. A direct method based on the identification of plasticizers excreted in the urine as a result of autologous blood transfusion was recently proposed as a reliable approach to detect such transfusions in athletes, although it has shown several drawbacks and is not currently implemented as a doping detection tool. However, an indirect approach, based on the assessment of Hb concentration and reticulocyte percentage (Ret%) over time, can be used and also combined in the so-called OFF-score (i.e. Hb-60/Ret%). These parameters are introduced into a statistical programme (i.e. the Athlete’s Biological Passport), which is aimed to unmask non-physiological perturbations of blood homeostasis such as those following a blood transfusion (abnormal Hb increase and a decrease in Ret%) or withdrawal of blood for the purpose of storage and subsequent re-administration (abnormal Hb decrease combined with an increase in Ret%). Absolute values of Hb and Ret% along with other derived parameters (mean corpuscular volume, Ret%, Hb, etc) might also be used for screening for blood transfusions, but the results provided so far have been rather controversial, despite the fact that the high sensitivity of the passport approach for detecting blood transfusion has been unequivocally proven in a blinded experiment in healthy subjects. Although the passport approach is useful in the case of “regular” amounts of re-infused blood (3 units), its low sensitivity in detecting smaller amounts (1 unit) still represents a major limitation. The leading issue in detecting autologous blood transfusion is the potential dilution of intravenously administered RBC and their early, partial removal [13290].

Adverse events

Homologous blood transfusions may trigger reactions characterized by fever, urticaria, and anaphylactic shock, and are significantly associated with transmission of blood-borne infectious diseases, including hepatitis, acquired immunodeficiency syndrome, malaria, cytomegalovirus and Creutzfeldt Jakob disease. Occasionally, patients may develop phlebitis, septicemia, bacterial infection, air/clot embolism and transfusion-associated graft-versus-host disease (TAGVHD), a lethal complication of infusion of non-irradiated cellular blood components into a susceptible recipient [06005].

Both blood transfusion and rhEPO will increase the hematocrit unless masked with a plasma volume expander. When the hematocrit rises above 0.46, there is a documented risk for cardio-vascular disease (CVD) in the normal population. However, this might not reflect the truth in athletes which represent a highly selected and younger cohort. A number of suspicious deaths in cyclists have, however, been reported, but it has not been identified any true hazard analysis for CVD and blood doping in the literature. Blood transfusions are safe in a hospital setting but adverse events like allergic and hemolytic transfusion reactions
do occur. However, in blood doping the strict regulation for labeling and storage of the components are likely not followed leading to a greater risk for adverse events [13005].

**Plasma markers for testing**

Several authors have highlighted the impact of blood manipulation on iron metabolism. With iron being the major substrate for Hb synthesis, it is evident that iron-related variables will be affected by any manipulations of erythropoiesis. Ferritin, the iron storage protein, was addressed as a potential marker as early as the 1980s. However, the association to inflammation and its susceptibility to be influenced by external iron administration limits its specificity. Hepcidin was recently investigated as another potential marker in this context. This molecule regulates the iron absorption by the body and was found in several studies to be a potential marker for blood manipulation. However, more data on the natural variation and the impact of exercise and other confounding factors are necessary [13009].

The athlete biological passport for the fight against doping is currently based on longitudinal monitoring for abnormal changes in cellular blood parameters. Serum parameters related to altered erythropoiesis could be considered for inclusion in the passport. The aim of one study was to quantify the changes in such parameters in athletes during a period of intense exercise. Twelve highly trained cyclists tapered for 3 days before 6 days of simulated intense stage racing. Morning and afternoon blood samples were taken on most days and analysed for total protein, albumin, soluble transferrin receptor, and ferritin concentrations. Plasma volume was determined via total haemoglobin mass measured by carbon-monoxide rebreathing. Percent changes in means from baseline and percent standard errors of measurement (analytical error plus intra-athlete variation) on each measurement occasion were estimated with mixed linear modelling of log-transformed measures. Means of all variables changed substantially in the days following the onset of racing, ranging from -13 percent (haemoglobin concentration) to +27 percent (ferritin). After the second day, errors of measurement were generally twice those at baseline. Plasma variables were affected by heavy exercise, either because of changes in plasma volume (total protein, albumin, haemoglobin), acute phase/inflammatory reactions (ferritin) or both (soluble transferrin receptor). These effects need to be taken into consideration when integrating a plasma parameter into the biological passport model for athletes [13304].

**Combination of biomarkers**

The main scientific focus has been on finding single biomarkers that will detect certain doping techniques with acceptable sensitivity and good specificity. Future research should explore complementary paths using mathematical methods for the combination of the multitude of markers that often display a good sensitivity but may lack specificity. Approaches such as support vector machines and other classification techniques that are in use in many other domains of science might be helpful. In this context, information from different sources (e.g. urine tests, blood tests) should be combined, as also for urine test information, a longitudinal monitoring has been proposed. By these means, specificity could be increased and distinct patterns for certain doping interventions or the identification of suspicious and potentially doping athletes could be warranted, which would then trigger follow-up testing with more specific methods. This would help to save resources and make the fight against doping more efficient. In this context, the application of newer diagnostic tools relating to the ‘omics’ technologies such as transcriptomics, proteomics, and metabolomics will nurture this area in the years to come. Thus, it may be possible to identify the biological fingerprint of many potential doping substances which would allow athletes to present themselves to a competition with physiological values and profiles that fall within their own personal limits. Additionally, the scope of analyses could be expanded to
Screening for homologous blood transfusions

Blood groups
The plasma membrane of red blood cells expresses a wide series of blood group antigens that are actually complex oligosaccharides that differ mainly in their biochemical structure. In addition to the traditional AB antigen cluster, RBCs are characterized by the rhesus (Rh) polymorphic polypeptides and the associated glycoprotein RhAG. That blood group system is the most polymorphic of the human blood groups, including at least 45 independent antigens. Owing to the complex combination of AB and Rh antigens on the RBC surface, hematopoiesis in the bone marrow produces erythrocytes characterized by a genetically determined, virtually unique antigen pattern. An antidoping method implemented by WADA is based on the quantification of antigenically distinct donor and recipient erythrocytes by flow cytometry, using standard blood-bank antisera combined with a fluorescent-labeled secondary antibody directed against human immunoglobulin. This strategy allows the detection of small populations (about 5%) of cells that are antigenically distinct from an individual’s own RBCs with a degree of sensitivity, provided there is at least one antigen mismatch between the donor and recipient. The test is based on phenotyping for several RBC antigens, including C, c, E, e, K, k, Fya, Fyb, Jka, Jkb, S and s. The chance of detecting two identical samples by this screening strategy is reportedly less than 1:500. Transfused erythrocytes remain in the circulation for up to 120 days in sedentary individuals and for 60–90 days in elite athletes, because RBCs typically display shorter survival in physically active individuals. The identification of a second RBC population in persons who are blood group chimeras may indicate either a transient (homologous RBC transfusion) or a life-long chimera phenomenon. In such a case, serial testing easily allows discrimination between these two situations. In other cases, enlargement of the antigen panel by Rh50 glycoprotein, CD47, glycophorin B, Duffy, LW glycoprotein, and Band 3 testing may represent a reliable solution. An alternative strategy that may soon become faster and cheaper is to screen blood for these antigens using genomic DNA and the associated single-nucleotide polymorphisms (SNPs). DNA analysis displays limited diagnostic efficiency in populations of athletes characterized by a higher prevalence of non-expressed RHD. In addition, it is important to mention that PCR-based assays are prone to different types of errors than those observed with the traditional phenotyping techniques, such as contamination with amplified products and the identification of particular antigen genotypes that are not necessarily expressed on the RBC membrane.

Screening for autologous blood transfusions

The detection of autologous blood transfusion is more challenging. A direct method based on the identification of plasticizers excreted in the urine as a result of autologous blood transfusion was recently proposed as a reliable approach to detect such transfusions in athletes, although it has shown several drawbacks and is not currently implemented as a doping detection tool. However, an indirect approach, based on the assessment of Hb concentration and reticulocyte percentage (Ret%) over time, can be used and also combined in the so-called OFF-score (i.e. Hb-60√Ret%). These parameters are introduced into a statistical programme (i.e. the Athlete’s Biological Passport), which is aimed to unmask non-physiological perturbations of blood homeostasis such as those following a blood transfusion (abnormal Hb increase and a decrease in Ret%) or withdrawal of blood for the purpose of storage and subsequent re-administration (abnormal Hb decrease combined with an increase in Ret%). Absolute values of Hb and Ret% along with other derived parameters (mean corpuscular volume, Ret%, Hb, etc.) might also be used for screening for blood
transfusions, but the results provided so far have been rather controversial, despite the fact that the high sensitivity of the passport approach for detecting blood transfusion has been unequivocally proven in a blinded experiment in healthy subjects. Although the passport approach is useful in the case of "regular" amounts of re-infused blood (3 units), its low sensitivity in detecting smaller amounts (1 unit) still represents a major limitation. The leading issue in detecting autologous blood transfusion is the potential dilution of intravenously administered RBC and their early, partial removal. It may also be mentioned that the organisation and management of the collection and long-term storage of blood bags is difficult, especially outside healthcare facilities, so that this practice is mostly limited to high-profile, wealthy athletes [12234].

Neither homologous nor autologous blood transfusions could be detected directly until 2003, when it was presented a test for the detection of homologous transfusion that identifies mixed red blood cell (RBC) populations using antisera against minor blood group antigens. In this assay, 12 blood group antigens were selected to identify donor RBC populations in patients who received between one to three units of homologous blood with a lower limit of detection around 5 percent of the total red cell population. Transfused erythrocytes survive for an average of 94 days after storage for 42-49 days, giving the test a wide detection window. The methods were further validated and improved by using a panel of eight primary blood groups antigens. Applying the technique of signal amplification, the authors improved the separation of those antigens with weak or heterozygous expression resulting in an identification of mixed RBC populations in samples containing 0.3-2.0 percent of donor blood. It has also been presented forensic standards of doping analyses for homologous blood transfusions in a single-blind and single-site study. A specificity of 100 percent was described as no false-positive results were observed in the analysis of 140 blood samples containing different percentages (0-5 %) of a minor RBC population. The sensitivity of the method was 78 percent and most samples were unambiguously detected. With their approach, the ISO-17025 accreditation and validation requirements were fulfilled. Examples of single and double population fluorescence intensity histograms (using anti-Jk\textsuperscript{a} antibody and the mix of fluorescein isothiocyanate (FITC)-coupled secondary antibodies) have been presented. Several athletes tested positive for homologous blood transfusion on the basis of this test. Obviously, this method is inappropriate for the detection of autologous blood transfusions, which continue to be undetectable by direct means. From the time before 2000, when EPO was similarly undetectable by a direct test, indirect biomarkers have emerged as a method to deter blood doping and possibly limit the extent of manipulations. The use of indirect biomarkers of doping has culminated in the introduction of the so-called athlete biological passport (ABP) which can be regarded as the best approach [13006].

The donor
Studies in a small number of donors have demonstrated that a temporary decrease in RBC mass is well tolerated when donors give twice the usual amount (170-225 mL) of erythrocytes in a standard 405-495 mL donation, causing no detectable symptoms of reduced oxygen-carrying capacity, as confirmed by negligible changes in heart rate or systolic and diastolic blood pressure. Nevertheless, some physiologic adjustments of the hematopoietic response to the acutely reduced RBC mass can be recorded. These include a typical erythropoietin response (serum erythropoietin increases four-fold within 1 day, declining exponentially thereafter), changes in erythrocyte and hemoglobin synthesis (reticulocyte count increases rapidly by 2.4-fold after 7 days, remaining elevated for another 7 days, whereas hemoglobin values remain reduced on average by about 15 % for 2 weeks) and changes in iron metabolism (serum ferritin sharply decreases, while the soluble transferrin receptor concentration increases by 60 % by day 14). Blood donation of 450 mL from healthy blood donors also leads to an increase in hypochromic reticulocytes from day 1 or 2, peaking on day 9 and reaching a maximum increase of 178 percent. Hypochromic
n erythrocytes and soluble transferrin receptor increase within the same period, whilst transferring saturation decreases. The reticulocyte count increases substantially from the first day, with a maximum increment of 55 percent [06005].

The recipient
Reinfusion of 1350 mL of blood generally leads to hemoglobin increases of 8 percent from the pre-phlebotomy level and of 14 percent from the pre-infusion level, with simultaneous reduction in serum erythropoietin in 24 h and a sharp increase in serum iron and bilirubin. Blood reinfusion also significantly increases both hemoglobin and ferritin concentrations by up to 18 percent and 68 percent, respectively. Such changes are associated with a stable and marked decline, approaching 50 percent of the baseline concentration, of serum erythropoietin. Levels of reticulocytes and soluble transferrin receptor also decline progressively from day 7 to day 21 following transfusion [06005].

The use of blood markers
The first attempt to develop a test to detect the use of autologous blood transfusions (ABT) was performed by Berglund et al in 1987. A volume of 1350 mL of blood, corresponding to 3U of blood, was withdrawn from each subject, stored at 4°C for 4 weeks, and then reinfused into the donor. The reinfusion of blood resulted in a 60 percent reduction in serum EPO within the first 24 hours as well as a marked increase in Hb, serum ferritin, and serum bilirubin. From these data, a test based on absolute thresholds of Hb (>12.0 g/dL), bilirubin (>30 micromol/L), ferritin (>33 micromol/L), and EPO (<15 mU/mL) yielded a sensitivity of 50 percent within the first week of transfusion. Nevertheless, a general problem with using such absolute thresholds is the small within-subject to between-subject variability ratio found for most erythropoiesis-sensitive markers. Therefore, an alternative “2-sample procedure” was suggested by Berglund et al, where an increase in Hb greater than 5 percent and a concomitant decrease in serum EPO greater than 50 percent served as the limits. This algorithm also yielded a sensitivity of 50 percent but with the window of detection now extending to two weeks. Another important aspect when developing a test for the detection of ABT is the applicability of the test across different transfusion regimens. Historical evidence has shown that athletes and their physicians adjust their doping regimes whenever a new testing technology is introduced [12009].

During the 1990s, different sports federations implemented upper hematocrit and Hb thresholds to discourage athletes from manipulating their blood. In addition, reticulocyte analysis and thresholds were implemented, but the sensitivity of these thresholds to detect ABT was not examined before 2006. The withdrawal of blood results in a significant increase in percentage of reticulocytes reaching peak levels of 3.0 percent 1 week after withdrawal in tandem with a significantly decreased Hb. Withdrawal of blood is the only blood manipulation that results in this specific test result pattern and is therefore highly indicative of ABT. After RBC reinfusion, only a modest decrease in percentage of reticulocytes, from 1.3 to 0.8 percent, was observed. Individual Hb values apply to an absolute cutoff threshold of 17.0 g/dL, but only 1 of 10 subjects exceeds this limit [12009].

mRNA
Leuenberger et al. investigated a different angle of detecting blood transfusion by measuring the impact on cell-free microRNA (miRNA) in plasma. In a controlled transfusion study with blood stored for 42 days, three miRNAs namely miR-30b, miR-30c, and miR-26b were significantly increased up to one day post re-infusion. Combining data from miRNA with ‘conventional’ blood parameters such as EPO serum concentration (which was shown to
decrease in the same study) could provide a complementary tool to determine autologous blood doping [13009].

**DNA-based method for detecting homologous blood doping**

Homologous (or allogeneic) blood doping, in which blood is transferred from a donor into a recipient athlete, is the easiest, cheapest, and fastest way to increase red cell mass (hematocrit) and therefore the oxygen-carrying capacity of the blood. Although thought to have been rendered obsolete as a doping strategy by the increased use of rhEPO to increased hematocrits, there is evidence that athletes are still using this potentially dangerous method to improve endurance performance. Current testing for homologous blood doping is based on identification of mixed populations of red blood cells by flow cytometry. This paper proposes that homologous blood doping could also be tested for by high-resolution qPCR-based genotyping and demonstrates that assays could be developed that would detect second populations of cells even if the "donor" blood was depleted of 99 percent of the DNA-containing leukocytes. Issues of test specificity and sensitivity are discussed as well as some of the ethical considerations that would have to be addressed if athletes' genotypes were to be used by the anti-doping authorities to prevent, or detect, the use of prohibited ergogenic practices [13302].

Aiming at an alternative methodology for the detection of homologous blood doping, it was studied the applicability of DNA analysis from whole blood to assess whether the genetic material of one or more individuals is present in one sample. Employing the typical 16 loci as used in forensic analyses, the presence of more than 2 allele lengths (in at least 7 different loci with a minimum relative abundance of 100 RFU) was considered proof for the presence of donor blood in the specimen. The sensitivity, as assessed by different mixtures of donor and acceptor blood ex vivo, was estimated to be a low as 2.5 percent (of donor blood), hence, representing a methodology with the potential to complement the currently routinely used flow cytometry [13009].

Homologous (or allogeneic) blood doping, in which blood is transferred from a donor into a recipient athlete, is the easiest, cheapest, and fastest way to increase red cell mass (hematocrit) and therefore the oxygen-carrying capacity of the blood. Although thought to have been rendered obsolete as a doping strategy by the increased use of rhEPO to increased hematocrits, there is evidence that athletes are still using this potentially dangerous method to improve endurance performance. Current testing for homologous blood doping is based on identification of mixed populations of red blood cells by flow cytometry. This paper proposes that homologous blood doping could also be tested for by high-resolution qPCR-based genotyping and demonstrates that assays could be developed that would detect second populations of cells even if the "donor" blood was depleted of 99 percent of the DNA-containing leukocytes. Issues of test specificity and sensitivity are discussed as well as some of the ethical considerations that would have to be addressed if athletes' genotypes were to be used by the anti-doping authorities to prevent, or detect, the use of prohibited ergogenic practices [13303].

**DNA testing**

Homologous blood transfusion is an illicit practice used by athletes to improve the delivery of oxygen to tissues and, as such, it is banned in sports. The current method of detection is based on the flow cytofluorimetric phenotypic identification of red blood cells mismatch of minor blood group antigens between the donor and the recipient. The selectivity of this
method to clearly identify transfused samples is related to the number of blood group antigens tested. Despite the fact that several different antigens are investigated, two individuals sharing the expression of the same minor blood group antigens pattern cannot be distinguished. We tested the possibility to use a different approach based on DNA forensic human identification techniques. Analysis of the DNA short tandem repeats (STRs) demonstrated its suitability in detecting mixed whole blood samples simulating homologous blood transfusion in 100 percent of the samples tested, ensuring the capability of clearly detecting mixed blood cell populations also on samples where the fraction of the minoritary population is as low as 2.5 percent [13323].

Response to foreign erythrocytes

Another interesting approach arises from the analysis of erythrocytes. In a pilot project, it was analyzed proteomic data (e.g. from 2-DE gels) from two volunteers and showed that RBC membrane proteome changes during storage could provide biomarkers for the detection of autologous transfusion in the future. In a similar small study, it was shown that the marker oxidative stress-related protein peroxiredoxin 2 and the RBC population characterized by density separation significantly changed after mixture of stored RBCs with freshly withdrawn RBCs in vitro. In another project supported by WADA one group hypothesized that a systemic response towards a supra-physiological red cell volume induced by autologous transfusion might be assessable in vivo by measuring the differences in red cell distribution by continuous density gradients and flow cytometry characterization of erythrocytes. Furthermore, as several molecular changes occur in stored RBCs, commonly referred to as the “storage lesion”, it was hypothesized that autologous transfusion will lead to a sudden exposure of cell detritus to the immune system causing a cellular and molecular immune response. A distinct immune reaction was found on a transcriptional level in T lymphocytes using whole genome microarrays and qRT-PCR confirmation. This concept was reassessed by evaluation of the immune response of peripheral blood mononuclear cells using cellular fluorescence markers in vitro and in vivo [13006].

Stability of hemoglobin

The stability of the most crucial variables, i.e. haemoglobin concentration and percentage of reticulocytes, under the defined pre-analytical conditions has been established to be at least 36 h, requiring rapid and cooled transport of doping control blood samples to an accredited laboratory. Extending the storage time to 168 h at +4°C, +6°C, and +12°C as done in this study demonstrated that these parameters were not significantly altered if the temperature was kept at 4-6°C, hence allowing to rely on analytical data up to seven days after blood collection when adequately cooled. Physiologically, the percentage of reticulocytes can vary depending on factors such as seasonal stress (training, competition, and recovery), sport discipline, diseases, etc. However, intra-individually, the parameter has proved extraordinary informative and has thus become a major pillar of the ABP. In order to support the significance of values measured from individuals and the comparison of analyses conducted at different laboratories and/or on different analytical systems of the same instrument type, a study to improve the between-instrument comparability by optimizing the calibration was conducted. By means of a stabilized whole blood matrix used as a calibrant, mean values of “authentic” samples were within 0.1 percent among the test instruments, thus allowing an improvement in the commonly observed bias between systems still operating within the manufacturers' specifications [13009].

Effects of blood withdrawal and reinfusion on biomarkers

In the 2000s, athletes have been tested positive for allogeneic blood transfusion by means of
flowcytometry indicating a return to former blood transfusion practices. However, an unequivocal detection method revealing autologous transfusion is still not available. Since accelerated or inhibited erythropoiesis leads to characteristic changes in peripheral blood parameters, irrespectively of the stimulating agent, an indirect detection method of recombinant human erythropoietin (rHu-EPO) use based on changes in certain hematological parameters has been implemented by some international sport federations. Despite its use, data on the effect of blood withdrawal and reinfusion in these hematological parameters have never been reported in full. It was hypothesized that autologous blood manipulations produce large variations in currently used biomarkers of erythropoiesis making individual hematological profiles an effective tool identifying this manipulation. It was measured hemoglobin concentration [Hb], hematocrit, reticulocytes, serum EPO and sTfR concentrations in 10 healthy, male subjects at baseline and after the withdrawal of 20 ± 3 percent (1.3 ± 0.2 L) of the subjects' blood volume on day 0, 1, 3, 7, 14, 21 and 28 and after reinfusion of 0.8 ± 0.1 L of packed RBCs on day 0, 1, 3, 7, 14 and 21. To maintain blood volume after blood withdrawal, 1.3 L of hydroxyl-ethyl-starch (Voluven®) was infused. All subjects were given daily oral iron supplementation (100 mg). [Hb], hematocrit, reticulocytes was measured. Serum EPO and sTfR concentrations were determined by ELISA. The indirect OFF-hr blood models for rHu-EPO detection were applied. It was found that following blood withdrawal, [Hb] remained reduced on average by about 15 percent for 2 weeks. Accordingly, s-EPO increased 4-fold within a day, declining exponentially thereafter. Reticulocyte count increased rapidly by 2.4-fold after 7 days, remaining elevated for another 7 days whereas sTfR increased by 60 percent by day 14 and remained elevated until 3 days after blood reinfusion. Following blood reinfusion, [Hb] increased acutely by 8 percent returning to the initial baseline value after 2 days. s-EPO remained unchanged whereas reticulocyte count was reduced by 25-37 percent from day 7 to 21. sTfR declined progressively after reinfusion. The highest OFFhr score was 126 at day 2 during the polycytemic period corresponding to a 1 in 1000 cut-off threshold. Evidence indicates that the hematocrit within-subject biological and seasonal variation in man shows a maximal relative change of 15 percent within a 95 percent confidence interval i.e. a change from 0.42-0.48.5 Data of a wider range of sports also show within subject estimates of variance in [Hb] of 1.6 g/dL,6 which agree with a10 percent fluctuation found in soccer players.7 In the present study, however, all 10 subjects exceeded the normal variation, with [Hb] ranging from 19-39 percent. Even when the control [Hb] value was taken as the mean [Hb] value, 9 of 10 subjects exceeded their individual upper limit based on a 7.5 percent addition to their control [Hb] value (range 3.0-11.5 %). Importantly, alterations in other hematological biomarkers (s-EPO, reticulocytes and sTfR) were observed at all times through this investigation, suggesting that determination of these biomarkers could be used as supportive evidence for erythropoietic manipulations with acute [Hb] increases of more than 7.5%. At present, only two blood tests are available. To protect the health of the athlete, World Anti Doping Agency has implemented an upper limit of 17.0 g/dL, which – if exceeded – elicits a no start sanction. However, only one subject in this study showed [Hb] values higher than 17.0 g/dL. In conclusion, autologous blood procedures induce a clear pattern of accelerated erythropoiesis during the anemic period. Within the limitations of the study, it is suggested that variations in [Hb] exceeding 15 percent between samples obtained in top ranked endurance athletes during the anticipated anemic period and shortly before any major competition would be indicative of autologous blood manipulation [06152].

Laboratory problems

Antidoping testing is currently exclusively based on haematochemical analysis performed in specialized laboratories accredited by WADA (World Anti-Doping Agency). Many of the analytical methods used for the determination of the parameters considered, such as haematological parameters (haemoglobin, hematocrit and reticulocytes), proteins (soluble
transferrin receptor and hepcidin) and hormones (erythropoietin and growth hormone) are often affected by lack of clear standardization and harmonization. The observed incongruity of the data deriving from different laboratories often results in the risk of false positive results in athletes. One review wanted to provide additional proofs in support of the need to improve the antidoping methodology involving different research and clinical institutions and skills [10354].

Robustness of measurements after storage of blood

With the setting up of the newly Athlete’s Biological Passport antidoping programme, novel guidelines have been introduced to guarantee results beyond reproach. It was investigated in this context, the effect of storage time on the variables commonly measured for the haematological passport. It was also wanted to assess for these variables, the within and between analyzer variations. Blood samples were obtained from top level male professional cyclists (27 samples for the first part of the study and 102 for the second part) taking part to major stage races. After collection, they were transported under refrigerated conditions (between 2 degrees and 12 degrees C), delivered to the antidoping laboratory, analysed and then stored at approximately 4 degrees C to conduct analysis at different time points up to 72 h after delivery. A mixed-model procedure was used to determine the stability of the different variables. As expected haemoglobin concentration was not affected by storage and showed stability for at least 72 h. Under the conditions of the investigation, the reticulocytes percentage showed a much better stability than previous published data (> 48 h) and the technical comparison of the haematology analyzer demonstrated excellent results. In conclusion, the data clearly demonstrate that as long as the World Anti-Doping Agency’s guidelines are followed rigorously, all blood results reach the quality level required in the antidoping context [10308].

Two main blood storage procedures can be used for storing red blood cells: refrigeration and freezing. Nevertheless, the efficiency of these procedures measured as the increase in hemoglobin after reinfusion compared with baseline has never been examined. The main objective was to examine which storage procedure yielded the largest increase in circulating haemoglobin after reinfusion compared to baseline. Equal volumes of blood from 15 men were withdrawn and stored either frozen or refrigerated as packed red blood cells. Serial measures of circulating hemoglobin by carbon monoxide rebreathing provided an opportunity to monitor recovery from anaemia, as well as the net increase in circulating haemoglobin after transfusion. The post-thaw yield of hemoglobin in the bags was 72 percent after refrigerated storage compared with only 52 percent after freezing. Nevertheless, frozen storage allowed hemoglobin to fully recover before reinfusion, while the haemoglobin was 10 percent lower in the refrigerated group compared with baseline. After reinfusion, the hemoglobin levels were 12 percent higher than the baseline values in the group reinfused with frozen blood, while for the refrigerated group, hemoglobin levels were only 5 percent higher than baseline. The relatively larger recovery from anemia in the frozen group during storage more than compensated for the larger loss of hemoglobin during freezing and resulted in a larger net gain in hemoglobin. Based on the average 23 g per week recovery of hemoglobin, extending refrigerated storage to 7-8 weeks may yield sufficient time for patients to fully replenish harvested hemoglobin from three bags of blood without reliance on frozen storage of RBC [11136].

One review examined the science and methodology of blood conservation in modern anaesthetic and surgical practice. Blood transfusion is associated with increased morbidity and mortality in all surgical patients, and the reduction or even elimination of transfusion has been and continues to be the subject of much research. Blood substitutes, despite extensive investigation, have not been proved successful in trials to date, and none have entered
clinical practice. Pharmacological treatments include antifibrinolytic drugs (although aprotinin is no longer in clinical use), recombinant factor VIIa, desmopressin, erythropoietin and topical haemostatic agents, and the role of each of these is discussed. Autologous blood transfusion has recently fallen in popularity; however, cell salvage is almost ubiquitous in its use throughout Europe. Anaesthetic and surgical techniques may also be refined to improve blood conservation. Blood transfusion guidelines and protocols are strongly recommended, and repetitive audit and education are instrumental in reducing blood transfusion [09155].

It should be mentioned that the organisation and management of the collection and long-term storage of blood bags is difficult, especially outside healthcare facilities, so that this practice is mostly limited to high-profile, wealthy athletes. The widespread availability and relatively low cost of blood for transfusions are paramount needs for modern healthcare systems. Blood requirements for surgical and medical treatment are increasing steadily while the availability is stable or even decreasing, so that the demand is already exceeding the supply. Current technology and increasing knowledge on tissue and cell engineering, especially those based on the use of haematological stem cells to obtain mature and efficient blood particles, is opening appealing strategies and approaches for obtaining safe and disposable blood to be used for clinical purposes. RBC have been widely investigated in the past, and their metabolic pathways and mechanical properties have been exhaustively described and also reproduced in vitro [13290].

**Peroxiredoxin 2 as a marker during storage**

Employing a different strategy at the proteome level of red blood cells (RBCs), a considerable increase of peroxiredoxin 2 (Prdx2) was observed upon ex vivo storage of erythrocytes. The study did not include transfusion experiments and it remains to be clarified whether the increased Prdx2 levels can be visualized once the stored RBCs have circulated in the bloodstream for a certain period of time; however, experiments with stored blood diluted tenfold with freshly sampled specimens allowed for the detection of Prdx2 using 1D gel electrophoresis and Western blotting [13012].

**Whole blood transfusion**

Hemoglobin mass (Hb\textsubscript{mass}) is a key factor for maximal exercise capacity. In aerobic sport disciplines, such as long-distance running, cycling, or cross-country skiing, the main factors determining performance are a high delivery of O\textsubscript{2} to the exercising skeletal muscles and its use. The rate of maximal O\textsubscript{2} uptake (O\textsubscript{2}\textsubscript{max}) is dependent on a high cardiac output (Q) and a wide difference for arterial-venous O\textsubscript{2} (a-vO\textsubscript{2}), that is, the Fick equation:

\[
\text{Oxygen consumption} = (\text{cardiac output} \times \text{oxygen concentration of arterial blood}) - (\text{cardiac output} \times \text{oxygen concentration of venous blood})
\]

Because maximal cardiac output is difficult (if not impossible) to manipulate to higher values during competitions, the distribution of Q during maximal exercise to the working skeletal muscles is close to 80 percent, and arterial O\textsubscript{2} extraction is already in the range of approximately 90 percent at maximal exercise, the only variable that remains open for manipulations in regards to increasing exercise performance is the arterial O\textsubscript{2} content. Accordingly, in a given person, changes in Hb concentration by either red blood cell (RBC) transfusion or hemodilution will increase or decrease O\textsubscript{2}\textsubscript{max}, respectively. On the group basis, however, concentration of hemoglobin is not predictive of O\textsubscript{2}\textsubscript{max}, whereas the total mass of Hb correlates very well. Indeed, a somewhat reduced hemoglobin concentration is sometimes, but not always, observed among athletes, whereas Hb\textsubscript{mass} is usually increased compared with normal healthy persons. It has been calculated that a change of 1 g in Hb\textsubscript{mass}...
will produce a change in $O_{2\text{max}}$ of 4 mL/min whereas the effects on submaximal exercise performance are probably variable according to competition distance. It should also be noted here that volume loading (i.e. plasma volume expansion) in itself does not lead to an improved exercise performance in elite athletes, again highlighting the role of Hb mass. If, however, a plasma volume expander is administrated simultaneously with increments in Hb mass, then performance will probably increase more than when just augmenting the total red cell volume. Some athletes apply prohibited techniques and substances with intent to increase Hb mass and physical performance, and this is often difficult to prove directly. A test for detection of allogeneic blood transfusion doping was implemented in 2004. The test uses blood group antisera to identify mixed RBC populations in blood samples by flow cytometry. Autologous RBC manipulations can at present only be detected via indirect measures, which represent a major problem in antidoping efforts. The CO rebreathing technique for detecting nonphysiologic increases in Hb mass is still investigational, and besides practical difficulties related to this method, its potential inclusion in the blood passport may be problematic, because the margin of variation when assessing Hb mass (biologic and measurement errors) would still allow athletes to manipulate with blood volumes that would increase exercise performance considerably. Finally, it should also be considered that, from an athlete’s viewpoint, it may not be desirable to breathe CO shortly before a competition as this may limit exercise performance. Novel erythropoietic substances, such as mimetics of erythropoietin (Epo) and activators of the Epo gene may soon enter the sports scene. In addition, Epo gene transfer maneuvers are imaginable. Effective since December 2009, the World Anti-Doping Agency (WADA) has therefore implemented “Athlete Biological Passport Operating Guidelines”, which are based on the monitoring of several parameters for mature RBC and reticulocytes. Blood doping may be assumed, when these parameters change in a non-physiological way. Hematologists should be familiar with blood doping practices as they may play an important role in evaluating blood profiles of athletes with respect to manipulations, as contrasted to the established diagnosis of clinical disorders and genetic variations [11116].

Traditional antidoping analyses are based on the detection of a substance in biologic fluids (“Adverse analytical finding”). This approach has major limitations in regard to blood doping. Autologous blood cannot be detected, there is a plethora of ESAs, the detection window is limited, and there is urine manipulation. Some sports federations earlier introduced upper hemoglobin concentration and Hct limits to escape from this dilemma. Athletes tested above the limits were declared unfit for competition (“No-start rule”). However, hemoglobin concentration and Hct are influenced by external factors, such as body posture, exercise, or residence at altitude. In addition, “clean” athletes can have naturally high hemoglobin concentrations and Hct values. A large retrospective study on male blood donors in Denmark revealed that 3.9 percent of nonathletes and 10.4 percent of elite rowers had Hct values more than 0.51 (i.e. above the recommended limits for athletic competition). In addition, the adoption of upper [Hb] and Hct limits may paradoxically generate more blood doping because, by ESA misuse, [Hb] and Hct can be manipulated with the aim of approaching the target values without exceeding it. Hematologic parameters depend on ethnicity, age, and gender. Even [Hb] values differ. Hence, it has been suggested to use longitudinal blood profiles together with heterogeneous factors, such as ethnicity and age, to develop models with improved sensitivity to detect blood doping. Some blood parameters, such as the concentration of Epo and reticulocytes (Ret), increase on administration of ESAs (ON-score), whereas they decrease after RBC transfusion or after the cessation of ESA administration (OFF-score). The “Abnormal Blood Profile Score” (not presently used for the assessment of abnormal blood profiles based on the passport data) regards additional red cell parameters, including the mean corpuscular Hb concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular Hb mass (MCH), Ret counts, serum Epo, and soluble transferrin receptor (sTfR). Having become effective in December 2009, the “Athlete Biologic Passport Operating
Guidelines" equip Anti-Doping Organizations with a framework in which to pursue antidoping rule violations in accordance with Article 2.2. of the World Anti-Doping (WAD) Code ("Use or Attempted Use by an Athlete of a Prohibited Substance or a Prohibited Method"). The guidelines include mandatory requirements for collection, transportation, analysis of blood samples, and results management. The following markers are considered in the Athlete Biologic Passport hematologic module: Hct, Hb, RBC count, reticulocyte percentage, reticulocyte number, MCV, MCH, and MCHC. In addition, parameters of interest can be the mean Ret cell volume (MCVr), Ret Hb concentration (MCHCr) and Ret Hb content (MCHr) [11116].

**RBC parameters associated with autologous transfusion**

The failure to obtain direct proof for autologous blood transfusion has prompted the search for indirect evidence. In a preliminary antidoping context it has been reported changes in hematologic parameters after blood withdrawal and reinfusion. Ten healthy men were subjected to withdrawal of 20 percent of their blood volume (and hence much more than common doping practice), which was replaced by 1.3 L of hydroxyl-ethyl starch. Circulating Epo increased 4-fold within a day, declining exponentially thereafter. Reticulocyte number increased 2.4-fold after 7 days, remaining elevated for another 7 days. Concentration of hemoglobin remained reduced on average by 15 percent for 2 weeks. Soluble transferrin receptor increased by 60 percent by day 14 and remained elevated until 3 days after reinfusion of 0.8 L of packed RBCs, which was performed one month later. Thereby, hemoglobin concentration increased acutely by 8 percent returning to the initial baseline value after 7 days. Epo concentrations remained unchanged, whereas reticulocyte numbers were reduced by approximately 30 percent from days 7 to 21. The loss of Hb\text{mass} of approximately 75 g (measured by CO rebreathing) after donation of 550 mL blood has been shown to be recovered after a mean of 36 days. After the retransfusion of one RBC unit Hb\text{mass} acutely increased by 51 g, showing a continuous decrease from week 2 until week 8, albeit Hb\text{mass} was still elevated compared with pre-reinfusion values. Based on the results of a retrospective longitudinal blinded study, the same group of investigators has reported that the use of an adaptive model incorporating hematologic measures allows for detection of autologous blood transfusion [11116].

Since no direct detection method for autologous blood transfusions exists, the most promising attempt is the Athlete Biological Passport (ABP) and its adaptive model that enables a longitudinal monitoring of hematologic measures to identify patterns of blood manipulations. The purpose therefore was to evaluate the performance of this adaptive model for the detection of autologous blood transfusions in a longitudinal blinded setting. Twenty-one subjects were divided into a doped group (multiple transfusions of 1-2 units of red blood cells, n=11) and a control group (n=10). The time course of a cycling season (42 weeks) was simulated including three major competitions (Classics, Grand Tour, World Championships). Up to 10 venous blood samples were ordered per subject by a blinded investigator mimicking the intelligent approach in obtaining hematologic data for the adaptive model (hemoglobin [Hb] concentration, reticulocyte percentage, OFF-score). Retrospective analysis allowed identification of four (probability >99 %) or three (probability >99.9 %) abnormal samples for Hb and eight (probability >99 %) or five (probability >99.9 %) abnormal samples for OFF-hr in doped subjects. Four doped subjects (36 %) presented an abnormal OFF-hr sequence and three doped subjects (27 %) an abnormal Hb sequence; there were no false-positive sequence results. The best possible sensitivity was 82 percent when a combination of all tests was used. The investigation provides evidence that the adaptive model allows detection of autologous blood transfusions with a good sensitivity. An intelligent testing approach and the adherence to World Anti-Doping Agency’s ABP operating guidelines are nevertheless determinants in the success [11135].
Detection of homologous blood transfusion

The aim of one study was to improve and validate a flow cytometric method for the detection of homologous blood transfusion in doping control analysis. A panel of eight different primary antibodies and two different phycoerythrin-conjugated secondary antibodies was used for the detection of different blood populations. The flow cytometer used in this study was the BD FACSArray instrument. Mixed red blood cell populations were prepared from phenotype known donors. Linearity, specificity, recovery, precision, robustness and interday-precision were tested for every primary antibody used in the presented assay. The technique of signal amplification was utilized for an improved separation of antigens with weak or heterozygous expression to improve the interpretation of histograms. The resulting method allowed to clearly identify mixed red blood cell populations in homologous blood transfusion samples containing 0.3-2.0 percent of donor blood [07114].

Subjects submitted to intravenous blood transfusions for medical reasons or blood doping to increase athletic performance are potentially exposed to the plasticizer di-(2-ethylhexyl)phthalate (DEHP) found in bags used for transfusens. Exposure to DEHP has been evaluated by measuring DEHP metabolites in selected groups of subjects. Urinary DEHP metabolites, mono-(2-ethylhexyl)phthalate, mono-(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP), and mono-(2-ethyl-5-oxohexyl)phthalate (MEOHP) were measured in a control group with no explicit known exposure to DEHP (n=30), hospitalized patients receiving blood transfusions (n=25), nontransfused hospitalized patients receiving other medical care involving plastic materials (n=39), and athletes (n=127). Patients were tested in the periods 0 to 24 and 24 to 48 hours after exposition. Urinary concentrations of all three DEHP metabolites were significantly higher in patients receiving blood transfusion than in nontransfused patients and the control group, except for MEHHP and MEOHP in the period 24 to 48 hours. Samples from four athletes showed increased concentrations of DEHP metabolites comparable to urinary concentrations of patients receiving blood transfusion. It was concluded that elevated concentrations of urinary DEHP metabolites represent increased exposure to DEHP. High concentrations of DEHP metabolites present in urine collected from athletes may suggest illegal blood transfusion and can be used as a qualitative screening measure for blood doping [09156].

A test for detection of allogeneic blood transfusion doping was implemented in 2004. The test uses blood group antisera to identify mixed RBC populations in blood samples by flow cytometry. It has been applied antisera against 12 blood group antigens and demonstrated that the presence of allogeneic cells can be assessed in the blood of subjects who had previously received at least one unit of allogeneic blood. No false-positive results were obtained in an analysis of 140 blood samples containing different percentages (0-5 %) of a minor RBC population, indicating a 100 percent specificity of the method. Most samples containing a 1.5 percent minor RBC population were unambiguously detected, yielding a 78 percent sensitivity. The method proved to fulfill the ISO-17025 accreditation and validation requirements. Athletes making use of allogeneic blood transfusion are thus very likely to be caught if tested. Autologous RBC manipulations can at present only be detected via indirect measures, which represents a major problem in antidoping efforts. The CO rebreathing technique for detecting nonphysiologic increases in Hb\text{mass} is still investigational and besides practical difficulties related to this method, its potential inclusion in the blood passport may be problematic, because the margin of variation when assessing Hb\text{mass} (biologic and measurement errors) would still allow athletes to manipulate with blood volumes that would increase exercise performance considerably. Finally, it should also be considered that, from an athlete's viewpoint, it may not be desirable to breathe CO shortly before a competition as this may limit exercise performance [11428].
Plasticizers from transfused blood

Despite the promising results and deterrence generated by the ABP, additional information enforcing anti-doping efforts concerning blood doping are desirable. The detection of atypically high concentrations of plasticizers in urine samples of athletes can be considered as indication for blood transfusion as demonstrated in several studies in the past. In a controlled blood transfusion study with 25 volunteers, blood re-infusion was conducted after 14 or 28 days with 12 and 13 participants, respectively. In a time-dependent manner, longer storage prior to re-infusion yielded higher urinary levels of plasticizer (here di(2-ethylhexyl)phthalate) metabolites, which were significantly elevated for more than 24 h post-infusion. In order to estimate the intra-individual variability of these urinary metabolites influenced by residential, dietary, or environmental exposure, a pilot study with seven volunteers was conducted over a period of seven days. Although the collective of individuals was rather small, no urinary values near those observed after blood transfusion were observed, supporting the idea of employing plasticizers for improved targeted doping controls [13012].

A totally different approach to detect ABT was recently proposed. This approach relies on increased levels of metabolites of di-2-ethyl hexyl phthalate (DEHP) in the urine. The DEHP is a chemical added to plastics such as polyvinyl chloride (PVC) to increase flexibility. The DEHP exists in the PVC matrix as a semisolid and is widely used in medical devices such as blood storage bags. The DEHP molecules migrate out of the PVC matrix into the blood products when they are kept in the blood bags. Considering the largest source of DEHP exposure in humans (ingestion), once DEHP enters the gastrointestinal tract in contaminated foods, it is rapidly hydrolyzed in the small intestine to mono(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol via pancreatic lipases. At low concentrations, most of the DEHP is absorbed as its metabolites, although at high doses, a limited amount of unmetabolized DEHP may also be absorbed. Intact DEHP that does cross into the bloodstream is rapidly converted by plasma and liver enzymatic actions to MEHP with a biologic half-life of less than 6 hours. Urinary concentrations of all 3 DEHP metabolites are significantly higher in patients receiving blood transfusions than in nontransfused patients and control subjects within the first 24 hours after transfusion. However, it must be recognized that the data collected are specific for a European population and might specifically reflect the commercial and regulatory trends in the European phthalate market. Another issue is the use of different blood products. Because of differences in solubility of DEHP in various blood products, an RBC unit would deliver more DEHP compared with a whole blood transfusion [12009].

Misuse of autologous blood transfusions in sports remains undetectable. The metabolites of the plasticizer di-(2-ethylhexyl)phthalate (DEHP) were recently proposed as markers of blood transfusion, based on high urinary concentrations of these compounds observed in patients subjected to blood transfusion. One study evaluated DEHP metabolites in urine for detecting autologous blood transfusion. One blood bag was drawn from moderately trained subjects and the red blood cells (RBCs) were reinfused after different storage periods. Group 1 (12 subjects) was reinfused after 14 days, and Group 2 (13 subjects), after 28 days of storage. Urine samples were collected before and after reinfusion for determination of the concentrations of three DEHP metabolites, mono-(2-ethylhexyl)phthalate, mono-(2-ethyl-5-hydroxyhexyl)phthalate, and mono-(2-ethyl-5-oxohexyl)phthalate. Concentrations of DEHP metabolites on the days before reinfusion were in agreement with those described after common environmental exposure. A few hours after the reinfusion a significant increase was observed for all metabolites in all volunteers. Concentrations 1 day later were still higher than before reinfusion. Variations in urine dilution supported normalization by specific gravity. Concentrations of DEHP metabolites tended to be higher after longer storage times of RBCs. It was concluded that autologous transfusion with RBCs stored in plastic bags provokes an
acute increase in the urinary concentrations of DEHP metabolites, allowing the detection of this doping malpractice. The window of detection is approximately 2 days. The method might be applied to urine samples submitted for antidoping testing [12237].

The category of illegal oxygen transfer enhancement includes several aspects such as various forms of blood doping and manipulation of oxygen uptake, transport, and delivery. Phthalate-derived plasticizers such as di(2-ethylhexyl)phthalate (DEHP) are commonly present PVC-based blood containers and released during storage into blood preparations. Upon infusion, DEHP blood concentrations increase causing elevated urine concentrations of DEHP metabolites, which were quantitatively assayed by means of isotope-dilution LC-MS/MS [12016].

Cytosolic red blood cell proteome analyze
The storage of packed red blood cells (RBCs) is associated with the development of morphological and biochemical changes leading to a reduced posttransfusion functionality and viability of the cells. Within this study, 2D DIGE and high-resolution/high-accuracy Orbitrap MS were used to analyze the storage-induced changes of the cytosolic RBC proteome and identify characteristic protein patterns and potential marker proteins for the assessment of RBC storage lesions. Leukodepleted RBC concentrates were stored according to standard blood bank conditions for 0, 7, 14, 28, and 42 days and analyzed by using a characterized and validated protocol. Following statistical evaluation, a total of 14 protein spots were found to be significantly altered after 42 days of ex vivo storage. Protein identification was accomplished by tryptic digestion and LC-MS/MS and three proteins potentially useful as biomarkers for RBC aging comprising transglutaminase 2, beta actin, and copper chaperone for superoxide dismutase were selected and validated by western blot analysis. These can serve as a basis for the development of a screening assay to detect RBC storage lesions and autologous blood doping in sports [12238].

Capillary electrophoretic separation
The use of autologous blood transfusions by endurance athletes has remained one of the most difficult doping practices to detect. The implementation of the Athlete's Biological Passport by some sporting bodies has proved to be effective; however, the analysis relies on the long-term monitoring of numerous biological markers, looking for abnormal variations in a number of biological markers to indicate doping. This work introduces an approach to identify autologous blood transfusions by examining the red blood cells (RBCs) directly. By using high-speed capillary electrophoretic separations, the relative distribution of the sizes of the RBCs in a sample can be established in under 3 min, following the preparation of the cells. As RBCs that have been stored for transfusions undergo vesiculation, the relative size of the transfused cells differs from the native cells. The capillary electrophoretic separation allows for a rapid examination of this distribution and the changes that are seen when transfused RBCs are mixed with native cells. In this work, the effectiveness of this approach is demonstrated in the identification of simulated (in vitro) autologous blood transfusions performed with blood samples from three highly trained cyclists; it was possible to rapidly identify when as little as 5% of the RBCs in the sample were from a simulated autologous transfusion [13073].

Intravascular hemolysis
Since the observation that mechanical stress causes red blood cell (RBC) destruction, foot-strike hemolysis has been used to explain sports anemia and RBC rejuvenation in athletes. Recently gained knowledge questions the importance of mechanical RBC trauma on RBC
hemolysis in athletes. Male athletes (n=90) and untrained male controls (n=58) were investigated for aerobic performance, hematological parameters, serum erythropoietin concentration (EPO), soluble transferrin receptor concentration (sTFR), and erythrocyte aspartate aminotransferase activity (eAST). On hard floor running disciplines (HFR, n=26, short- and long-distance runners, triathletes) showed a lower eAST and thus no younger RBC population than not on hard floor running athletes (NHFR, n=64, cyclists, soccer players, others) or the untrained control group (n=58). HFR had higher but still normal EPO and no higher sTFR. It was concluded that because intravascular hemolysis occurs in swimmers, cyclists, and runners, and mean RBC age is not reduced in runners, mechanisms other than foot-strike hemolysis have to be considered as well. Possible reasons are intramuscular destruction, osmotic stress, and membrane lipid peroxidation caused by free radicals released by activated leukocytes. Intravascular hemolysis can even be regarded as physiological means to provide heme and proteins for muscle growth [06148].

**Evaluation of blood parameters**

Information derived from blood analyses can assist in the detection and/or deterrence of blood doping in sport. It was investigated whether comparing an athlete's hematologic values against his or her own historical baseline rather than population-derived thresholds enhanced the ability to detect blood doping. It was developed an approach whereby an athlete's true baseline value could be estimated with just one prior blood test. It was also estimated a universal value for within-subject variability for key hematologic parameters using the highest value obtained among four separate cohorts of male athletes including 80 elite rowers, 124 endurance-trained or team-sport subjects, 288 professional football players and 630 athletes competing at national or international level. The (individual) baseline and (universal) variability were then incorporated so as to define expected thresholds for subsequent blood tests. The sensitivity of our approach was obtained by analyzing data from 49 recreational athletes administered either recombinant human erythropoietin (n=37) or placebo (n=12). It was found that removing within-subject variability by comparing new results against an historical baseline heightened the capacity to detect blood doping. It was possible to delineate the longitudinal changes in either hemoglobin (Hb) or the OFF-hr model score (an algorithm using both Hb and percent reticulocytes) caused by recombinant human erythropoietin treatment from the natural biological fluctuations found in subjects treated with placebo. The objective data supported the intuitive belief that longitudinal monitoring of athletes' blood profiles will help detect blood doping. This information could be used to instigate target-testing of suspicious athletes, or even warrant the exclusion from competition of athletes with aberrant variations in key hematologic values [06149].

Hematological manipulation to optimize aerobic performances is a serious problem in elite and professional sports and the approach to identify blood doping is as yet challenging. In most cases, the current strategy contemplates a first stage of analysis, based on the application of arbitrary threshold for hemoglobin or hematocrit, followed by second-generation blood tests, or the adoption of an individual hematological passport. To establish the influence of preanalytical variables on the athletes' hematological profile, we compared hemoglobin, hematocrit, and reticulocytes count in 27 male professional cyclists after a mean time of 2.30 ± 0.12 tourniquet holding. Statistically significant differences were observed for hematocrit (+ 2.4 %) and hemoglobin measurements (+ 1.4 %), but not for the reticulocytes count (- 1.9 %). In 4 out of 27 cases (15 %), the variability of the hematocrit measurement exceeded the 4.1 percent desirable analytical quality specification for total error. Results of the present investigation further highlight the risk that unfulfillment of rigorous and standardized procedures for collection of blood specimens might increase the number of false positive testing and might lead to inappropriate sanctioning of a minority of clean athletes with hematocrit or hemoglobin values naturally elevated. Owing to the minor
biological variability and the lesser susceptibility to variation of the preanalytical phase, the hemoglobin concentration might be a more suitable parameter than hematocrit for inclusion within laboratory testing to identify blood doping [06150].

Evaluation over time
It was assessed haematological parameters and possible modifications in elite rugby players throughout a competitive season for increasing the knowledge of physiological characteristics of these sportsmen. Blood samples were collected from the members of the Italian National rugby team at four consecutive training camps during a whole competitive season. Forty-four athletes were recruited for the first camp, 36 for the second, 30 for the third and 32 for the fourth. Due to turnover of the subjects during the season only 13 athletes could be examined at all four camps, and another six in the first three camps. Therefore, it was selected the data of these 19 athletes. Iron and transferrin saturation were stable, whilst ferritin increased at the end of the season. The modifications of the soluble transferrin receptor (sTFR) were linked to those of haematocrit: sTFR increased after training and during the competition period when haemoglobin and haematocrit decreased, and decreased at the end of the season. Haemoglobin and haematocrit showed slightly higher levels during the first part of the season and decreased in the second half, when physical demand was high, as demonstrated by biochemical additional tests. Leucocytes and platelets were stable throughout the season. Haematological and iron metabolism parameters in the elite rugby players examined during a whole season fall within physiological range of values. The variability of the parameters during the season is related to training and competition workload. Reticulocytes and sTFR are the most sensitive parameters for studying the iron metabolism of the athletes [06151].

Erythropoietin (EPO)

Overview
The synthesis of recombinant human erythropoietin has marked a turning point in the treatment of anaemia secondary to chronic kidney disease. However, the potentially fatal cardio- and cerebrovascular complications of the intake of high-doses of ESAs (erythropoiesis-stimulating agents), such as those observed in athletes who resort to doping, reason out the ever-prevalent debate concerning the balance between the risks and benefits of ESA administration for therapeutic purposes. Hence, there is still a discussion as to what values haemoglobin should ideally be maintained at. Additional concerns arise in cancer patients due to the ability of erythropoietin to act as an angiogenic and, in general, as a cell growth factor, because this might favour the progression of neoplastic disease. It was summarized the prominent points of the latest guidelines on the management of anaemia in nephropathic patients, also identifying the possible risks that may result from the tendency to aim at too low haemoglobin levels [13306].

Erythropoietin (EPO) is the main hormonal regulator of red blood cell production. Recombinant EPO has become the leading drug for treatment of anaemia from a variety of causes; however, it is sometimes misused in sport with the aim of improving performance and endurance. This paper presents an introductory overview of EPO, its receptor, and a variety of recombinant human EPOs/erythropoiesis stimulating agents (ESAs) available on the market (e.g. epoetins and their long acting analogs - darbepoetin alfa and continuous erythropoiesis receptor activator). Recent efforts to improve on EPO's pharmaceutical properties and to develop novel replacement products are also presented. In most cases, these efforts have emphasized a reduction in frequency of injections or complete elimination
of intravenous or subcutaneous injections of the hormone (biosimilars, EPO mimetic peptides, fusion proteins, endogenous EPO gene activators and gene doping). Isoelectric focusing (IEF) combined with double immunoblotting can detect the subtle differences in glycosylation/sialylation, enabling differentiation among endogenous and recombinant EPO analogues. This method, using the highly sensitive anti-EPO monoclonal antibody AE7A5, has been accepted internationally as one of the methods for detecting misuse of ESAs in sport [12241].

Erythropoietin (EPO) is a peptide hormone and another compound that is found in the blood. EPO occurs naturally in the human body. To boost the amount of EPO in the human body with the aim of improving endurance performance or to improve recovery from anaerobic exercise, some athletes (e.g. cyclists) may use recombinant EPO. Recombinant EPO is prohibited both in and out of competition under the World Anti-Doping Code Prohibited List, although raising endogenous EPO in an athlete's body through the method of high altitude training is not prohibited. Recombinant EPO use, traditionally difficult to detect in the athlete, has recently been successfully tested in athletes as a result of collaboration of WADA and the pharmaceutical companies in uncovering a molecular marker of the drug. This uncovering process helps differentiate naturally occurring EPO and those artificially introduced [12015].

Recombinant human erythropoietin (rhEPO) is arguably the most successful therapeutic application of recombinant DNA technology till date. Humoral regulation of hematopoiesis was first identified in 1906 and endogenous EPO was isolated in 1977, with its gene cloned in 1985. A series of initial clinical trials were performed to assess its effectiveness in correcting anemia of chronic kidney disease (CKD). After it proved to abrogate the transfusion requirements and improve the well-being of patients, it was granted license in 1988 as a therapeutic agent for CKD patients. Since then, it has found varied applications, especially in stimulating erythropoiesis in anemia due to chronic conditions like renal failure, myelodysplasia, infections like HIV, in prematurity, and in reducing peri-operative blood transfusions. EPO, a member of the type I cytokine superfamily, was first identified as the hormone that stimulates erythroid progenitors within the bone marrow to mature into erythrocytes. The main site of production of EPO is from the kidney and to a much lesser extent from the liver. In the kidney, certain interstitial fibroblasts appear to be a major source of EPO; however, other studies suggest an important role of proximal tubular cells as well. The human erythropoietin gene is located at chromosome 7q11-22, and consists of five exons and four introns, which produce a post-transcriptional single polypeptide backbone containing 193 amino acids. This undergoes post-translational modification with the addition of three N-glycosylation and one O-glycosylation sites and removal of 28 amino acids, resulting in a 165 amino acid polypeptide chain which is the primary structure of the mature EPO. The molecular mass of the polypeptide backbone and the glycosylated form of erythropoietin is estimated to be 18 and 30 kDa, respectively. EPO acts synergistically with other cytokines to promote the proliferation, differentiation, and survival of progenitor cells in the erythroid lineage and boosts the production of erythrocytes. It does not influence the fate of the pluripotent stem cell, but acts on the colony forming unit-erythroid (CFU-E) cells to prevent their apoptosis and induce expression of erythroid specific proteins. The EPO-R polypeptide is a 66-kDa membrane protein belonging to the cytokine receptor superfamily. The EPO binding to its receptor results in homodimerization of the receptor, followed by activation of several signal transduction pathways: JAK2/STAT5 system, G-protein (RAS), calcium channel, and kinases. A gain of function mutation in JAK2 has been reported in patients with polycythemia vera and other myeloproliferative diseases. EPO also acts on angiogenesis, vasculogenesis, regulation of vascular resistance, and neuroprotection. Various types of rhEPO are commercially available today with different dosage schedules and modes of delivery.
- erythropoietin alpha: Epoetin alpha is an isoform of recombinant DNA-derived erythropoietin (rEPO), synthesized in Chinese hamster ovary (CHO) cells. It differs from the beta isoform in its migration on isoelectric focusing (IEF) and in a range of lectin-binding assays.

- erythropoietin beta: Epoetin beta is also synthesized by CHO cell lines and differs from epoetin alpha in containing:
  - a greater proportion of more basic isoforms
  - a greater proportion of EPO binding to Erythrina cristagalli agglutinin
  - isoforms with higher in vivo: In vitro bioactivity

Their efficacy in stimulating erythropoiesis is dose dependent and differs according to the patient's disease and nutritional status. Apart from the generally recommended subcutaneous (sc) route of administration, intravenous (iv) and intraperitoneal routes have been used to administer EPO. With the increasing reports of pure red cell aplasia (PRCA) with SC route, the Department of Health in UK recommends a change EPO-alpha administration from subcutaneous to intravenous route. However, sc route has several advantages over iv route like ease of administration, non-requirement of venous access, and up to 30 percent reduction in weekly rhEPO dose on hemodialysis patients. Although patients on peritoneal dialysis may benefit from intraperitoneal route, a larger dose may be required to maintain the same hemoglobin level. Outside the uremic setting, both iv and sc rhEPO have been employed, but the sc route was used in the majority of the studies. However, there have been no studies to compare the efficacy of these routes. rhEPO can be given once, twice, or thrice weekly depending on the clinical status of the patient as per the level of hemoglobin maintained. EPO should be used carefully according to guidelines as unsolicited use can result in serious adverse effects. Because of its capacity to improve oxygenation, it has been abused by athletes participating in endurance sports and detecting this has proved to be a challenge [12242].

Stimulation of erythropoiesis is one of the most efficient ways of doping. This type of doping is advantageous for aerobic physical exercise and of particular interest to endurance athletes. Erythropoiesis, which takes place in bone marrow, is under the control of EPO, a hormone secreted primarily by the kidneys when the arterial oxygen tension decreases. In certain pathological disorders, such as chronic renal failure, the production of EPO is insufficient and results in anemia. The pharmaceutical industry has, thus, been very interested in developing drugs that stimulate erythropoiesis. With this aim, various strategies have been, and continue to be, envisaged, giving rise to an expanding range of drugs that are good candidates for doping. Anti-doping control has had to deal with this situation by developing appropriate methods for their detection. One article presented an overview of both the drugs and the corresponding methods of detection, and thus follows a roughly chronological order [12243].

Among the peptide hormone-derived therapeutics, ESAs and predominantly erythropoietin (EPO) have been subject of extensive studies concerning improved or newly established traceability as well as pure/fundamental research elucidating and later exploiting the small but significant differences between the natural human EPO and its recombinant analogs. In order to probe for the capability of routine doping control methodologies, i.e. isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) and sodium dodecyl sulfate-PAGE (SDS-PAGE), to detect epoetin kappa in human urine, an administration study was conducted. Three male volunteers received 3000 IU intravenously and urine samples were monitored for up to 48 h. Both approaches (IEF- and SDS-PAGE) allowed for the detection of epoetin kappa with SDS-PAGE being superior in terms of the detection window (24 h). While the benefits of SDS-PAGE concerning EPO analyses have been recognized several years
ago, its utility for third-generation EPO drugs was established only recently with the introduction of sarcosyl-PAGE (also referred to as SAR-PAGE). Using sarcosyl instead of SDS, enhanced antibody-antigen binding as well as improved band focusing was accomplished, which allowed for a significantly lowered detection limit of the prohibited compound in plasma and urine sports drug testing samples. The different electrophoretic behaviors of recombinant human EPO products and their natural analogs in serum and urine, which are essential to all routinely applied doping control methods, have been attributed to minor but analytically relevant modifications within the glycosidic moiety. Consequently, elucidating the nature of these modifications was of particular interest to research groups.

Focusing on recombinant human EPO, glycopeptides derived from enzymatic digests with trypsin and Glu-C were separated by capillary electrophoresis and analyzed by means of ESI-TOF MS. Here, comprehensive glycoform analysis was conducted for both N- and O-glycopeptides, allowing (among others) the identification of a sulfated sialoform of N83 in recombinant human EPO. As dictated by the employed analytical technique, only accurate masses (errors varied up to 30 ppm) of the protonated intact analytes, as well as respective adduct ions, served for characterization purposes. Following a different strategy, namely sequential deglycosylation by exoglycosidase treatment and subsequent SDS-PAGE analysis, human urinary and serum EPO as well as recombinant EPO were investigated concerning their glycosylation pattern. While EPO from all three sources was amenable to degradation by b-N-acetylglucosaminidase, the subsequent incubation with alpha- or betamannosidase did affect only recombinant EPO, demonstrating a distinct difference in the glycosidic moiety potentially offering a new angle for future doping control assays. Aiming at a fast alternative to conventional EPO doping control tests, the utility of the recently introduced membrane-assisted isoform immunoassay (MAIIA) combined with wheat germ agglutinin (WGA)-based chromatographic separation of recombinant as well as human urinary and serum EPO was evaluated. Employing immuno-magnetic beads-based extraction (IMBE) combined with capillary zone electrophoresis and deep UV laser-induced fluorescence detection, the highly resolved glycoform profiling of EPO was accomplished for pharmaceutical preparations. The approach can support studies concerning isoform composition studies in recombinant EPO products but the sensitivity was not found sufficient for the analysis of urine or serum and the method considered prone to matrix interference; hence, an introduction into sports drug testing programs is not expected [12017].

Due to the complexity of detecting and differentiating recombinant EPO from its natural analog in human urine or blood, alternative indirect approaches have been subject of various recent studies. One of these elucidated the impact of EPO administration on circulating and/or renally eliminated microRNAs (miRNA) and their potential as long-term biomarkers for ESA doping. Following the intravenous or subcutaneous administration of Mircera (200 mg), plasma samples were collected for up to 27 days and analyzed for miRNA affected in a statistically significant manner. Among a variety of marker candidates, miR-144 was the most influenced parameter, which was significantly elevated 27 days post-administration of Mircera and thus possibly representing a valuable alternative marker for illicitly administered ESAs. These preliminary findings will require in-depth elucidation and validation but possess the potential for expanded complementary doping control assays. Hepcidin has been considered as another potential marker for EPO administration due to its decreased serum concentration following subcutaneous EPO injections. Whether the effect also prevails upon i.v. applications of 50 IU/kg bodyweight was subject of another study. Despite significantly elevated (4 h post administration) and subsequently decreased (24 h post administration) serum hepcidin levels, the dynamics and variability of the marker essentially excluded its utility as indicator for doping control purposes [12017].

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Erythropoietin (EPO) is the main hormonal regulator of red blood cell production. Recombinant EPO has become the leading drug for treatment of anaemia from a variety of causes; however, it is sometimes misused in sport with the aim of improving performance and endurance. This paper presents an introductory overview of EPO, its receptor, and a variety of recombinant human EPOs/erythropoiesis stimulating agents (ESAs) available on the market (e.g. epoetins and their long acting analogs – darbepoetin alfa and continuous erythropoiesis receptor activator). Recent efforts to improve on EPO's pharmaceutical properties and to develop novel replacement products are also presented. In most cases, these efforts have emphasized a reduction in frequency of injections or complete elimination of intravenous or subcutaneous injections of the hormone (biosimilars, EPO mimetic peptides, fusion proteins, endogenous EPO gene activators and gene doping). Isoelectric focusing (IEF) combined with double immunoblotting can detect the subtle differences in glycosylation/sialylation, enabling differentiation among endogenous and recombinant EPO analogues. This method, using the highly sensitive anti-EPO monoclonal antibody AE7A5, has been accepted internationally as one of the methods for detecting misuse of ESAs in sport [12240].

Although the concept of a humoral regulator of erythropoiesis was introduced more than 100 years ago, its existence was first firmly established a little more than 50 years ago. One review briefly describes the historical development of information about erythropoietin. It then describes our current understanding of where erythropoietin is produced; the factors that regulate its rate of production; how erythropoietin acts at the cellular level to stimulate erythropoiesis; and its role in the regulation of the rate of erythropoiesis. Finally, it discusses the clinical uses of erythropoietin in the diagnosis and therapy of hematopoietic diseases [09157].

A review covered landmarks or milestones in the development of erythropoietin (EPO). Thirty-nine landmark advances have been identified, which cover the period 1863-2003. Several reports are included that directly support these original landmark advances. The areas of EPO research updated are: sites of production; purification, assay and standardization; regulation; action; use in anemias; extraerythropoietic actions; adverse effects; and blood doping. The new reports on the use of EPO in the therapy of myocardial infarction; stroke and other neurological diseases; diabetic retinopathy and other retinal diseases are also covered [10467].

Erythropoietin. EPO, is a 165 amino acid, 34 kDa glycoprotein hormone produced by the kidney in response to low blood oxygenation which causes an increase in circulating red blood cells by stimulating the division and differentiation of erythroid progenitor cells in the
bone marrow. EPO consists of various isoforms which are made up of different complex sugars that are linked by a single polypeptide chain. The carbohydrate moieties make up 40 percent of its molecular weight. Epoetin, the recombinant human erythropoietin (rhEPO) was approved by the Food and Drug Administration for clinical use in 1989. The various EPO pharmaceutical preparations are based on differences in their carbohydrate moieties [07002].

To overcome the limitation of the currently adopted direct method to detect recombinant Human Erythropoietin (rHuEpo) abuse in sport, indirect analysis of blood parameters are increasingly used as part of the anti-doping strategies. The aim of one work was to identify whether immunophenotype modifications on erythroid cells may be indicative of previous rHuEPO administration. The study was conducted on dialyzed patients under treatment with rHuEPO (DPT). Dialyzed patients without rHuEPO therapy (DP) and volunteer donors (H) were used as controls. The analysis of erythroid cells immunophenotype, performed using a multiparametric flow cytometry technique, showed a peculiar pattern of CD71 expression following rHuEPO treatment. In particular CD71 showed an increased expression in mature and intermediate reticulocytes and a surprisingly decreased expression in immature reticulocytes. In conclusion, the analysis of reticulocyte maturation stages with TO/CD71 double staining may be considered as a valid alternative indirect method for the detection of rHuEPO abuse [07115].

Endogenous and exogenous erythropoietin (EPO) present in urine can be distinguished according to their isoelectric profiles. This methodology requires urine samples to be concentrated about 200 to 1000 times with manipulations that should remove most of the cells occurring in the original sample. In one study, it was tried to obtain DNA profiles from 10 ultrafiltered urines (retentates) in order to evaluate whether a formal genetic identification was technically feasible. No nuclear DNA profiles could be established from retentates, despite 34 PCR-cycles amplifications. Contrastingly, mitochondrial DNA (mtDNA) profiles were obtained for 9 out of the 10 retentates. Apart from some particularities, retentate mtDNA profiles were all distinct and matched mtDNA profiles of corresponding reference samples [07116].

Epo is a naturally occurring hormone. Its existence was suspected almost a century ago by Carnot and Deflandre, who postulated that a humoral factor, which they called “hemopoietine,” regulates RBC production. The definitive existence of Epo was demonstrated by Krumdieck in 1943. The sequence encoding for human Epo is located on chromosome 7. Human Epo, a 165-amino acid glycoprotein with a molecular weight of 34 kDa, is derived from a 193-amino acid transcript that has been modified by the cleavage of a 27-amino acid leader sequence and the loss of the carboxyl-terminal arginine by post-translational processing by an intracellular carboxypeptidase. After synthesis, N-linked carbohydrate chains with terminal sialic acids prevent the immediate hepatic clearance of the molecule. Despite few sequence homologies, the tertiary structure of Epo is similar to that of other growth hormones and cytokines such as growth hormone, prolactin, interleukin 6, and granulocyte colony-stimulating factor. The human erythropoietin gene was originally cloned in the mid 1980s, and rHuEpo was first approved for marketing in France in 1988. Its therapeutic use for the treatment of several forms of chronic anemia was shown to be effective and safe. Thus far, several mammalian Epo genes have been cloned, sequenced, and expressed. Epo is highly conserved among mammals, and there is a high degree of sequence homology in the coding region of the mature secreted protein. More than 63 percent of the molecule is composed of invariant amino acids (106 residues); human and monkey Epos display 94 percent and 91 percent sequence homology in nucleotides and amino acid composition, respectively. In contrast, human and mouse Epos are 76 percent identical in nucleotide sequence and 80 percent identical in amino acid sequence. There is also increasing evidence that many non-hematopoietic organs and tissues, such as stem
Genetically engineered erythropoietin (Epo) has been systematically administered to anemic chronic renal failure patients for the past 15 years. By the mid-1990s, it had also become clear that Epo was the drug of choice among endurance athletes seeking to improve performance. From a technical perspective, Epo has several advantages over blood transfusion, including no complex logistically challenging maneuvers such as blood withdrawal, storage, and reinfusion. In addition, there is no decay in performance or training after a period of blood withdrawal, and there is limited “detectability,” as Epo is a naturally occurring peptide hormone. A considerable amount of clinical evidence supported the use of rHuEpo for doping purposes, as it provided significant erythropoietic and ergogenic benefits due to substantial increases in hemoglobin, hematocrit, maximal oxygen uptake, and exercise endurance time. As testing strategies for Epo are as important as the development of reliable analytical procedures, detailed knowledge of the pharmacokinetic characteristics of rHuEpo is essential both for interpreting changes observed in indirect markers of administration and for planning suitable methods of testing for direct identification. Following subcutaneous administration, a “flip-flop” phenomenon can be observed; the pharmacokinetics appear to be linear from 50 to 1000 U/kg, but not for a lower dose of 10 U/kg. The mean half-life of 50 IU/kg daily repeated subcutaneous administration of rHuEpo is about 36 h, and the total clearance is 17 ml/h/kg. Remarkably, the total clearance appears nearly three times higher in athletes than in untrained subjects (6.5 ml/h/kg). The half-life is five times longer following subcutaneous than intravenous administration (from 4 to 7 h). The administration of rHuEpo is typically accompanied by a delayed increase in hemoglobin (up to 9.6 %), hematocrit (up to 8.3 %), reticulocytes, macrocytes, serum Epo, and soluble transferrin receptor (sTfr) concentrations. The increase of these markers becomes significant from the third to the tenth day following initial administration, and it is always followed by a negative and transitory feedback loop of endogenous Epo production, which can be interpreted as an indirect marker of rHuEpo administration. In clinical practice, rHuEpo administration produces a remarkable acceleration of the dynamic response of maximal oxygen uptake to submaximal exercise, increasing exercise capacity [10353].

Several variants of rHuEpo are currently available. The term “erythropoietin” conventionally refers to rHuEpo preparations that have an amino acid sequence homologous to that of the naturally occurring hormone, whereas the appropriate Greek letters, “alpha,” “beta,” “gamma,” and “delta” designate preparations that differ in composition and/or the nature of the carbohydrate moieties. Epoetin alfa (Epogen®, Eprex®, Epoxitin®, Erypo®, Espo®, Globuren®, Procrit®) and epoetin beta (Epogen®, Marogen®, NeoRecormon®, Recormon®), which are produced by transfected Chinese hamster ovary cells and are nearly biochemically and immunologically identical to their naturally occurring counterpart, display similar molecular and pharmacokinetic characteristics, although the latter has a higher molecular weight, a lower number of sialylated glycan residues, and, possibly, slight pharmacokinetic advantages, such as a longer terminal elimination half-life. Epoetin beta has been the subject of studies aimed at proving efficacy with a reduced administration frequency, but results are rather controversial. Epoetin gamma is produced in a different host cell than the other erythropoietic agents; therefore, its glycosylation pattern and pharmacokinetics are different. Small-scale clinical studies found epoetin gamma to be slightly more biologically active than epoetin alfa. Epoetin delta (Dynepo®) is a recently approved rHuEpo produced by human cells engineered to transcribe and translate the Epo gene under the control of a newly introduced regulatory DNA sequence. Epoetin delta has the same amino acid sequence and glycosylation pattern as human Epo. Because it has become available recently, only limited clinical data are available. The pharmacodynamic response to two to three times weekly 40 to 100 IU/kg epoetin delta administration appears to be consistent with known Epo cells; the embryo proper, including its developing nervous system; brain; uterus; and ovary express Epo [10353].
pharmacological activity and includes a dose-dependent increase in hematocrit (between 0.12 and 0.18 %/day), hemoglobin, reticulocytes, and RBCs for at least 3 to 4 weeks, followed by a decline toward baseline values with exposure cessation. A tendency for efficacy to fall with time, which may be associated with the development of neutralizing antibodies or tolerance, has been observed. The mean half-life of subcutaneous administration is 18 to 20 h, compared to 7 to 12 h following intravenous injection [10353].

As an increase in the carbohydrate content of Epo results in a longer plasma half-life and enhanced biological activity, more effective analogs have been developed. Darbepoetin alfa, a novel erythropoiesis-stimulating protein or NESP (Aranesp®), is a glycosylation analog of rHuEPO that has been synthesized using DNA technology. Its molecular weight is 38.5 kDa, and its total carbohydrate content is 52 percent. The amino acid sequence of NESP differs from that of native human Epo at five positions (Ala30Asn, His32Thr, Pro87Val, Trp88Asn, and Pro90Thr); this allows the attachment of additional oligosaccharides at asparagine residue positions 30 and 88. Therefore, NESP contains five N-linked oligosaccharide chains and 22 sialic acid residues, whereas native Epo contains three oligosaccharide chains and 14 sialic acid residues. NESP binds to the Epo receptor in the same fashion as native Epo and induces intracellular signaling. In comparing the pharmacokinetics of NESP and rHuEPO, the mean half-life for NESP is nearly two to three times longer than that of rHuEPO (25 and 49 h when administered intravenously and subcutaneously, respectively). Despite a similar volume of distribution of nearby 50 ml/kg, the clearance of NESP is significantly reduced as compared to that of rHuEPO (1.6 ± 0.3 versus 4.0 ± 0.3 ml/h/kg). Dosage requirements of darbepoetin do not differ between the intravenous and subcutaneous routes of administration. The less frequent administration of darbepoetin alfa in comparison to the other epoetins may reduce drug costs in the long term, but the variability in dosage or dosage frequency required within a single patient is much higher [10353].

CERA (continuous erythropoietin receptor activator) is the latest erythropoiesis-stimulating protein that has become commercially available. Administered at a considerably less frequent dosing (once every 3 or 4 weeks), CERA induces enhanced and sustained erythropoietic effects through continuous modulated stimulation of erythropoiesis and offers greater flexibility and convenience as compared to other conventional blood-boosting substances. Postulated mechanisms of action include a weaker binding to and a more rapid dissociation from the Epo receptor and an extended half-life in plasma, which has been reported to be seven-fold greater than that of standard epoetins. At doses of 5 to 8 microg/kg, CERA increases reticulocytes and hemoglobin by 262 percent and 16 to 23 g/L (over 50 % of patients had a response ≥ 20 g/L), respectively; these levels were maintained over the next 12 weeks. Levels of sTfr also increase in a dose-dependent manner, whereas serum ferritin and serum iron levels decrease, reaching a nadir 5 to 10 days after administration before returning to baseline. The mean elimination half-life is 133 to 137 h for both intravenous and subcutaneous administration. Side effects of CERA therapy are generally mild, and no serious adverse events have been reported thus far. CERA is currently in phase III clinical trials [10353].

Approximately 90 percent of endogenous Epo is produced by the kidney in response to tissue oxygen sensors that register oxygen depletion. The recent discovery of a novel family of proteins called “hypoxia inducible factors” (HIF) has increased our understanding of the complex mechanism of response to hypoxia and Epo production that occurs when the human body has to deal with increased oxygen demand, such as in hard-working muscles. Epo is the paradigm of oxygen-regulated genes controlled by transcription factor HIF-1, well recognized as the key regulator of cellular and systemic oxygen homeostasis. Despite its clinical effectiveness in selected clinical settings, concern has been expressed about complications and side effects of rHuEpo therapy. In both controlled and uncontrolled clinical
studies, the most frequent adverse events per 100 patient-years of exposure to epoetin delta were hypotension, muscle cramps, upper respiratory infections, headache, thrombosis, and hypertension. An important concern is that the cardiovascular system of an athlete misusing erythropoiesis-stimulating proteins may be in jeopardy. In some clinical trials, thrombotic events such as myocardial infarction, cerebrovascular disease, transient ischemic attack, and venous thromboembolism occurred at a rate of 0.04 events per patient-year. In addition, there have been occasional reports of serious or unusual thromboembolic events, including migratory thrombophlebitis, microvascular thrombosis, and thrombosis of the cerebral sinus, retinal artery, and temporal and renal veins. Endothelial activation, alterations of blood rheology (namely, increased blood viscosity), increased systolic blood pressure at submaximal exercise, and platelet reactivity were acknowledged as important mechanisms involved in the thrombotic potential of Epo. Although an unequivocal causal relationship with rHuEpo therapy has not been established yet, polycythemic conditions characterized by hematocrit values exceeding 60 percent, as were anecdotally achieved in the mid-1990s when rHuEpo abuse was largely uncontrolled, when compounded by dehydration, may have predisposed athletes to thromboembolic complications. Far more threatening is the onset of red cell aplasia, a rare congenital or acquired condition characterized by an arrest in RBC production. A final concern arises from the evidence that the Epo receptor pathway may be somehow involved in the growth, viability, and angiogenesis of malignant tumors. Increasing evidence has accumulated to show that Epo has pleotropic effects beyond regulation of the RBC mass. In the embryo, Epo acts as a major regulator of vascular formation and organ growth, and Epo receptors are found in almost every embryonic tissue.

As exogenous recombinant Epo is less negatively charged than the naturally occurring hormone, isoelectric focusing has emerged as a reliable approach for direct detection of rHuEpo and analogs in urine. Electropherograms from urinary rHuEpo differ substantially from those of endogenous Epo, as the former produce four or five bands in the basic region, whereas the latter gives rise to up to 14 bands that overlap with and are parallel to those of rHuEpo in the basic region but that are also clustered in the acidic region. A preliminary electrophoretic technique for discriminating rHuEpo from its endogenous counterpart in urine was described in 1995. The method was improved by using isoelectric focusing that reduced the non-specific binding traditionally accompanying immunoblotting. This isoelectric focusing technique detects rHuEpo in urine samples collected 3 days after nine doses of epoetin alfa with both 100 percent sensitivity and specificity. However, 7 days after the last dose, the overall sensitivity of the techniques falls to 50 percent. The indirect approach, based on multiple markers of enhanced erythropoiesis, appears to be a valid and reliable strategy when complemented by the urine confirmatory test. The combination of blood and urine tests represented the strategy implemented by the International Olympic Committee for detecting rHuEpo misuse at the Sydney Olympics. However, in analogy with other performance-enhancing drugs, during-competition testing has led to much wasted testing effort, as the pharmacodynamic properties of rHuEpo and its analogs discourage misuse near or at the time of competition. Thus, direct testing methods, such as the rHuEpo urine test or the ON-model, will likely fail due to the almost complete elimination of rHuEpo before the test and the return to baseline of most biochemical parameters of erythropoiesis, unless international sporting federations use the information gathered to assist in targeted out-of-competition testing. However, as the performance-enhancing effect is greater than variation of hematologic changes and persistence of rHuEpo in blood, the indirect OFF model, based on the expression of specific genes following rHuEpo administration, should partially overcome this limitation. Fortunately, Epo analogs appear to be more easily detectable, and some athletes were sanctioned for use of NESP at the 2002 Winter Olympics. Most techniques measure the increased immunoactivity of NESP in serum following desialylation with neuraminidase, as measured by conventional immunoassays for Epo. The method requires a
small amount of blood, is simple to perform, is reliable (up to 100% sensitivity), and allows
detection of NESP from 2 to 14 days after the last injection [10353].

A method was described to isolate human erythropoietin (hEPO) from plasma using an EPO-
specific immunoaffinity micro well plate (IAP). The operating conditions of the method
(binding, blocking and elution) were optimised to avoid isoform discrimination and cross-
contamination with other glycoproteins. The overall hEPO recovery was about 56 percent
and significant clean-up for plasmatic hEPO was achieved. Polyvinylpyrrolidone (PVP) was
used as a blocking reagent and elution took place at pH 11.0. Under these conditions all
isoforms from recombinant human EPOs (rhEPOs) and analogues were uniformly recovered
guaranteeing lack of discrimination. The resulting procedure allowed isolating erythropoietin
from plasma in conditions amenable to hEPO analysis by other techniques such as SDS-
PAGE or IEF. Moreover, avoiding contamination with other glycosylated material allowed the
identification in human plasma samples of the non-human N-glycolyl-neuraminic acid
(Neu5Gc) using HPLC-FLD. Neu5Gc is present as 1-2 percent of the sialic acid content in
rhEPO so this approach could be used to unequivocally detect abuse of rhEPOs or
analogues as part of the doping control [10358].

Numerous factors involved in general homeostasis are able to modulate ventilation.
Classically, this comprises several kinds of molecules, including neurotransmitters and
steroids that are necessary for fine tuning ventilation under different conditions such as
sleep, exercise, and acclimatization to high altitude. Recently, however, it has been found
that erythropoietin, the main regulator of red blood cell production, influences both central
(brainstem) and peripheral (carotid bodies) respiratory centers when the organism is
exposed to hypoxic conditions [09158].

Erythropoietin is a naturally occurring hormone produced by the kidney that stimulates red
blood cell production in the bone marrow in response to low circulating oxygen levels. It was
not until 1977 that it was identified and extracted from human urine. This was concurrent with
the development of recombinant DNA technology, and in 1989 Epogen® was released in the
United States and approved for the treatment of anemia. Procrit® was licensed in 1991 for
the treatment of chemotherapy-induced anemia. European formulations include Aranesp®,
Eprex® and NeoRecormon®. Erythropoietin used for medical treatments is administered by
intravenous or subcutaneous injection [08006].

Endurance can be dramatically enhanced by artificially elevating the red blood cell mass, as
seen in the “blood-doping” strategies used by some athletes. By reinfusing previously stored
red blood cells, or by stimulating erythropoiesis with the use of synthetic erythropoietin,
known as EPO, performance can be surreptitiously increased. Detecting such manipulation
is not easy. Erythropoietin products leave the system quickly, and autologous transfusions of
blood cells are difficult to identify. Attempts to identify elevated hematocrit levels in the hours
before competition led to the masking of such practices with intravenous hemodilution
techniques. What has not gone unnoticed is the catastrophic loss of life attributed to
erythropoietin in many sports – particularly cycling, which many have come to regard as a
veritable incubator of doping techniques [08218, 08219].

Erythropoietin abuse in sport was believed to start as soon as the drug was available as a
replacement for the more complex and dangerous doping technique referred to as blood
doping [011]. When a network of blood transfusion for doping purposes was detected in
Spain in 2006, it led to the seizure of samples of whole blood, plasma, and red blood cell
concentrates for autologous blood transfusion, allegedly belonging to elite sportsmen from
different countries. High (supra-physiological) concentrations of erythropoietin were found in
some plasma samples. The finding of rEPO in samples connected to a blood transfusion
doping network shows that to administer recombinant erythropoietin prior to blood withdrawal and storage may be current practice also for doping [08220].

Erythropoietin leads to the production of red blood cells. Since these carry oxygen to active muscles, one would consider enhanced endurance performance because of the additional flux of oxygen. EPO is a circulating glycosylated protein hormone that is the principal regulator of erythropoiesis. It is produced primarily by the kidney inversely related to the concentration of O$_2$ in the blood. Following administration, there is a direct relationship between haemoglobin levels and increased performance following administration of rHuEPO in rats and humans. Methods used in doping include hypoxia and hypoxia-mimetics. One such method is to train at altitude; however, one cannot necessarily train as hard as at lower altitude because of hypoxic-mediated fatigue. Variations on this theme include living at altitude and training at sea level or sleeping in a tent or chamber with diminished oxygen tension (at lower altitude) and training at the same elevation. These methods are not considered doping [10001].

Erythropoietin (EPO), a glycoprotein hormone, stimulates the growth of red blood cells and as a consequence it increases tissue oxygenation. This performance enhancing effect is responsible for the ban of erythropoietin in sports since 1990. Especially its recombinant synthesis led to the abuse of this hormone, predominantly in endurance sports. The analytical differentiation of endogenously produced erythropoietin from its recombinant counterpart by using isoelectric focusing and double blotting is a milestone in the detection of doping with recombinant erythropoietin. However, various analogous of the initial recombinant products, not always easily detectable by the standard IEF-method, necessitate the development of analytical alternatives for the detection of EPO doping [10114].

rHuEPO and its follow-on biological relatives can provide an effective mechanism to stimulate erythropoiesis as noted above; however, the baseline hematocrit increases and may rise even more to dangerous levels, likely due to dehydration, in athletes during and after training and competition. The rheology of blood changes exponentially as the hematocrit rises above 55 percent and accelerates even more rapidly as it rise above 60 percent. Deaths in competitive cyclists have been directly linked to changes in the flow properties of blood, as the hematocrit rises. There are no studies of rHuEPO, or its related proteins in child or adolescent athletes, but theoretically the responses should be no different from older adolescents or young adults. The major issues are those that relate to increased hematocrit- sluggish blood flow in the small vessels of critical organs and pulmonary emboli [10001].

Haemoglobin concentration ([Hb]), reticulocyte percentage (retic%) and OFF(hr score) are well-implemented screening tools to determine potential recombinant human erythropoietin (rHuEpo) abuse in athletes. Recently, the International Cycling Union implemented the OFF(z score) and the Hb(z score) in their anti-doping testing programme. The aim of one study was to evaluate the sensitivity of these indirect screening methods. Twenty-four human subjects divided into three groups with eight subjects each were injected with rHuEpo. Group 1 and group 2 received rHuEpo for a 4-week period with 2 weeks of "boosting" followed by 2 weeks of "maintenance" and a wash-out period of 3 weeks. Group 3 received rHuEpo for a 10-week period (boost in 3 weeks; maintenance in 7 weeks; wash out in 1 week). Three, seven and eight of the 24 volunteers exceeded the cut-off limits for OFF(hr score), [Hb] and retic%, respectively. One subject from group 1, nobody from group 2, and seven subjects from group 3 exceeded the cut-off limit for Hb(z score.) In total, ten subjects exceeded the cut-off limit for the OFF(z score); two subjects from group 1, two subjects from group 2 and six subjects from group 3. In total, indirect screening methods were able to indicate rHuEpo injections in 58 percent of subjects, i.e. 42 percent of our rHuEpo-injected subjects were not
detected. It should be emphasised that the test frequency in real world anti-doping is far less than the present study, and hence the detection rate will be lower [10115].

Stimulation of erythropoiesis by the third-generation erythropoietin drug CERA, a pegylated derivative of epoetin beta, has provided valuable therapeutic benefits to patients suffering from renal anemia, but has also rapidly found application as an illicit performance-enhancing strategy in endurance sports. It was presented a novel method for selective determination of CERA in serum, based on polyethylene glycol precipitation followed by a commercial homogeneous immunoassay. The developed method was highly discriminating between serum samples from CERA-treated patients and control subjects, as the covalently linked polyethylene glycol chain in CERA strongly enhanced the solubility of the protein in a polyethylene glycol-containing medium. Intravenous administration of CERA could be detected for several weeks in the majority of subjects tested. This assay outperforms the currently available CERA detection methods in terms of simplicity, convenience, cost, and throughput, making it ideal as a screening tool for doping control [10116].

The tampering of athlete's urine samples by the addition of proteolytic enzymes during the doping control sampling procedure was reported recently. The aim of one study was the application of a stabilization mixture in urine samples to chemically inactivate proteolytic enzymes and improve the electrophoretic signal of erythropoietin (EPO) in human urine. The stabilization mixture applied was a combination of antibiotics, antimycotic substances and protease inhibitors. A series of incubation experiments were conducted under controlled conditions in the presence and absence of the stabilization mixture in urine aliquots spiked with six proteases. Two different analytical techniques were applied for the qualitative and quantitative EPO measurement: isoelectric focusing (IEF) and chemiluminescent immunoassay respectively. The addition of the chemical stabilization mixture into urine aliquots substantially improved EPO detection in the presence of proteolytic enzymes following incubation at 37 degrees C or storage at -20 degrees C. The results of this study indicated that the stabilization of urine prior to the sample collection procedure with the proposed chemical mixture might prove to be a useful tool for the preservation of anti-doping samples [10117].

A screening method able to differentiate recombinant human erythropoietins (rhEPOs) and analogues like CERA from human urinary erythropoietin (uhEPO) is described. The method is based on the discrimination between isoforms observed when the protein is eluted under acidic followed by basic conditions from immunoaffinity microtiter wells. From a comparison with the complex IEF protocol currently applied in anti-doping analysis, the newly developed assay procedure is amenable to wide screening application and presents good resolving power between rhEPOs and uhEPO [10118].

Erythropoietin (EPO) is an endogenous hormone produced primarily by the kidney which controls the production of erythrocytes. The main stimulus to production is low tissue oxygen (hypoxia) and EPO triggers the formation of red blood cells by binding to a receptor on erythroid progenitor target cells. Alteration in the EPO regulatory system produces a change in circulating EPO in a variety of disease states, such as renal anaemia and polycythaemia. The availability of recombinant EPO in the 1980s transformed the treatment of anaemia, particularly anaemia of end stage renal disease, and led to the development of more sensitive and specific assays for the measurement of EPO. There are more widespread uses for EPO and preliminary studies indicate that EPO may be useful as a neuroprotective agent by reducing inflammation near the site of injury. The use of EPO to boost endurance in athletes has attracted unwanted publicity, although analytical techniques are now available that can differentiate between endogenous and recombinant EPO. Different types of erythropoietic agents have been developed with a longer plasma half-life and the ability to
In recent years the use of erythropoietin has exploded, and the anemia of patients with chronic renal failure has been practically resolved with the administration of rHuEpo (recombinant human, Erythropoietin). However, as a result of an intense commercial campaign, strong therapies with this growth hormone, prescribed to achieve surprising sporting performances, got athletes to run the risk of thrombosis and vascular accidents because of red blood cells increase. Erythropoietin represents a significant subject of research. In fact, besides the ability of stimulating erythrocyte production, it has many pleiotropic effects. Several studies allow the assertion that EPO, in different concentrations, has protective effects mainly on central nervous system and cardiovascular system through various mechanisms, among which a key role seems to be held by the ability to stimulate angiogenesis. The consequent problem is that anaemia therapy with rHuEpo in patients with cancer may accelerate the progression of neoplastic disease by promoting tumour angiogenesis and, thus, metastasization. The study of angiogenic process in tumours led to the synthesis of drugs that, blocking VEGF, exert an anti-angiogenic action, contrasting cancer spread. However, benefits are relatively modest. Is erythropoietin perhaps the further angiogenic hormone to block in tumour pathology? Therefore, Epo plays a role in regenerative medicine since it intervenes in a persistent natural regenerative activity of humans: angiogenesis. The understanding of the regeneration mechanisms of complex structures in the adult salamander has opened original lines of research. Regenerative medicine tries to develop therapeutic pathways through the stimulation of natural regenerative processes in humans [09159].

**Physiology**

In addition to its function as a regulator of hematopoiesis, the cytokine erythropoietin (Epo) initiates adaptive cellular responses to both moderate environmental challenges and tissue damaging insults in various non-hematopoietic mammalian tissues. Epo's neuroprotective and neuroregenerative functions mediated through janus kinases (JAK)/signal transducers and activators of transcription (STAT) transduction pathways and regulation of Epo and Epo receptor expression in the nervous system by hypoxia inducible factor (HIF) have been documented in a variety of in vitro and in vivo studies and homologs of the human Epo gene are present in fish, amphibians and mammals. One study reproduced the hallmarks of Epo-mediated mammalian neuroprotection in the grasshopper nervous system. Recombinant human Epo (rhEpo) increases the survival of dissociated grasshopper brain neurons under normoxic and hypoxic conditions and promotes the regeneration of neurites in vitro. In addition, reestablishment of sound source localization after unilateral tympanic nerve crush injury was accelerated and more complete after application of rhEpo, demonstrating in vivo support of auditory receptor cell axon regeneration. Immunoblots of central nervous tissue extracts from mouse, grasshopper, crayfish and leech labeled protein bands of about 38 kDa, fitting to the molecular weight of Epo reported in earlier studies. These results indicate that a ligand/receptor system that shares structural and functional similarities with mammalian Epo and Epo receptor exerts neuroprotective and neuroregenerative effects in insects. With both upstream (HIF system) and downstream (JAK/STAT pathway) elements of the mammalian Epo system being present in insects and other invertebrates, Epo-like signaling involved in tissue protection appears to be an ancient beneficial function shared by vertebrates and invertebrates [11338].

Erythropoietin (EPO) is a 30 400 molecular weight glycoprotein hormone produced mainly in the kidney, and also in the liver (<10 %) and, in very little quantities, in the brain. The
physiological stimulus for EPO production is tissue hypoxia, which, in the large majority of instances, is directly related to the number of circulating erythrocytes. Thus, EPO and erythropoiesis are part of a negative feedback cycle that keeps tissue oxygen delivery within a narrow range by controlling the number of erythrocytes circulating in the blood. In a normal individual, any loss of erythrocytes, such as by bleeding or haemolysis, decreases delivery of oxygen to the tissues. When this tissue hypoxia is sensed by cells in the kidney and liver capable of producing EPO, they produce and secrete EPO into the plasma. EPO is carried to the bone marrow, where it binds to specific cell surface receptors on its target cells: the CFU-E, pro-erythroblasts, and basophilic erythroblasts. The binding of EPO by these cells increases their ability to survive and reach the reticulocyte stage and thereby contribute to the population of circulating erythrocytes. The increased numbers of circulating erythrocytes in turn deliver more oxygen to the tissues. This increased oxygen delivery is sensed by the EPO producing cells, which then reduce EPO production so that the normal steady state number of erythrocytes is restored [06145].

Effects of hypoxia
The response of the kidneys to hypoxia has been shown to be exponential; that is, in individuals with a normal capacity to produce EPO, a linear decline in haematocrit is accompanied by an exponential increase in plasma EPO levels. This exponential increase is not based on the release of stored, preformed EPO. Rather, the hypoxia is sensed by an intracellular molecule that interacts with an enhancer element of the Epo gene and thereby induces transcription of the gene. The increase in EPO production in the hypoxic kidney is achieved by recruitment of more cells to produce EPO. The EPO producing cells of the kidney are a minor subset of cortical interstitial cells. Under normal conditions, only a few scattered cells produce EPO. When a threshold level of hypoxia is achieved, the cells capable of producing EPO do so at a maximal rate. The greater the areas of renal cortex in which the hypoxia threshold is met, the greater the number of cells that produce EPO [06145].

Mechanism of action of erythropoietin
In the bone marrow, EPO binds to receptors displayed on the cell surface of CFU-E, proerythroblasts, and basophilic erythroblasts. The mature EPO receptor, with a molecular weight of approximately 72,000, is a transmembrane glycoprotein, a member of a much larger family of receptors of cytokines and haematopoietic growth factors. The effect of EPO binding to its receptor, in terms of cellular physiology, has been shown to be the prevention of programmed cell death (apoptosis). In multiple systems of erythropoiesis, EPO has been shown to be a survival factor for the erythroid cells in the later stages of differentiation from the CFU-E through basophilic erythroblasts. Although an effect of EPO on mitosis has been reported for BFU-E and an EPO dependent cell line, EPO is required only for CFU-E and later stages, and apoptosis appears to result when EPO signalling cannot occur [06145].

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*Different ways of action*

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Erythropoietin (EPO), the main hemopoietic hormone synthesized by the kidney as well as by the liver in fetal life, is implicated in mammalian erythropoiesis. Production and secretion of EPO and the expression of its receptor (EPO-R) are regulated by tissue oxygenation. EPO and EPO-R, expressed in several tissues, exert pleiotropic activities and have different effects on nonhemopoietic cells. EPO is a cytokine with antiapoptotic activity and plays a potential neuroprotective and cardioprotective role against ischemia. EPO is also involved in angiogenesis, neurogenesis, and the immune response. EPO can prevent metabolic alterations, neuronal and vascular degeneration, and inflammatory cell activation. Consequently, EPO may be of therapeutic use for a variety of disorders. Many tumors express EPO and/or EPO-R, but the action of EPO on tumor cells remains controversial. It has been suggested that EPO promotes the proliferation and survival of cancer cells expressing EPO-R. On the other hand, other reports have concluded that EPO-R plays no role in tumor progression. One review provided a detailed insight into the nonhemopoietic role of EPO and its mechanism(s) of action which may lead to a better understanding of its potential therapeutic value in diverse clinical settings.

*Mechanisms of erythropoietin doping*

The wide-spread assumption that doping with erythropoietin or blood transfusion is only effective by increasing arterial blood \( O_2 \) content because of rising hematocrit is not self-evident. "Natural blood dopers" (horses, dogs) increase both hematocrit and circulating blood volume during exercise by releasing stored erythrocytes from the spleen. Improvement of aerobic performance by augmenting hemoglobin concentration may be expected until the optimal hematocrit is reached; above this value maximal cardiac output declines due to the steep increase of blood viscosity. Therefore an enlarged blood oxygen content might only be useful if the normal hematocrit of man during exercise is suboptimal. However, recent studies suggest that cardiac power rises after erythropoietin allowing an unchanged cardiac output in spite of increased viscosity. Other factors underlying improved performance after blood doping might be: augmented diffusion capacity for oxygen in lungs and tissues, increased percentage of young red cells with good functional properties (after erythropoietin), increased buffer capacity, increase of blood volume, vasoconstriction, reduced damage by radicals, mood improvement by cerebral effects of erythropoietin. Also the importance of placebo is unknown since double-blind studies are rare. It is suggested that blood doping has multifactorial effects not restricted to the increase in arterial oxygen content.

*Receptor biology*

The two erythropoiesis stimulating agents (ESAs), short acting recombinant human erythropoietin (EPO) and long acting continuous erythropoietin receptor activator (CERA),
have been hypothesized to share an in vivo elimination pathway that involves binding to erythropoietin receptor (EPOR) and subsequent internalization. A physiologically based recirculation model and a pharmacokinetic tracer interaction methodology (TIM) were used to compare the in vivo interaction kinetics with EPOR between the two ESAs in adult sheep. Animals treated with EPO experienced a greater EPOR up-regulation than those treated with CERA, as evidenced by an eightfold-higher initial EPOR normalized production rate constant versus a twofold-larger EPOR degradation rate constant. In agreement with in vitro studies, EPO had a lower in vivo equilibrium dissociation constant from EPOR than CERA. The internalization and/or degradation of the EPO-EPOR complex was faster than that of the CERA-EPOR complex. The adopted model enables a mechanism-based explanation for CERA’s slower elimination and greater erythropoietic activity in vivo. As predicted by the model, the slower elimination of CERA is due to less EPOR up-regulation induced by CERA administration; slower binding of CERA to EPOR; and reduced internalization and/or degradation rate of surface-bound CERA. Slower CERA/EPOR complex elimination explains the greater in vivo erythropoiesis reported for CERA, despite its lower affinity to EPOR. A sensitivity analysis showed that the model parameters were reliably estimated using the TIM methodology [11139].

**Erythropoietin and the heart**

Erythropoietin (Epo) has been thought to act exclusively on erythroid progenitor cells. The identification of Epo receptor (EpoR) in non-haematopoietic cells and tissues including neurons, astrocytes, microglia, immune cells, cancer cell lines, endothelial cells, bone marrow stromal cells, as well as cells of myocardium, reproductive system, gastrointestinal tract, kidney, pancreas and skeletal muscle indicates that Epo has pleiotropic actions. Epo shows signals through protein kinases, anti-apoptotic proteins and transcription factors. In light of interest of administering recombinant human erythropoietin (rhEpo) and its analogues for limiting infarct size and left ventricular (LV) remodelling after acute myocardial infarction (AMI) in humans, the foremost studies utilising rhEpo are reviewed. The putative mechanisms involved in Epo-induced cardioprotection are related to the antiapoptotic, anti-inflammatory and angiogenic effects of Epo. Thus, cardioprotective potentials of rhEpo are reviewed in the article by focusing on clinical applicability. An overview of non-haematopoietic Epo analogues, which are a reliable alternative to the classic EpoR agonists and may prevent undesired side effects, is also provided [13312].

**A neuroprotective agent**

The tissue protective functions of the hematopoietic growth factor erythropoietin (EPO) are independent of its action on erythropoiesis. EPO and its receptors (EPOR) are expressed in multiple brain cells during brain development and upregulated in the adult brain after injury. Peripherally administered EPO crosses the blood-brain barrier and activates in the brain anti-apoptotic, anti-oxidant and anti-inflammatory signaling in neurons, glial and cerebrovascular endothelial cells and stimulates angiogenesis and neurogenesis. These mechanisms underlie its potent tissue protective effects in experimental models of stroke, cerebral hemorrhage, traumatic brain injury, neuroinflammatory and neurodegenerative disease. The preclinical data in support of the use of EPO in brain disease have already been translated to first clinical pilot studies with encouraging results with the use of EPO as a neuroprotective agent [09160].

**Neovascularization**
Doping with erythropoietic proteins such as erythropoietin (EPO) is a serious issue in sport. There is little information on the possible ophthalmologic alterations followed by frequent EPO abuse in athletes. EPO is a potent retinal angiogenic factor and is capable of stimulating retinal angiogenesis and neovascularization in the presence of ischemia. Systemic and intravitreal EPO concentrations are highly correlated. A linkage between EPO doping and retinal proliferation is possible and further studies are warranted. Gathering and analyzing data on retinal findings from these athletes, either retrospectively or prospectively might yield preliminary information to support the safety of those athletes. Implications of this hypothesis cover other kinds of neovascularizations and angiogenesis [12246].

**Psychological effects**

One study examined the time course of mean self-esteem and physical self scores in three groups: male endurance athletes treated with recombinant human erythropoietin (rHuEPO group, n=6), a placebo group (n=5) injected with a sodium chloride solution and a control group who did not receive any injection (n=6). Each participant completed the Physical Self Inventory twice a day (between 07.00 and 09.00 h and between 19.00 and 21.00 h). Using a 10 cm visual analog scale, the participants assessed global self-esteem, physical self-worth and the sub-domains of physical condition, sport competence, attractive body and physical strength. This was conducted over three consecutive periods: in the 2 weeks before the course of injections, during the 6 weeks of injections and for 4 weeks after the injections. Aerobic capacity was assessed before and after 4 weeks of treatment. The results showed a significant increase in aerobic physical fitness in the rHuEPO group and a significant increase in perceived physical condition and physical strength scores at the end of treatment. The main psychological result was that endurance athletes were highly sensitive to the effects of rHuEPO on physical fitness. The perception of increased physical condition may lead to a stronger commitment to training. The rHuEPO injections presented a dangerous hedonic effect linked to endurance training. These results confirm the need to tackle rHuEPO abuse at any time during the training season [06158].

**Influence of N-acetylcysteine**

The aim of one study was to follow up whether the modification of pro-antioxidant status by 8-day oral application of N-acetylcysteine (NAC) in healthy men affects the hematological response, whether there is a direct relationship between antioxidant defences and erythropoietin (EPO) secretion and whether NAC intake enhances exercise performance. Fifteen healthy men were randomly assigned to one of two groups: control or NAC (1,200 mg per day for 8 days prior to and 600 mg on the day of exercise trial). To measure the ergogenic effectiveness of NAC, subjects performed incremental cycle exercise until exhaustion. NAC administration significantly influenced the resting and post-exercise level of glutathione (+31 %) as well as the resting activity of glutathione enzymes (glutathione reductase, -22 %; glutathione peroxidase, -18 %). The oxidative damage markers, i.e. protein carbonylation and lipid peroxidation products (thiobarbituric acid reactive substance) were reduced by NAC by more than 30 percent. NAC considerably affected the plasma level of EPO (+26 %), haemoglobin (+9 %), haematocrit (+9 %) and erythrocytes (-6 %) at rest and after exercise. The mean corpuscular volume and the mean corpuscular haemoglobin increased by more than 12 percent. Plasma total thiols increased by 17 percent and directly correlated with EPO level. NAC treatment, contrary to expectations, did not significantly affect exercise performance. The study has shown that 8-day NAC intake at a daily dose of 1,200 mg favours a pro-antioxidant status and affects haematological indices but does not enhance exercise performance [10119].
Kinetics of erythropoietin

The mean half-life of recombinant erythropoietin following repeated 50 IU/kg per day subcutaneous administrations is ~36 h (with a average clearance of 17 mL/h/kg). However, half life is reduced to 4-7 h following intravenous administration and total clearance is nearly three times higher in athletes than in untrained subjects (6.5 mL/h/kg) [08221]. Due to this particular kinetics, intravenous administration of recombinant erythropoietin in athletes would lead to undetectable plasma levels after 2-3 h. Furthermore, due to the long lasting effects of rEPO and derivatives on erythrocyte biology, it is unlikely that athletes will use rEPO or similar compounds close to competitions [08222]. Finally, athletes preparing to dope with autologous transfusions can do without rEPO administration by extracting blood without prior "boosting". Another specific concern on the analytical efficiency of the new method is the use of epoetin delta and CERA (continuous erythropoietin receptor activator). Due to innovative production technology, these new erythropoietin analogues are as yet undetectable by the traditional isoelectric focusing approach, both in urine and in plasma. Therefore, it is consider that in- or near-competition testing for plasma rEPO would primarily waste human and economical resources, whereas the revenues in terms of doping prevention are as yet unpredictable. Even in out-of-competition settings, the specific kinetics of rEPO would make detection rather unlikely, provided that blood is collected in the immediate period following rEPO administration [08045].

Interactions with androgens

Association between androgens and erythropoiesis has been known for more than seven decades. Androgens stimulate hematopoietic system by various mechanisms. These include stimulation of erythropoietin release, increasing bone marrow activity and iron incorporation into the red cells. Before the discovery of recombinant erythropoietin (rhEpo), androgens were used in the treatment of anemia associated with renal disease, bone marrow suppression and hypopituitarism. Anabolism is an additional advantage of androgen therapy. Furthermore, in light of recent reports regarding adverse effects of rhEpo, the role of androgen therapy in various types of anemias should be readdressed. Polycythemia remains a known side effect of androgen therapy. In one review, it was briefly discussed the initial animal and human studies which demonstrated the role of androgens in the treatment of anemia, their mechanism of action, a detailed account of the efficacy of androgens in the treatment of various anemias, the erythropoietic side effects of androgens and finally, the relationship between hematocrit levels and cardiovascular disease [09162].

Effects of exercise on erythropoietin molecules

The anti-doping control of recombinant erythropoietin relies on the detection, in the urine, of its isoelectric pattern, which differs from that of the corresponding natural hormone, the latter being typically more acidic than the former. However, a small number of natural urinary patterns, referred to as "atypical patterns," are less acidic than the dominant form. Based on anecdotal evidence, the occurrence of such patterns seems to be related to particular strenuous exercises. One study aimed to demonstrate this relation using a strenuous exercise protocol. Seven top-level athletes (cyclists) took part in a training protocol including a series of supramaximal short-duration exercises. Erythropoietin (EPO) isoelectric patterns were obtained by submitting blood and urine samples to isoelectric focusing. Additional protein dosages were performed. Supramaximal short-duration exercises induced the transformation of typical urinary natural EPO patterns into atypical ones. None of the obtained atypical patterns fulfilled the 3 criteria mandatory for reporting an adverse analytical finding. Serum EPO patterns were not affected by the exercises that caused the
transformation of urinary patterns. An exercise-induced transient renal dysfunction is proposed as a hypothetic explanation for these observations that rely on parallel investigations of proteinuria in the same samples [09161].

**Effect of endurance training**

Erythropoietin (Epo) administration improves aerobic exercise capacity and insulin sensitivity in renal patients and also increases resting energy expenditure (REE). Similar effects are observed in response to endurance training. The aim was to compare the effects of endurance training with erythropoiesis-stimulating agent (ESA) treatment in healthy humans. Thirty-six healthy untrained men were randomized to 10 weeks of either: 1) placebo (n=9), 2) ESA (n=9), 3) endurance training (n=10), or 4) ESA and endurance training (n=8). In a single-blinded design, ESA/placebo was injected one time weekly. Training consisted of biking for 1 h at 65 percent of Wattmax three times per week. Measurements performed before and after the intervention were as follows: body composition, maximal oxygen uptake, insulin sensitivity, REE, and palmitate turnover. Uncoupling protein 2 (UCP2) mRNA levels were assessed in skeletal muscle. Fat mass decreased significantly after training, whereas ESA induced a small but significant increase in intrahepatic fat. Serum free fatty acid (FFA) levels and palmitate turnover decreased significantly in response to training, whereas the opposite pattern was found after ESA. REE corrected for lean body mass increased in response to ESA and training, and muscle UCP2 mRNA levels increased after ESA. Insulin sensitivity increased only after training. In conclusion: 1) insulin sensitivity is not improved after ESA treatment despite improved exercise capacity, 2) the calorigenic effects of ESA may be related to increased UCP2 gene expression in skeletal muscle, and 3) training and ESA exert opposite effects on lipolysis under basal conditions, increased FFA levels and liver fat fraction was observed after ESA treatment [13313].

Erythropoietin (EPO) and soluble transferrin receptor (sTfR) in serum have been proposed as indirect biomarkers for the detection of recombinant human EPO (rhEPO) misuse in sport. The purpose of one study was to investigate the influence of different levels of physical fitness, sport, different training workload during the sport season, and endurance exercise in the concentrations of these serum biomarkers for their application into mathematical models to indirectly detect rhEPO misuse. Serum EPO and sTfR concentrations were measured in 96 elite athletes of various sports along the sport season, in 21 recreational athletes at baseline (non-exercising) conditions and in 129 other recreational athletes before and after long-distance races (10 and 21 km). In elite athletes, hemoglobin concentrations and percentage of reticulocytes were also measured, and indirect detection models applied. In recreational athletes, for EPO and sTfR, significant differences were only observed after the 21-km race. In baseline conditions, no differences were observed between recreational and elite athletes for EPO and sTfR. In elite athletes, individual EPO and sTfR concentrations slightly changed over the sport season, with coefficients of variation (CV) of 26.1 % and 9.0 %, respectively. Hemoglobin and reticulocytes were influenced by sport, but their individual variation over the sport season was not physiologically relevant (CV of 3.7 % and 21.3 %, respectively). When applying mathematical models for detection of rhEPO administration, only one elite athlete obtained an individual model score above the established thresholds. Physical fitness, sport and different training workload during the sport season had no substantial effect on serum EPO and sTfR concentrations, except in recreational athletes after a 21-km race. Variations observed in mathematical models to detect EPO administration were mainly due to fluctuation in hemoglobin concentrations, commonly observed in elite athletes [06156].

**Effect of marathon**

Erythropoietin (EPO) was studied in 13 female marathon runners before and up to 8 days
after a competition marathon run. The median baseline control value was 13.7 U/l. No change in EPO concentration was found immediately (15 min.) and one day after the run. However, a median increase in EPO concentration (18.1 U/l) was found on day three post-exercise. On day 8 no change was found compared to pre-exercise values. This late increase in EPO concentration would seem to be responsible for the well known increase of red blood cell mass in long distance runners [06157].

Use of erythropoietin in cycling

Imagine a medicine that is expected to have very limited effects based upon knowledge of pharmacology and (patho)physiology, is studied in the wrong population, with low quality studies that use a surrogate endpoint that relates to the clinical endpoint in a partial manner at most. Such a medicine would surely not be recommended. Recombinant human erythropoietin (rHuEPO) use to enhance performance in cycling is very common. A qualitative systematic review of the available literature was performed to look at the evidence for these ergogenic properties of this drug normally used to treat anaemia in chronic renal failure patients. The results of this literature search show there is no scientific basis to conclude rHuEPO has performance enhancing properties in elite cyclists. The reported studies have many shortcomings regarding translation of the results to professional cycling endurance performance. Additionally, the possibly harmful side-effects have not been adequately researched for this population but appear to be worrying at least. It was concluded that rHuEPO use in cycling is rife but scientifically unsupported by evidence and its use in sports is medical malpractice. What its use would have been, if the involved team physicians had been trained in clinical pharmacology and had investigated this properly, remains a matter of speculation. A single well controlled trial in athletes under real life circumstances would give a better indication of the real advantages and risk factors of rHuEPO use, but it would be an oversimplification that this would eradicate its use [12244].

Effect on performance

All kinds of blood manipulations aim to increase the total hemoglobin mass (tHb-mass). To establish tHb-mass as an effective screening parameter for detecting blood doping, the knowledge of its normal variation over time is necessary. The aim of one study, therefore, was to determine the intraindividual variance of tHb-mass in elite athletes during a training year emphasizing off, training, and race seasons at sea level. tHb-mass and hemoglobin concentration were determined in 24 endurance athletes five times during a year and were compared with a control group (n=6). An analysis of covariance was used to test the effects of training phases, age, gender, competition level, body mass, and training volume. Three error models, based on a total percentage error of measurement, the combination of a typical percentage error of analytical origin with an absolute SD of biological origin, and between-subject and within-subject variance components as obtained by an analysis of variance, were tested. In addition to the expected influence of performance status, the main results were that the effects of training volume and training phases on tHb-mass were not significant. It was found that within-subject variations mainly have an analytical origin (typical error approximately 1.4 %) and a very small SD (7.5 g) of biological origin. It was concluded that tHb-mass shows very low individual oscillations during a training year (<6 %), and these oscillations are below the expected changes in tHb-mass due to erythropoetin application or blood infusion (approximately 10 %). The high stability of tHb-mass over a period of 1 year suggests that it should be included in an athlete’s biological passport and analyzed by recently developed probabilistic inference techniques that define subject-based reference ranges [20823].

The performance enhancing (ergogenic) effect of recombinant human Epo (rhEpo) in aerobic sports was investigated shortly after the medicine became available. It soon became clear
that subcutaneous administration of rhEpo at doses of 60 to 350 U/kg body weight and week for 4 to 6 weeks increases \(O_2\text{max}\) and the time to exhaustion substantially. More recent studies in which rhEpo was applied to healthy volunteers in lower dosages demonstrated that \(O_2\text{max}\) is increased by 6-12 percent when the hematocrit (Hct) is increased to approximately 0.50 but also demonstrated that time to exhaustion (in the laboratory) at a given level of \(O_2\text{max}\) is increased by up to 50 percent. A challenge for antidoping work is that when rhEpo administration is discontinued in healthy volunteers, \(O_2\text{max}\) remains elevated for at least 3 weeks. Although Epo is reported to activate several nonhematologic factors (i.e. in addition to stimulating erythropoiesis), which are usually also associated with improvements in aerobic performance, the primary mechanism by which Epo increases exercise performance in humans is through augmented erythropoiesis. Erythropoiesis-stimulating agents are particularly effective in combination with iron supplementation. The administration of iron results in increased ferritin levels in athletes. Ferritin levels more than 1000 g/L have been observed. There are as yet no reports on physical performance in healthy humans with increased circulating Epo and Hb mass because of the administration of compounds stimulating the expression of the endogenous Epo gene or after Epo gene transfer [11116].

Treatment with recombinant human erythropoietin (rhEpo) induces a rise in blood oxygen-carrying capacity (\(\text{CaO}_2\)) that unequivocally enhances maximal oxygen uptake (\(\text{VO}_2\text{max}\)) during exercise in normoxia, but not when exercise is carried out in severe acute hypoxia. This implies that there should be a threshold altitude at which \(\text{VO}_2\text{max}\) is less dependent on \(\text{CaO}_2\). To ascertain which are the mechanisms explaining the interactions between hypoxia, \(\text{CaO}_2\) and \(\text{VO}_2\text{max}\) it was measured systemic and leg \(O_2\) transport and utilization during incremental exercise to exhaustion in normoxia and with different degrees of acute hypoxia in eight rhEpo-treated subjects. Following prolonged rhEpo treatment, the gain in systemic \(\text{VO}_2\text{max}\) observed in normoxia (6-7 %) persisted during mild hypoxia (8 % at inspired \(O_2\) fraction of 0.173) and was even larger during moderate hypoxia (14-17 % at \(O_2\) fraction 0.153-0.134). When hypoxia was further augmented to \(O_2\) fraction 0.115, there was no rhEpo-induced enhancement of systemic \(\text{VO}_2\text{max}\) or peak leg \(\text{VO}_2\). The mechanism highlighted by the data is that besides its strong influence on \(\text{CaO}_2\) rhEpo was found to enhance leg \(\text{VO}_2\text{max}\) in normoxia through a preferential redistribution of cardiac output toward the exercising legs, whereas this advantageous effect disappeared during severe hypoxia, leaving augmented \(\text{CaO}_2\) alone insufficient for improving peak leg \(O_2\) delivery and \(\text{VO}_2\). Finally, that \(\text{VO}_2\text{max}\) was largely dependent on \(\text{CaO}_2\) during moderate hypoxia but became abruptly \(\text{CaO}_2\)-independent by slightly increasing the severity of hypoxia could be an indirect evidence of the appearance of central fatigue [08224].

After 13 weeks of erythropoietin application with an increase in hemoglobin concentration from 142 ± 4 to 156 ± 4 g/l at rest, eight physically active men underwent a graded cycle test to exhaustion. Following erythropoietin application (Post-rHuEpo measurements) maximal work rate and oxygen uptake increased significantly by 9 percent (337 to 368 W) and 8 percent (3,950 to 4,255 ml/min), respectively. Then the extra red cell volume was withdrawn and replaced by a 5 percent albumin solution. After only 2 hours, the cycle test with all measurements was repeated. The performance decreased to 339 W (not significant) and maximal oxygen uptake to 4,060 ml/min. This result is not convincing evidence for a pure effect of Hb concentration. Interestingly the lactate concentrations after hemodilution are the lowest in all tests including the pre-Epo values, whereas arterial norepinephrine concentrations are the highest, hinting at reduced glucose availability or increased strain but lower exhaustion. Also the arrangement of the experiment relative to circadian rhythm has to be considered; in early afternoon the intention to work hard is reduced. Might blood loss for the measurements have influenced maximal work rate? It has also been argued that the enlarged heart work because of rising blood viscosity does not limit maximal cardiac output and thus exercise capacity. A better measure of mechanical work of the heart, however, is
cardiac power estimated as the product of stroke volume times pressure times heart rate. It increases from Pre-rHuEpo (6.66 W) to Post-rHuEpo (7.84 W) but tends also to slightly higher values after hemodilution (6.94 W). In the latter case the stroke volume is clearly higher and the blood pressure lower than Post-rHuEpo (blood pressures at maximal performance are surprisingly low in this investigation). One might speculate that a fully compensating increase of the stroke volume is restricted mechanically during the diastole (e.g. by heart wall stiffness or by too low filling pressure) [08225].

The performance enhancing (ergogenic) effect of recombinant human Epo (rhEpo) in aerobic sports was investigated shortly after the medicine became available. It soon became clear that subcutaneous administration of rhEpo at doses of 60 to 350 U/kg body weight and week for 4 to 6 weeks increases $O_{2max}$ and the time to exhaustion substantially. More recent studies in which rhEpo was applied to healthy volunteers in lower dosages demonstrated that $O_{2max}$ is increased by 6-12 percent when the hematocrit (Hct) is increased to approximately 0.50 but also demonstrated that time to exhaustion (in the laboratory) at a given level of $O_{2max}$ is increased by up to 50 percent. A challenge for antidoping work is that when rhEpo administration is discontinued in healthy volunteers, $O_{2max}$ remains elevated for at least 3 weeks. Although Epo is reported to activate several nonhematologic factors, which are usually also associated with improvements in aerobic performance, the primary mechanism by which Epo increases exercise performance in humans is through augmented erythropoiesis. ESAs are particularly effective in combination with iron supplementation. The administration of iron results in increased ferritin levels in athletes. Ferritin levels more than 1000 microg/L have been observed. There are as yet no reports on physical performance in healthy humans with increased circulating Epo and Hb mass because of the administration of compounds stimulating the expression of the endogenous Epo gene or after Epo gene transfer [11428].

**Effects of repetitive training at low altitude**

Classical altitude training can cause an increase in total hemoglobin mass if a minimum "dose of hypoxia" is reached (altitude ≥2,000 m, ≥3 weeks). It was investigated if repetitive exposure to mild hypoxia during living and training at low altitude (<2,000 m) for several weeks, often performed by elite athletes, might also have significant effects on erythropoiesis. Total hemoglobin mass, erythropoietin (EPO), soluble transferrin receptor (sTfR) and ferritin were determined in 8 elite runners before and after each of 2 training camps at low altitude interspersed by 3 weeks of sea-level training and at the same time points in a control group of 5 well-trained runners. EPO, sTfR and ferritin were also repeatedly measured during the altitude training camps. Repeated measures ANOVA revealed significant increases in EPO- and sTfR-levels during both training camps and a significant decrease in ferritin indicating enhanced erythropoietic stimulation during living and training at low altitude. Furthermore, significant augmentation of total hemoglobin mass by 5.1 percent occurred in the course of the 2 altitude training camps. In conclusion, repetitive living and training at low altitude leads to a hypoxia-induced increase in erythropoietic stimulation in elite 400 m and 800 m runners and, apparently, might also cause a consecutive augmentation of total hemoglobin mass [10120].

**Effect of erythropoietin of scuba diving**

Erythropoiesis is affected during deep saturation dives. The mechanism should be related to a downregulation of serum Erythropoietin (s-EPO) concentration or to a toxic effect of the hyperbaric hyperoxia. It was evaluated s-EPO and other haematological parameters in 6 scuba divers before, during and after a 14-days guinness saturation dive (8-10 m). Athletes were breathing air at 1.8-2 ATA, under the control of a team of physicians. Serum parameters were measured before diving (T0) and: 7 days (T1), 14 days (T2) after the beginning of the dive and 2 h (T3) and 24 h (T4) after resurfacing. Hgb, and many other
haematological parameters did not change whereas Ht, s-EPO, the ratio between s-EPO predicted and that observed and reticulocytes (absolute, percent) declined progressively from T0 to T3. At T4 a significant rise in s-EPO was observed. Hgb did not vary but erythropoiesis seemed to be affected as s-EPO and reticulocyte counts showed. All these changes were statistically significant. The experiment, conducted in realistic conditions of dive length, oxygen concentration and pressure, allows us to formulate some hypotheses about the role of prolonged hyperbarism on erythropoiesis. The s-EPO rise, 24 h after resurfacing, is clearly documented and related to the "Normobaric Oxygen Paradox". This evidence suggests interesting hypotheses for new clinical applications such as modulation of s-EPO production and Hgb content triggered by appropriate O₂ administration in pre-surgical patients or in some anemic disease [13314].

**Effects of erythropoietin on serum hepcidin and serum iron bioavailability**

Hepcidin regulates plasma iron bioavailability and subsequently iron availability for erythropoiesis. rHuEPO has been reported to decrease hepcidin expression in case of repeated subcutaneous injections. Thus, hepcidin level measurement could be a candidate marker for detection of rHuEPO abuse. However, when used for doping, rHuEPO can be injected intravenously and the scheme of injection is unknown. The aim of one study was to evaluate the early effects of a single intravenous rHuEPO injection on serum hepcidin levels. Fourteen male healthy volunteers received one intravenous injection of 50 U/Kg of rHuEPO during a placebo-controlled, randomized, double-blind, cross-over study. Serum hepcidin, quantified by a competitive ELISA method and iron parameters was then evaluated for 24 h. Serum levels of hepcidin were significantly increased 4 h after rHuEPO injection when compared with placebo injection, whereas iron and transferrin saturation dramatically decreased 12 h after rHuEPO injection when compared with placebo injection. In addition, 12 and 24 h after rHuEPO injection serum hepcidin levels were lower compared with placebo injection. Intravenous injection of recombinant EPO induces a precocious and transient increase of serum hepcidin leading to a transient decrease of iron bioavailability. The transitory increase and dynamics of its concentration make difficult the practical use of hepcidin to detect rHuEPO doping [12247].

**New biomarkers**

Erythropoietin (Epo) is produced primarily in the kidneys upon low blood oxygen availability and stimulates erythropoiesis in the bone marrow. Recombinant human Epo (rHuEpo), a drug developed to increase arterial oxygen content in patients, is also illicitly used by athletes to improve their endurance performance. Therefore, a robust and sensitive test to detect its abuse is needed. The aim of one study was to investigate potential human serum biomarkers of Epo abuse employing a proteomic approach. Eight healthy male subjects were injected subcutaneously with rHuEpo (5,000 IU) every second day for a 16-day period. Serum was collected before starting the treatment regime and again at days 8 and 16 during the treatment period. Samples were homogenized and proteins separated by two-dimensional gel electrophoresis (2DE). Spots that changed significantly in response to rHuEpo treatment were identified by mass spectrometry. Both the number of reticulocytes and erythrocytes increased throughout the study, leading to a significant increase in hematocrit and hemoglobin content. In addition, transferrin levels increased but the percentage of iron bound to transferrin and ferritin levels decreased. Out of 97 serum proteins, seven were found to decrease significantly at day 16 compared with pre-Epo administration, and were identified as four isoforms of haptoglobin, two isoforms of transferrin, and a mixture of hemopexin and albumin. In support, total serum haptoglobin levels were found to be significantly decreased at both days 8 and 16. Thus a 2DE proteomic approach for discovery of novel markers of
Epo action appears feasible [11140].

Theoretical aspects of detection blood doping with erythropoietin

Peptidic ESAs

Currently available rhEpo preparations (epoetins) are produced in Epo complementary DNA (cDNA) transfected Chinese hamster ovary (CHO) or baby hamster kidney (BHK) cell cultures. The only therapeutic rhEpo engineered in human cells (epoetin-delta) is off market since the beginning of 2009. Because the patents of the originator epoetins have died, biosimilar products have been approved in many parts of the world. Furthermore, various copied CHO cell-derived rhEpos are available in countries without a regulated market. Endogenous Epo and the epoetins have an invariant sequence of 165 amino acids, but they differ in glycosylation. Compared with the epoetins, endogenous Epo isoforms are more acidic and smaller in size. Epo can be separated by isoelectric focusing (IEF) or electrophoresis of urine samples. After IEF, a double-blotting procedure is performed. The mutein darbepoetin-alfa migrates more in the acidic range than Epo on IEF. The WADA has established criteria to achieve harmonization in the performance of the test for epoetin and darbepoetin in urine. Actually, when urine samples from rhEpo-treated subjects were submitted to two WADA-accredited laboratories, the results were not fully consistent, which, as claimed by the laboratories, was apparently the result of methodologic differences. A recent detection problem has arisen with the addition of proteases by athletes to their urinary samples, which destroys the erythropoietic proteins. The adulteration of urine with proteases is a prohibited method, and techniques have been developed for the detection of their misuse. Another difficulty relates to the fact that once hemoglobin concentrations has been raised in athletes by the administration of recombinant ESAs, only microdoses or less frequent injections of the drugs are needed to maintain [Hb] at the high level. In this situation, the window of detection of rhEpo in urine is only 12-18 hours, compared with about 3 days on regular dosing (50 U/kg body weight 3 times a week). Thus, although the detection of rhEpo in urine is effective if the injection frequency is high, this is certainly not the case when the injection rate is reduced to weekly injections. Because darbepoetin-alfa has a 3- to 4-fold longer half-life (24-26 hours) than the epoetins (6-8 hours), the window of detection of darbepoetin-alfa is prolonged to approximately 7 days. CERA has an even longer half-life of approximately 6 days. IEF of CERA yields bands in the less acidic area compared with native Epo. IEF for investigation of doping with CERA has also been applied to blood samples. In addition, CERA can be detected by ELISA. Epo mimetic peptides (EMPs) are synthetic cyclic peptides of about 20 amino acids. A potent pegylated EMP dimer (INN: peginesatide; Hematide®) proved to stimulate erythropoiesis in experimental animals and in healthy male volunteers. In an alternative approach, EMPs have been constructed onto human IgG1-based scaffolds by recombinant DNA-technology [11428].

Drugs activating the endogenous erythropoietin gene

The Epo enhancer is under the control of HIFs, heterodimeric proteins composed of subunits alpha and beta. HIF-2 is the main factor inducing Epo expression. The C-terminus of HIF-alpha is composed of proline residues that are hydroxylated in the presence of O2. Prolylhydroxylated HIF-alpha binds the von Hippel-Lindau tumor suppressor protein in complex with an E3-ligase and undergoes immediate proteasomal degradation. The transcriptional activity of the HIFs is suppressed by O2-dependent hydroxylation of an asparagine residue. The HIF-alpha hydroxylases contain Fe2+ and are inactivated by Fe2+ removal. However, iron chelators are not suited for stimulation of erythropoiesis in the long-term because iron is required for heme synthesis. HIF-dependent Epo expression is augmented by divalent transition metals, such as cobalt or nickel. It has been known that cobalt increases erythropoiesis in experimental animals. Cobalt is a very potent inducer of Epo transcription. Indeed, the international Epo unit (IU) was originally defined as the dose
eliciting the same erythropoiesis stimulating response in rats as 5 micromol of cobaltous chloride. However, cobalt may be misused by athletes as a proper means to enhance Epo levels and Hb<sub>mass</sub>. Cobalt is very potent, inexpensive, and not comprehended in the WADA’s “Prohibited List” [11428].

**Other erythropoietic hormones**

Several hormones may stimulate the renal and/or hepatic production of Epo, including prostanoids, thyroid hormone, angiotensin II, growth hormone (GH), and testosterone. The latter are of particular interest regards blood doping. The fact that the rise in plasma Epo occurred earlier than the rise in insulin-like growth factor-1 (IGF-1) indicates that GH directly stimulates Epo production. IGF-1 was earlier shown to promote the growth of erythrocytic progenitors. The hormone Epo, which derives from kidneys and liver, stimulates the survival, proliferation, and differentiation of the erythrocytic progenitors in hemopoietic tissues. The enhanced release of reticulocytes leads to an increase in the blood hemoglobin concentration and, thus, the O<sub>2</sub> capacity of the blood and the total Hb mass. Epo gene expression in the kidneys and the liver is controlled at the transcriptional level [11428].

**RBC parameters associated with ESA doping**

There are no major differences in basal hemoglobin concentration RBC count, Hct, and MCHC values in elite athletes compared with healthy nonathletes. An increase in hypochromic red cells has been seen on rhEpo therapy despite the use of parenteral or oral iron. Reticulocyte number was not affected by intravenous iron administration in healthy humans subjected to a bolus injection of rhEpo (300 U/kg intravenously). However, MCHr and Ret [Hb] were increased in the intravenous iron/rhEpo group compared with the group receiving rhEpo alone. Thus, intravenous iron increases the hemopoietic response to rhEpo in normal subjects, and this therapy is probably practiced by cheating athletes. Note that parenteral iron alone did not produce a change in Hb<sub>mass</sub>, hemoglobin concentration, or specific RBC parameters in young female athletes, despite their low baseline hemoglobine (128 g/L) and serum ferritin (35 g/L) levels. There is fair stability of reticulocyte number in top-level athletes, although decreases were observed in some athletes during competition periods. High and middle fluorescence (immature) Ret with a high RNA content (IRF) are relatively frequent in athletes because of continuous bone marrow stimulation linked to hemolysis, which is typical of sports activities. Bolus rhEpo injections (150 U or 300 U/kg body weight) further increase the IRF. The increase in immature Ret starts 36 hours after a single dose of rhEpo, reaching a peak after 3-4 days and normalizing within 7 days. A pharmacodynamic model calculation has revealed that rhEpo transiently increases the life span of circulating Ret from the baseline value of 1.7 days to 3.4 days. Thus, the treatment with rhEpo appears to increase Ret values 2-fold: by increased Ret release from the bone marrow and by prolonged maturation time of circulating Ret. It has also been studied the time course of reticulocyte number after repeated subcutaneous injections of rhEpo (50 U/kg body weight every day) in athletes. Reticulocyte numbers were increased from day 10 to 24 and remained elevated for 7 days after cessation of rhEpo therapy. Reticulocyte numbers were significantly lower than the baseline values 14 and 25 days after the last rhEpo injection. During treatment up to 14 days after the last rhEpo injection, soluble transferring receptor and the sTfr/serum protein ratio were elevated above baseline [11116].

**Micro dosing**

Athletes can illicitly use the effects of microdose recombinant human erythropoietin regimens (rHuEPO) to boost red cell mass and thereby oxygen carrying capacity and endurance performance. There are persistent suggestions that athletes have learnt to use rHuEPO, but test negative, by titrating rHuEPO dosage regimens in order to minimize the appearance of
basic isoforms in urine samples (rHuEPO can be detected via electrophoresis because rHuEPO isoforms are more basic than endogenous erythropoietin isoforms). It is vital for antidoping agencies to determine whether existing deterrent strategies have been circumvented. To establish whether it is possible to confound detection strategies by titrating rHuEPO dosages, a study simulated a so-called ‘microdose’ rHuEPO regimen and measured the level of basic isoforms in urine collected during and after the administration protocol. Two well-trained male subjects (28 years old, 74 kg, 177 cm, regional level triathlete; 31 years old, 62 kg, 170 cm, national level long distance runner) gave informed consent to participate in this study which was reviewed and approved by the Regional Ethics Committee. Initially red cell production was rapidly accelerated in both subjects using high doses of rHuEPO (about 260 IU/kg injections on days 0, 2, 4, 7, 9 and 11) in conjunction with a single intravenous iron treatment (100 mg), with the goal to elevate hemoglobin (Hb) concentration to approximately 170 g/L. Over the next 3 weeks, injections were given every 2-3 days (injections on days 15, 17, 19, 22, 24, 26, 29, 31 and 33) and dosages were adjusted by a pharmacologist guided only by basic hematologic information (blood and reticulocyte counts, no urine profiles were provided as feedback). Microdosages were less than 10 percent of the initial dose (exact dosage undisclosed to prevent replication by athletes). Urine samples were collected three times per day during the microdose phase (7-9h, 11-13h, 19-21h), and analyzed for the presence of rHuEPO at the French national antidoping laboratory. As expected high dose rHuEPO treatment rapidly elevated Hb concentrations within 2 weeks (140 to 166 g/L; 148 to 174 g/L; subjects 1 and 2, respectively). It was found that it was possible to maintain elevated Hb values using microdoses of rHuEPO. After 3 weeks of the microdose regimen Hb concentrations were still 164 g/L and 170 g/L respectively (and 164 g/L and 162 g/L 1 week after all injections ceased). During the microdose phase reticulocyte percentages ranged in value from 0.8-1.2 percent and 0.4-1.1 percent for the two subjects. Urine samples collected more than 24 hours after a microdose injection typically had less than 80 percent basic isoforms, which until recently was the criterion used to declare a sample positive. In some instances samples collected just 12-18 hours after the last injection fell below the 80 percent threshold. It is noteworthy that the pharmacologist was able to quickly devise an effective microdose regimen utilizing limited feedback and with few prior attempts. Interestingly, isoelectric profiles showed the reappearance of endogenous erythropoietin bands during the microdose phase. This is in contrast to the existing paradigm which holds that endogenous erythropoietin production is suppressed when the red cell mass has been increased beyond the homeostatic set point. The implications of this remain unclear, however it can be speculated that were an athlete to receive microdoses of rHuEPO for an extended period (>2-4 weeks), it is conceivable that reappearance of endogenous bands of erythropoietin would be of sufficient magnitude to further reduce the effective window of detection of the test for rHuEPO. The results show that it is conceivable for athletes to maintain illicit rHuEPO doping even during multiday endurance events when competitors may be tested at the end of each day (i.e. at 24 hour intervals). The electrophoretic test has proven legally defensible and remarkably robust. The fact that microdoses of rHuEPO disappear rapidly from the circulation could be exploited by athletes to evade detection. This implies that authorities should supplement the urine test with an approach providing greater reach-back. This research also sharpens awareness that, to be efficient, urine tests should be based on out of competition testing [06154].

**Enzymatic desialylation**

Recombinant erythropoietin (rhEPO) has been misused for over two decades by athletes, mainly but not only in endurance sports. A direct rhEPO detection method in urine by isoelectric focusing (IEF) was introduced in 2000, but the emergence of third-generation erythropoiesis-stimulating agents and so-called biosimilar rhEPOs, together with the sensitivity of human endogenous EPO (huEPO) pattern to enzymatic activities and its
modification following short strenuous exercise, prompted the development of a complementary test based on SDS-PAGE analysis. While Mircera and NESP are easily detected with the existing IEF and SDS-PAGE methods, some samples containing both epoetin-α/β and huEPO present profiles that are still difficult to interpret. As doping practices have moved to micro-dosing, these mixed patterns are more frequently observed. We investigated the impact of enzymatic desialylation on the urinary and serum EPO profiles obtained by SDS-PAGE with the aim of improving the separation of the bands in these mixed EPO populations. It was observed that the removal with neuraminidase of the sialic acid moieties from the different EPOs studied reduced their apparent molecular weight (MW) and increased the migration distance between huEPO and rhEPO centroids, therefore eliminating the size overlaps between them and improving the detection of rhEPO [13310].

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Excessive dosing

Mice
To investigate the consequences of inborn excessive erythrocytosis, we made use of our transgenic mouse line (tg6) that constitutively overexpresses erythropoietin (Epo) in a hypoxia-independent manner, thereby reaching hematocrit levels of up to 0.89. It was detected expression of human Epo in the brain and, to a lesser extent, in the lung but not in the heart, kidney, or liver of tg6 mice. Although no acute cardiovascular complications are observed, tg6 animals have a reduced lifespan. Decreased swim performance was observed in 5-months-old tg6 mice. At about 7 months, several tg6 animals developed spastic contractions of the hindlimbs followed by paralysis. Morphological analysis by light and electron microscopy showed degenerative processes in liver and kidney characterized by increased vascular permeability, chronic progressive inflammation, hemosiderin deposition, and general vasodilatation. Moreover, most of the animals showed severe nerve fiber degeneration of the sciatic nerve, decreased number of neuromuscular junctions, and degeneration of skeletal muscle fibers. Most probably, the developing demyelinating neuropathy resulted in muscular degeneration demonstrated in the extensor digitorum longus muscle. Taken together, chronically increased Epo levels inducing excessive erythrocytosis leads to multiple organ degeneration and reduced life expectancy. This model allows investigation of the impact of excessive erythrocytosis in individuals suffering from polycythemia vera, chronic mountain sickness, or in subjects tempted to abuse Epo by means of gene doping [06155].
Laboratory techniques

One study was based on the assumption that changes in an ADMA-DDAH-NOS (ADMA-asymmetrical dimethylarginine; DDAH-dimethyl-arginine dimethylaminohydrolase; NOS-nitric oxide synthase) system could be employed as indirect markers for recombinant human erythropoietin (rHuEPO) administration in doping control. It was assessed a predictive value of four proposed new markers for rHuEPO abuse. Preliminary data showed that concentrations of ADMA, symmetrical dimethylarginine (SDMA), citrulline and arginine in human urine were increased after administration of a single intravenous erythropoietin injection (2000 U/day, Epocrine, St-Petersburg, Russia). The study of variations of ADMA, SDMA, arginine and citrulline levels before and after rHuEPO administration was performed with two healthy male volunteers. Urine samples were collected before rHuEPO administration and urinary concentrations of ADMA and SDMA were determined at 10.0-40 microg/mL and of arginine and citrulline at 0.5-10 microg/mL. A single dose injection of rHuEPO caused an increase in ADMA, SDMA, arginine and citrulline concentrations up to 40-270 microg/mL, 40-240 microg/mL, 10-60 microg/mL and 12-140 microg/mL, respectively. These preliminary results indicated that an indirect approach could be used as a pre-screening of urine samples in order to decrease the number of samples with a low probability of rHuEPO abuse and, thus, save costs and human workload [08226].

Identification of post-translational modifications of proteins in biological samples often requires access to preanalytical purification and concentration methods. In the purification step high or low molecular weight substances can be removed by size exclusion filters, and high abundant proteins can be removed, or low abundant proteins can be enriched, by specific capturing tools. In one paper is described the experience and results obtained with a recently emerged and easy-to-use affinity purification kit for enrichment of the low amounts of EPO found in urine and plasma specimens. The kit can be used as a pre-step in the EPO doping control procedure, as an alternative to the commonly used ultrafiltration, for detecting aberrantly glycosylated isoforms. The commercially available affinity purification kit contains small disposable anti-EPO monolith columns (6 μL volume, Ø7 mm, length 0.15 mm) together with all required buffers. A 24-channel vacuum manifold was used for simultaneous processing of samples. The column concentrated EPO from 20 mL urine down to 55 μL eluate with a concentration factor of 240 times, while roughly 99.7% of non-relevant urine proteins were removed. The recoveries of Neorecormon (epoetin beta), and the EPO analogues Aranesp and Mircera applied to buffer were high, 76 percent, 67 percent and 57 percent, respectively. The recovery of endogenous EPO from human urine was 65 percent. High recoveries were also obtained when purifying human, mouse and equine EPO from serum, and human EPO from cerebrospinal fluid. Evaluation with the accredited EPO doping control method based on isoelectric focusing (IEF) showed that the affinity purification procedure did not change the isoform distribution for rhEPO, Aranesp, Mircera or endogenous EPO. The kit should be particularly useful for applications in which it is essential to avoid carry-over effects, a problem commonly encountered with conventional particle-based affinity columns. The encouraging results with EPO propose that similar affinity monoliths, with the appropriate antibodies, should constitute useful tools for general applications in sample preparation, not only for doping control of EPO and other hormones such as growth hormone and insulin but also for the study of post-translational modifications of other low abundance proteins in biological and clinical research, and for sample preparation prior to in vitro diagnostics [10468].

The sample preparation method preceding the urinary erythropoietin (EPO) doping test is based on several concentration and ultrafiltration steps. In order to improve the quality of isoelectric focusing (IEF) gel results and therefore, the sensitivity of the EPO test, new sample preparation methods relying on affinity purification were recently proposed. One
article focused on the evaluation and validation of disposable immunoaffinity columns targeting both endogenous and recombinant EPO molecules in two World Anti-Doping Agency (WADA) accredited anti-doping laboratories. The use of the columns improved the resolution of the IEF profiles considerably when compared with the classical ultrafiltration method, and the columns’ ability to ensure the isoform integrity of the endogenous and exogenous EPO molecules was confirmed. Immunoaffinity columns constitute therefore a potent and reliable tool for the preparation of urine samples and their use will significantly improve the sensitivity and specificity of the actual urinary EPO test [10469].

An ultra-sensitive quantitative EPO (erythropoietin) lateral flow immunochromatographic test with a detection limit of 1.2 fM (10^{-15} M), 0.035 ng EPO/L, which is 50-100 times more sensitive than a corresponding enzyme based immunoassay, was presented. In comparison with commercial lateral flow tests for other analytes, like cardiac troponins that also require high sensitivity, the detection limit achieved in the presented test is about three orders of magnitude lower. The thin zone for capture and concentration of the analyte, the carbon black nano-strings used as label and the use of a conventional image scanner for the quantitative determination are the key components that enable the high sensitivity obtained. The convective flow in the lateral flow monolith creates short diffusion distances between immobilised antibody, analyte and labelled antibody thus enhancing the binding efficiency. This rapid and sensitive EPO test procedure can be used both to process hundreds of samples in 1 h and be utilized as a 15-minute dipstick test for single determinations. The technique was demonstrated by measuring EPO in urine. EPO, like many of the other urine proteins, is often found in the urine precipitates and the specimens are therefore treated with a urine precipitate dissolution buffer before analysis. It was shown that EPO in urine from normal individuals occurs in low concentration in a wide range between 1.7 and 51 ng/L. The concentration was however subjected to a wide variation during the day due to the EPO production variation and the urine concentration by the kidneys. It was also shown that the presented lateral flow device can be used as a miniaturized affinity column to distinguish an analyte (EPO) from its analogue (darbepoetin), directly by comparing the affinity profiles obtained after interaction with the immobilised antibody. The method for measuring the amount of EPO present in urine, the possibility to rapidly check the amount of EPO after a pre-treatment concentration step, and the potential to identify affinity differences between EPO and its analogues should make the presented method a valuable tool in the fight against EPO doping [08227].

To overcome the limitation of the currently adopted direct method to detect recombinant Human Erythropoietin (rHuEpo) abuse in sport, indirect analysis of blood parameters are increasingly used as part of the anti-doping strategies. The aim of one work was to identify whether immunophenotype modifications on erythroid cells may be indicative of previous rHuEPO administration. The study was conducted on dialyzed patients under treatment with rHuEPO. Dialyzed patients without rHuEPO therapy and volunteer donors were used as controls. The analysis of erythroid cells immunophenotype, performed using a multiparametric flow cytometry technique, showed a peculiar pattern of CD71 expression following rHuEPO treatment. In particular CD71 showed an increased expression in mature and intermediate reticulocytes and a surprisingly decreased expression in immature reticulocytes. In conclusion, the analysis of reticulocyte maturation stages with TO/CD71 double staining may be considered as a valid alternative indirect method for the detection of rHuEPO abuse [08228].

The test used by anti-doping laboratories to detect the misuse of recombinant erythropoietin (rhEPO) is based on its different migration pattern on isoelectric focusing (IEF) gel compared with the endogenous human erythropoietin (hEPO) that can possibly be explained by structural differences. While there is definitely a need to identify those differences by LC-
MS/MS, the extensive characterization that was achieved for the rhEPO was never performed on human endogenous EPO because its standard is not available in sufficient amount. The goal of one study was therefore to develop an analytical method to detect pmol amounts of N-linked and O-linked glycopeptides of the recombinant hormone as a model. Using a nanoflow HPLC-Chip electrospray ionization/ion trap mass spectrometer, the diagnostic ion at m/z 366 of oligosaccharides was monitored in the product ion spectra to identify the four theoretical glycosylation sites, Asn24, Asn38, Asn83 and Ser126, respectively, on glycopeptides 22-37, 38-55, 73-96 and 118-136. With 3 pmol of starting material applied on Chip, only the desialylated N-glycopeptides 22-37 and 38-55/38-43 could be observed, and of all the glycan isoforms, those with the smaller structures were predominantly detected. While the preservation of the sialic acid moieties decreased the detection of all the N-glycopeptides, it allowed a more extensive characterization of the O-linked glycopeptide 118-136. The technique described herein provides a mean to detect glycopeptides from commercially available pharmaceutical preparations of rhEPO with the sensitivity required to analyze pmol amounts of hEPO, which could ultimately lead to the identification of structural differences between the recombinant and the human forms of the hormone 08229.

Recombinant human erythropoietin (rhEPO), generally produced in Chinese hamster ovary (CHO) cells, can be used as not only a therapeutic protein but also a doping agent in sports. Profiling of EPO glycoforms is a critical means for quality control in pharmaceutical industrial and anti-doping analysis of misuse in sports. However, the existing methods for the analysis of EPO are associated with either time consuming or poor resolution. In one work, a rapid and high-resolution glycoform profiling method was presented based on capillary isoelectric focusing (cIEF) with whole column imaging detection (WCID). Experimental conditions that influence the separation were investigated. Under optimized conditions, rhEPO from three different sources were resolved into distinct populations within 5 min with excellent reproducibility. As compared with existing methods, the presented method exhibited the advantages of speed and high resolution. If combined with an effective sample enrichment step and a much more sensitive WCID version, the method can be a potential alternative for the detection of rhEPO misuse in sports 08230.

A screening method able to differentiate recombinant human erythropoietins (rhEPOs) and analogues like CERA, continuous erythropoietin receptor activator, from human urinary erythropoietin (uhEPO) was described. The method is based on the discrimination between isoforms observed when the protein is eluted under acidic followed by basic conditions from immunoaffinity microtiter wells. From a comparison with the complex IEF protocol currently applied in anti-doping analysis, the newly developed assay procedure is amenable to wide screening application and presents good resolving power between rhEPOs and uhEPO 09163.

The measurement of serum erythropoietin (EPO) is important for the detection of recombinant human EPO misuse in sports. It was developed a sensitive and rapid CE immunoassay with enhanced chemiluminescence detection of EPO, in which silica dioxide nanoparticles (SiO₂ NPs) were used as the pseudostationary phase to improve the separation efficiency of analytes. By adopting SiO₂ NPs in the CE immunoassay, the separation can be successfully performed in neutral running buffer solution. However, running buffer of extreme pH was still necessary for the conventional CZE mode. Neutral phosphate buffer (pH 7.4) containing SiO₂ NPs and poly(ethylene oxide) (PEO) was chosen as running buffer. The influences of SiO₂ NPs, PEO and buffer concentration on the separation efficiency and EPO detection were investigated. EPO-horse radish peroxidase (HRP) and immunocomplex were baseline separated in a 10 mM phosphate buffer (pH 7.4) consisting of 0.08 percent PEO and 0.08 percent SiO₂ NPs. The linear range for EPO was
1.8-158.0 ng/mL and the detection limit was 0.9 ng/mL. The assay was successfully applied for the quantification of EPO in human sera and the results correlated well with those obtained using chemiluminescence immunoassay kits, thus demonstrating that the present method was a potential powerful tool for EPO misuse detection and clinical diagnosis [09164].

The tampering of athlete’s urine samples by the addition of proteolytic enzymes during the doping control sampling procedure was reported recently. The aim of one study, funded by the World Anti-Doping Agency (WADA), was the application of a stabilization mixture in urine samples to chemically inactivate proteolytic enzymes and improve the electrophoretic signal of erythropoietin (EPO) in human urine. The stabilization mixture applied was a combination of antibiotics, antimycotic substances and protease inhibitors. A series of incubation experiments were conducted under controlled conditions in the presence and absence of the stabilization mixture in urine aliquots spiked with six proteases. Two different analytical techniques were applied for the qualitative and quantitative EPO measurement: isoelectric focusing (IEF) and chemiluminescent immunoassay respectively. The addition of the chemical stabilization mixture into urine aliquots substantially improved EPO detection in the presence of proteolytic enzymes following incubation at 37 degrees C or storage at -20 degrees C. The results of this study indicated that the stabilization of urine prior to the sample collection procedure with the proposed chemical mixture might prove to be a useful tool for the preservation of anti-doping samples [09165].

Mass spectrometry-based proteomic approaches have been used to develop methodologies capable of detecting the abuse of protein therapeutics such as recombinant human erythropoietin and recombinant human growth hormone. Existing detection methods use antibody-based approaches that, although effective, suffer from long assay development times and specificity issues. The application of liquid chromatography with tandem mass spectrometry and selected reaction-monitoring-based analysis has demonstrated the ability to detect and quantify existing protein therapeutics in plasma. Furthermore, the multiplexing capability of selected reaction-monitoring analysis has also aided in the detection of multiple downstream biomarkers in a single analysis, requiring less sample than existing immunological techniques. The flexibility of mass spectrometric instrumentation has shown that the technique is capable of detecting the abuse of novel and existing protein therapeutics, and has a vital role in the fight to keep sports drug-free [10123].

The sample preparation method preceding the urinary erythropoietin (EPO) doping test is based on several concentration and ultrafiltration steps. In order to improve the quality of isoelectric focusing (IEF) gel results and therefore, the sensitivity of the EPO test, new sample preparation methods relying on affinity purification were recently proposed. One article focused on the evaluation and validation of disposable immunoaffinity columns targeting both endogenous and recombinant EPO molecules in two World Anti-Doping Agency (WADA) accredited anti-doping laboratories. The use of the columns improved the resolution of the IEF profiles considerably when compared with the classical ultrafiltration method, and the columns’ ability to ensure the isoform integrity of the endogenous and exogenous EPO molecules was confirmed. Immunoaffinity columns constitute therefore a potent and reliable tool for the preparation of urine samples and their use will significantly improve the sensitivity and specificity of the actual urinary EPO test [10124].

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of antibiotics, antimycotic substances and protease inhibitors. A series of incubation experiments were conducted under controlled conditions in the presence and absence of the stabilization mixture in urine aliquots spiked with six proteases. Two different analytical techniques were applied for the qualitative and quantitative EPO measurement: isoelectric focusing (IEF) and chemiluminescent immunoassay respectively. The addition of the chemical stabilization mixture into urine aliquots substantially improved EPO detection in the presence of proteolytic enzymes following incubation at 37 degrees C or storage at -20 degrees C. The results of this study indicated that the stabilization of urine prior to the sample collection procedure with the proposed chemical mixture might prove to be a useful tool for the preservation of anti-doping samples [10125].

One report summarized the main analytical strategies developed to identify the presence of recombinant erythropoietin (EPO) administered as a doping agent. Indirect evidence is based on the analysis of blood parameters (haemoglobin, haematocrit, reticulocytes, macrocytes, etc.) and serum markers (concentration of EPO and serum transferrin receptors, etc.). The problem of intertechnique comparison for reliable results evaluation is emphasized, especially for serum markers. Charge differences between isoforms of recombinant EPO and native urinary EPO are the grounds for the isoelectric focusing-double blotting-chemiluminescence detection method presently approved for doping control. Works addressing its advantages and limitations are presented and commented on. The chemical bases of the differential detection are highlighted and some future approaches for detection are also presented. The appearance and detectability of EPO analogues and mimetics susceptible for abuse are also addressed [07117].

Erythropoietin (EPO) promotes the production of red blood cells, the key factor in the regulation of the oxygen transport, and has been abused by athletes for performance enhancement in endurance sports. Current methods to detect EPO misuse are based on isoelectric focussing (IEF), double blotting, and chemiluminescence detection. A new approach utilizing SDS-PAGE mobilities of target analytes is presented. Employing two internal standards (novel erythropoiesis stimulating protein and recombinant rat EPO), the assay provides a tool which allows the calculation of relative mobility values for endogenous urinary EPO and recombinant epoetins (e.g. Dynepo) and, thus, the distinction of these analytes in doping control samples. A reference group of 53 healthy volunteers and samples originating from a Dynepo (epoetin delta) excretion study conducted with a single person were analyzed and led to a significant discrimination of endogenous urinary and recombinant EPO. A clear differentiation was accomplished over a period of four days post-administration of a single injection of 50 IU/kg body weight. Hence, the method may be useful as a screening procedure in doping control or as complementary confirmation tool to the established IEF assay [07118].

The detection of recombinant human erythropoietin (rhEPO) is difficult and becomes more challenging when only microdoses are administered intravenously. Twenty-three subjects were divided into two groups: EPO group (n=7) and CONTROL group (n=16). Seven urine and blood samples per subject were collected at least 5 days apart to determine within- and between-subject standard deviations in the percentage of migrating isoforms by the MAIIA test. Six injections of 50 IU/kg bw (boosting dosage) of epoetin beta (Neorecormon) were performed intravenously during a 3-week period, followed by two microinjections of only 10 IU/kg bw. Blood and urine samples were collected 2, 6, 12, and 72 h after the microinjection, as well as 72 h after the last boosting dose. Sensitivities and specificities of the MAIIA test were examined by absolute and passport thresholds. Sensitivity was 100 percent for at least 12 h after the microinjection, with 30 percent of plasma samples still exceeding the 99.9 percent passport threshold 72 h after a microinjection. The specificity was higher for the passport approach compared to the absolute approach, but there were no
differences in sensitivities between approaches or between specimens (urine and plasma). It was concluded that the MAIIA test shows potential for detecting very small doses of rhEPO [13315].

Misuse of recombinant human erythropoietin (rhEPO) is a major concern in competitive sports, and the implementation of tests allowing for higher detection rates than what current tests are capable of is required. In this study, a novel lateral flow EPO isoform test kit, EPO WGA MAIIA, is evaluated on the basis of plasma and urine samples obtained from eight healthy males in connection with a 28-day rhEPO injection period. rhEPO was injected every other day during the first 14 days of the study, and the method proved to be 100% effective in detecting rhEPO in the concomitantly obtained samples. Seven days after the last injection, three positive (>99.99% confidence limit (CL)) subjects were found. When using 99% CL as the cut-off limit, six of the eight subjects (75%) were found to be suspected of doping. Samples obtained 14 and 21 days after the last injection showed no detectable trace of rhEPO. A previous study using indirect methods to determine EPO doping on the same samples indicated only that two of the subjects had suspicious values 7-21 days after the last injection. We propose implementing the easy to-use EPO WGA MAIIA test as an initial screening procedure in anti-doping work to increase the detection rate of potential rhEPO doping athletes and allow for a 10- to 20-fold higher analytical rate than what is possible today [13316].

Among the peptide hormone-derived therapeutics, ESAs and predominantly erythropoietin (EPO) have been subject of extensive studies concerning improved or newly established traceability as well as pure/fundamental research elucidating and later exploiting the small but significant differences between the natural human EPO and its recombinant analogs. In order to probe for the capability of routine doping control methodologies, i.e. isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) and sodium dodecyl sulfate-PAGE (SDS-PAGE), to detect epoetin kappa in human urine, an administration study was conducted. Three male volunteers received 3000 IU intravenously and urine samples were monitored for up to 48 h. Both approaches (IEF- and SDS-PAGE) allowed for the detection of epoetin kappa with SDS-PAGE being superior in terms of the detection window (24 h). While the benefits of SDS-PAGE concerning EPO analyses have been recognized several years ago, its utility for third-generation EPO drugs was established only recently with the introduction of sarcosyl-PAGE (also referred to as SAR-PAGE). Using sarcosyl instead of SDS, enhanced antibody-antigen binding as well as improved band focusing was accomplished, which allowed for a significantly lowered detection limit of the prohibited compound in plasma and urine sports drug testing samples. The different electrophoretic behaviors of recombinant human EPO products and their natural analogs in serum and urine, which are essential to all routinely applied doping control methods, have been attributed to minor but analytically relevant modifications within the glycosidic moiety. Consequently, elucidating the nature of these modifications was of particular interest to research groups. Focusing on recombinant human EPO, glycopeptides derived from enzymatic digests with trypsin and Glu-C were separated by capillary electrophoresis and analyzed by means of ESI-TOF MS. Here, comprehensive glycoform analysis was conducted for both N- and O-glycopeptides, allowing (among others) the identification of a sulfated sialoform of N83 in recombinant human EPO. As dictated by the employed analytical technique, only accurate masses (errors varied up to 30 ppm) of the protonated intact analytes, as well as respective adduct ions, served for characterization purposes. Following a different strategy, namely sequential deglycosylation by exoglycosidase treatment and subsequent SDS-PAGE analysis, human urinary and serum EPO as well as recombinant EPO were investigated concerning their glycosylation pattern. While EPO from all three sources was amenable to degradation by β-N-acetylglucosaminidase, the subsequent incubation with alpha- or beta-
mannosidase did affect only recombinant EPO, demonstrating a distinct difference in the glycosidic moiety potentially offering a new angle for future doping control assays [13012].

Aiming at a fast alternative to conventional EPO doping control tests, the utility of the recently introduced membrane-assisted isoform immunoassay (MAIIA) combined with wheat germ agglutinin (WGA)-based chromatographic separation of recombinant as well as human urinary and serum EPO was evaluated. Nine different recombinant EPO preparations expressed in hamster or human cell lines were differentiated using a “dip-stick test” that allows the completion of a set of samples within 1 h necessitating a minimum of 0.7 pg of EPO (absolute) in the test sample (immunoaffinity-purified extract of 5 ml of urine). In authentic administration study samples, the subcutaneous administration of recombinant EPO was determined up to seven days in urine specimens of patients with renal dysfunction. The same analytical strategy was applied to a set of plasma samples collected from healthy volunteers having received an intravenous “microdose” (10-40 IU/kg BW) 72-96 h post-administration. In contrast to subcutaneously administered recombinant EPO analyzed in urine, only two out of nine plasma samples tested positive under the given administration protocol; however, it was concluded that an intra-individual comparison of Percentage-of-Migrated-Isoform (PMI)-values significantly improves the assay’s sensitivity and thus can enhance its fitness-for-purpose for future doping control [13012].

Employing immuno-magnetic beads-based extraction (IMBE) combined with capillary zone electrophoresis and deep UV laser-induced fluorescence detection, the highly resolved glycoform profiling of EPO was accomplished for pharmaceutical preparations. The approach can support studies concerning isoform composition studies in recombinant EPO products but the sensitivity was not found sufficient for the analysis of urine or serum and the method considered prone to matrix interference; hence, an introduction into sports drug testing programs is not expected [13012].

Development of a mass spectrometric method for the unambiguous detection of doping with recombinant human erythropoietins (rhEPO) has been attempted for many years. Unfortunately, progress in this field was hampered by the unavailability of highly purified human endogenous EPOs (urinary[uhEPO], serum/plasma EPO) – a prerequisite for generating detailed mass spectrometric glycosylation data necessary for revealing significant differences between uhEPO and rhEPOs. The paper presents the worldwide first analytical data on purified human urinary EPO generated with a high resolution high accuracy mass spectrometer (LTQ-Orbitrap). The focus is on the tryptic O-glycopeptide (E117-R131) and its degree of sialic acid O-acetylation. Data are compared with results obtained from 40 rhEPO pharmaceuticals. It could be demonstrated that the O-glycopeptide of uhEPO (ca 100 IU) contains only trace amounts of mono-acetylated mono- and di-sialylated O-glycans but no other O-acetylated structures and in this respect significantly differs from all rhEPOs. Moreover, Dynepo – a rhEPO previously thought to be not O-acetylated - also contains small amounts of O-acetylations within the O-glycan structure. The results might be useful for antidoping purposes as well as the development of EPO pharmaceuticals with closer structural similarity to the endogenous hormone [13317].

The misuse of microdoses of performance enhancing drugs like erythropoietin (EPO) constitutes a major challenge in doping analysis. When injected intravenously, the half-life of recombinant human EPO (rhEPO) like epoetin alfa, beta, and zeta is only a few hours and hence, the window for direct detection of rhEPO in urine is small. In order to investigate the detection window for rhEPO directly in blood and urine with a combined affinity chromatography and lateral flow immunoassay (EPO WGA MAIIA), we recruited nine healthy people who each received six intravenously injected microdoses (7.5 IU/kg) of NeoRecormon (epoetin beta) over a period of three weeks. Blood and urine samples were collected in the
days following the injections and analyzed with EPO WGA MAIIA as well as the current validated methods for rhEPO; isoelectric focusing (IEF) and sarcosyl polyacrylamide gel electrophoresis (SAR-PAGE). For samples collected 18 h after a microdose, the sensitivity of the EPO WGA MAIIA assay was 100 percent in plasma and 87.5% in urine samples at the respective 98% specificity threshold levels. In comparison, the sensitivity in plasma and urine was 75 and 100 percent, respectively, with IEF, and 88 percent in plasma and 100 percent in urine when analyzed with SAR-PAGE. It was concluded that EPO WGA MAIIA is a sensitive assay for the detection of rhEPO, with the potential of being a fast, supplemental screening assay for use in doping analysis [13318].

**Recommended methods for detection of abuse in 2013**

The current technical document published by WADA in 2013 stipulates IEF or SAR both as the initial test and for confirmation purposes. The recommended method used for confirmation depends on the type of substance found in the initial assessment. New developments for ESA screening in urine include the use of liquid chromatography mass spectrometry (LCMS). This technique, commonly used in anti-doping settings, showed promising results but still needs to be validated in the field. Another advance in the field of ESA detection is the MAIIA (membrane-assisted isoform immunoassay) method for the detection of ESAs. It combines immunoassay with chromatography technology and can be used for both urine and blood samples. The first results have been promising, especially for the detection of very low doses of ESA. Another advantage of this new approach is the relatively easy analytical handling of the test and very little time requirements, allowing a high throughput of samples within a short time. On the other hand, the high costs of the MAIIA method may be considered a drawback. As for the LCMS method, validation is still pending [13006].

**Possible origins of undetectable EPO in urine samples**

In order to determine the possible origins of undetectable EPO profiles in athletes’ urine, it was analyzed the data obtained from a large number of official anti-doping urine tests aimed at detecting recombinant erythropoietin. The following variables were considered as potential causes for lack of EPO detection: athlete’s gender, competition effect, urine specific gravity as well as possible usage of proteasic adulterants to evade doping detection. Statistical analyses indicated that undetectable EPO profiles were clearly related to urine properties such as low EPO concentrations or extreme specific gravities. The addition of very small quantities of protease was shown to remove all traces of EPOs in urine. This finding led to the development of a simple, specific and sensitive test that reveals proteasic activity based on albumin digestion. It was concluded that urine characteristics clearly affect the detectability of an EPO profile. At the same time, addition of anti-proteases prevents the adulteration of urine. These two findings have clear practical implications with regards to the timing of urine collection as well as the entire anti-doping control procedure [07119].

**Lateral flow isoform test**

Misuse of recombinant human erythropoietin (rhEPO) is a major concern in competitive sports, and the implementation of tests allowing for higher detection rates than what current tests are capable of is required. In this study, a novel lateral flow EPO isoform test kit, EPO WGA MAIIA, is evaluated on the basis of plasma and urine samples obtained from eight healthy males in connection with a 28-day rhEPO injection period. rhEPO was injected every other day during the first 14 days of the study, and the method proved to be 100 percent effective in detecting rhEPO in the concomitantly obtained samples. Seven days after the last injection, three positive (>99.99% confidence limit (CL)) subjects were found. When using 99 percent CL as the cut-off limit, six of the eight subjects (75%) were found to be suspected of doping. Samples obtained 14 and 21 days after the last injection showed no detectable trace of rhEPO. A previous study using indirect methods to determine EPO doping on the same
samples indicated only that two of the subjects had suspicious values 7-21 days after the last injection. We propose implementing the easy to-use EPO WGA MAIIA test as an initial screening procedure in anti-doping work to (1) increase the detection rate of potential rhEPO doping athletes and (2) allow for a 10- to 20-fold higher analytical rate than what is possible today [13319].

**Erythrocyte aspartate aminotransferase activity**
A reliable and cost-effective screening test for erythropoietin (EPO) doping is still unavailable. Thus a new approach by estimating mean red blood cell (RBC) age by means of erythrocyte aspartate aminotransferase activity (eAST) was developed. We investigated 201 women and 169 men residing at low altitude for peak oxygen uptake, EPO, and eAST. Additionally, it was investigated 63 women and 42 men residing at 2600 m above sea level for EPO and eAST. Furthermore, 22 female and 28 male patients with renal failure receiving recombinant human EPO (rhEPO) were investigated for eAST levels. There was no difference in eAST between trained, moderately trained, and untrained subjects (women and men) at low altitude. Participants receiving rhEPO had a significant dose-dependent increase in eAST. Trained high-altitude residents had higher eAST than untrained high-altitude residents. Since eAST was sensitive to RBC rejuvenation, eAST elevation could indicate EPO use in lowlanders. eAST values above the 95 percent confidence interval (>3.3 U/gHb for men; >4.1 U/gHb for women) are suspected of EPO use [07120].

**Discriminant analysis**
The detection in urine of recombinant human erythropoietin (rHuEPO), a hormone misused by endurance athletes as a doping agent, is based on the differentiation of its isoelectric pattern from that of the corresponding natural hormone. Different empirical criteria have been proposed for discriminating the images of the patterns but none of them have been elaborated from a rational statistical approach. Discriminant analysis was applied to a dataset of profiles defined as positive (116 profiles from 26 subjects) (presence of rHuEPO and possibly residual natural endogenous hormone) and negative (131 profiles from 131 subjects) (presence of natural endogenous hormone only). The different bands were numbered according to a template of 16 possible positions and their relative intensities constituted the 16 variables of the statistical analysis. This method was then tested with data from an administration trial of low doses (6.7-10 IU/kg) following high-dose (265 IU/kg) injections (71 profiles from one subject). The analysis of the dataset clearly separated the negative and positive profiles. A cross-validation procedure confirmed that the analysis was extremely stable: with ten-fold cross-validation, no false positives were observed even with 100,000 simulations. Furthermore, the detection of rHuEPO in the profiles from the low-dose trial was greatly improved in comparison with a previously validated empirical criterion [07121].

**Bayesian inference approach for the detection of abnormal values**
Sports authorities exclude athletes with abnormal levels of blood parameters. Here, the consideration of longitudinal blood profiles together with heterogeneous factors such as ethnicity and age produces a model with enhanced sensitivity to detect blood doping. In sports, rHuEPO, the recombinant form of erythropoietin, is illicitly used to improve physical performance. Some sports federations with a fairly homogeneous population of athletes have discouraged rHuEPO doping by introducing hematocrit (Hct) and hemoglobin (Hb) limits. Athletes tested above these limits are declared unfit for competition: the so-called no-start rule. Unfortunately, since Hct and Hb present elevated between-subject variations and can easily be manipulated, the efficiency of both variables remains limited. Models using multiple hematological variables, such as Abnormal Blood Profile Score (ABPS), were proposed to improve sensitivity/specificity. Along with this, the idea of a hematological passport was also suggested. Sportsmen with significant differences between new test results and an individual
historical baseline could be excluded from competition. Hematological variables depend on various factors such as gender, ethnicity and age. Even though the effects of these factors on hematological parameters have been well described, they have not been taken into consideration in the formulation of a blood model. This leads to an unpredictable number of false-positive findings for heterogeneous populations. Unsurprisingly, sports federations with highly heterogeneous populations refrained from introducing a no-start rule. It was, therefore, propose a blood test that combines a multiparametric approach for increased specificity, a hematological passport for individual longitudinal monitoring, and the formal integration of various factors for heterogeneous populations. The blood test is based on a global Bayesian inference approach for the detection of abnormal values over time. Hb and ABPS3 (with Hct, Hb, RBC count, reticulocytes percentage, mean corpuscular volume, mean corpuscular Hb, mean corpusular Hb concentration variables) markers are used. Effects of gender [male, female], ethnicity [Caucasian, Asian, African, Oceanian], age [<19 years, 19-24 years, >24 years], altitude [<610, 610–1730, >1730], sport [endurance, non-endurance] on the mean of each parameter were taken from published data. Except for gender, the variance of blood parameters is considered independent of the factors and modeled as a log-normal distribution with parameters estimated from data collected on control subjects. Firstly, 135 blood profiles were collected from 22 top-level elite endurance athletes, 11 males and 11 females, participating in a study commissioned by the Swiss Federal Office for Sport to promote drug-free sports. Regular anti-doping tests were conducted on each athlete over a period of 2 years (6 blood and 11 urine samples in average) returning only negative test results. Secondly, 572 blood profiles were collected from 47 male amateur athletes over a period of two months. To summarized, 32 healthy volunteer males participated in this study. The aim was to reproduce rHuEPO doping habits practised in some sports. Eight subjects received subcutaneous injections of 40 IU/kg of EPREX three times a week (group R40), 8 subjects 80 IU/kg doses (group R80), 8 subjects received placebo (group P), and the last 8 subjects (group NT) had no treatment. rHuEPO administration in R40 and R80 groups was nevertheless individualized. A full dosage was administered for Hct below 45 percent, half doses for Hct between 45 and 50 percent, and substitution by isotonic saline when Hct exceeded 50 percent. Five hundred and ten blood samples (178 from doped subjects) were analyzed. In total, 1,039 samples collected in the three studies were used to estimate the specificity. Consideration of a new individual Hb value induces a new distribution of possible values of Hb, and, therefore, new individual reference range. The significant decrease between the first (a population-based threshold) and the last cut-off limit (an individual threshold) can be associated to a larger between-than within-subject variance of the hematological variable. All three aspects of the model (multiparametric approach, longitudinal analysis and consideration of heterogeneous factors) lead to a decrease of the overall variance of hematological data. A heightened sensitivity to discontinuous rHuEPO treatment was observed. A population-based strategy on Hb gives only 3 true positives for a specificity of 0.999, whereas the Bayesian model returned as much as 11 times more true positives. With the ABPS marker, sensitivity was even further improved [07124].
detection models applied. In recreational athletes, for EPO and sTfR, significant differences were only observed after the 21-km race. In baseline conditions, no differences were observed between recreational and elite athletes for EPO and sTfR. In elite athletes, individual EPO and sTfR concentrations slightly changed over the sport season, with coefficients of variation (CV) of 26 percent and 9 percent, respectively. Hemoglobin and reticulocytes were influenced by sport, but their individual variation over the sport season was not physiologically relevant. When applying mathematical models for detection of rhEPO administration, only one elite athlete obtained an individual model score above the established thresholds. Physical fitness, sport and different training workload during the sport season had no substantial effect on serum EPO and sTfR concentrations, except in recreational athletes after a 21-km race. Variations observed in mathematical models to detect EPO administration were mainly due to fluctuation in hemoglobin concentrations, commonly observed in elite athletes [07122].

The effects of recombinant human erythropoietin (rHuEpo) treatment on aerobic power (VO\textsubscript{2max}) are well documented, but little is known about the effects of rHuEpo on submaximal exercise performance. The present study investigated the effect on performance (ergometer cycling, 20-30 min at 80 % of maximal attainable workload), and for this purpose eight subjects received either 5,000 IU rHuEpo or placebo every second day for 14 days, and subsequently a single dose of 5,000 IU/placebo weekly/10 weeks. Exercise performance was evaluated before treatment and after 4 and 11 weeks of treatment. With rHuEpo treatment VO\textsubscript{2max} increased by 13 and 12 percent in week 4 and 11, respectively, and time-to-exhaustion (80 % VO\textsubscript{2max}) was increased by 54 and 54 percent after 4 and 11 weeks of treatment, respectively. However, when normalizing the workload to the same relative intensity (only done at time point week 11), TTE was decreased by 27 percent as compared to pre rHuEpo administration. In conclusion, in healthy non-athlete subjects rHuEpo administration prolongs submaximal exercise performance by about 54 percent independently of the approximately 12 percent increase in VO\textsubscript{2max} [07123].

False-positive detection following strenuous physical exercise
Erythropoietin (Epo) is a glycoprotein hormone that promotes the production of red blood cells. Recombinant human Epo (rhEpo) is illicitly used to improve performance in endurance sports. Doping in sports is discouraged by the screening of athletes for rhEPO in urine. The adopted test is based on a combination of isoelectric focusing and double immunoblotting, and distinguishes between endogenous and recombinant human Epo. It was shown that this widely used test can occasionally lead to the false-positive detection of rhEpo (epoetin-beta) in postexercise, protein-rich urine, probably because the adopted monoclonal anti-Epo antibodies are not monospecific [06161].

Effects of high intensity training on measurement techniques
Exercise induced proteinuria is a common phenomenon in high performance sports. Based on the appearance of so called "effort urines" in routine doping analysis the purpose of this study was to investigate the influence of exercise induced proteinuria on IEF profiles and SDS-PAGE relative mobility values (rMVs) of endogenous human erythropoietin (EPO). Twenty healthy subjects performed cycle-ergometer exercise until exhaustion. VO\textsubscript{2max}, blood lactate, urinary proteins and urinary creatinine were analysed to evaluate the exercise performance and proteinuria. IEF and SDS-PAGE analyses were performed to test for differences in electrophoretic behaviour of the endogenous EPO before and after exercise. All subjects showed increased levels of protein/creatinine ratio after performance (8.8 ± 5.2 to 26.1 ± 14.4). IEF analysis demonstrated an elevation of the relative amount of basic band areas. Using SDS-PAGE analysis it was observed a decrease in rMVs after exercise and no shift in direction of the recombinant human EPO (rhEPO) region. Following identification criteria of the World Anti Doping Agency all samples were negative. The implementation of
the SDS-PAGE method represents a good solution to distinguish between results influenced by so-called effort urines and results of rhEPO abuse. Thus, this method can be used to confirm adverse analytical findings [10126].

New laboratory technique

The constant development of new erythropoiesis-stimulating agents (ESAs), since the first introduction of recombinant erythropoietin (rhEpo) for clinical use, has also necessitated constant development of methods for detecting the abuse of these substances. Doping with ESAs is prohibited according to the World Anti-Doping Code and its prohibited list of substances and methods. Since the first publication of a direct and urine-based detection method in 2000, which uses changes in the Epo isoform profile as detected by isoelectric focusing in polyacrylamide slab gels (IEF-PAGE), the method has been constantly adapted to the appearance of new ESAs (e.g., Dynepo, Mircera). Blood had to be introduced as an additional matrix, because Mircera (a PEGylated Epo) is best confirmed in serum or plasma after immunoaffinity purification. A Mircera ELISA was developed for fast screening of sera. With the appearance of Dynepo and copy epoetins, the additional application of sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE or equivalent) became necessary. The hematological module of the Athlete Biological Passport is the latest development in multivariable indirect testing for ESA doping [11141].

The biochemical actions and side effects of recombinant erythropoietins (rhEPOs), their analogs and mimetics, their misuse as doping agents, and the principal analytical strategies developed to identify them in athletes' biologic fluids are reviewed. Patients who experience a range of pathologies have benefited from the administration of rhEPOs to correct severe anemia. Currently, monitoring the biologic effect of rhEPO in patients under treatment is by measuring the hemoglobin concentration. However, it may be valuable to directly monitor the actual levels of the administered drug and determine a dose-dependent correlation with any clinical adverse effect observed. This may permit the adoption of a patient-specific administration regime. Currently, the method of detecting EPO approved for doping control is an isoelectric-focusing, double-blotting, chemiluminescence assay based on charge differences between isoforms of rhEPOs and endogenous EPO in urine. The advantages and limitations of this method were presented. A new approach using sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a complementary tool to the established method is discussed. The application of matrix-assisted laser desorption/ionization mass spectrometry and liquid chromatography combined with tandem mass spectrometry for the direct detection of the rhEPO molecules may prove to be promising. Indirect evidence of rhEPO abuse by athletes is based on the analysis of blood parameters (hemoglobin, hematocrit, reticulocytes, macrocytes, etc) and serum markers (concentration of EPO and serum transferrin receptors, etc). Enrichment of the screened parameters with gene or biochemical markers revealing altered erythropoiesis and adoption of longitudinal monitoring of athletes' hematologic and biochemical parameters could also be a complementary approach in the fight against doping [11142].

Isoelectric focusing

IEF can be used to differentiate human urinary erythropoietin (uEPO), recombinant human erythropoietin or epoetin (rEPO) and darbepoetin (novel erythropoiesis stimulating protein (NESP)). This is the basis of the method currently used to detect misuse of rEPO and NESP by elite athletes. Recently, an unknown activity has been attributed to some urine samples (denominated “unstable” urine by the World Anti-Doping Agency; WADA). This activity has shown to give rise to artefactual profiles for both rEPO and NESP when incubated with such urine and, thus, raised concerns with respect to doping control. It was evaluated which charges produce the characteristic IEF profiles of uEPO, rEPO and NESP and how these profiles respond to distinct enzymatic reactions. From sialidase digestions it became evident
that only uEPO contains charges different from sialic acid, and a comparison of all substances after complete de-N-glycosylation localized these charges in the carbohydrate moiety. Partial desialylation, or digestion with arylsulfatase from Helix pomatia yielded profiles for recombinants species similar to those observed for unstable urine samples. The contributions from our studies to the anti-doping problem include: protocols that may corroborate the potential misuse of rEPO or NESP based on the particular enzymatic activity of an arylsulfatase preparation, or a broad-specificity sialidase; assurance that the instability observed in some urine samples may only result from false-negatives, but not from false-positive testing; and a simple remedy to prevent an unstable urine from altering the IEF profile by adding selective competitive substrates [06159].

**Band segmentation**

In recent years, the development of methodology and laboratory techniques for doping control of recombinant erythropoietin (rEpo) has become one of the most important topics pursued by doping control laboratories accredited by World Anti-Doping Agency (WADA). The software system GASepo has been developed within the international WADA project as a support for Epo doping control. Although a great number of functions for automatic image processing have been involved in this software, for Epo images with considerably distorted bands additional effort is required from the user to interactively correct the results of improper band segmentation. In this paper a problem of geometrically distorted bands is addressed from the viewpoint of how to transform the lanes in distorted Epo images in order to reach better band segmentation. A method of band straightening via column shift transformation has been proposed that is formulated as an optimization procedure with cost functions. The method involves several novel approaches: two-stage optimization procedure, four cost functions and selection of relevant columns. The developed band straightening algorithm has been tested on real Epo images with distorted bands. Based on the evaluation scheme involving the GASepo software itself a recommendation is made for implementation of the method with the cost function based on correlation matrix. Estimates of computational complexity of the individual steps of BSA are also given [06160].

**DNA testing**

Endogenous and exogenous erythropoietin (EPO) present in urine can be distinguished according to their isoelectric profiles. This methodology requires urine samples to be concentrated about 200 to 1000 times with manipulations that should remove most of the cells occurring in the original sample. In one study, it was tried to obtain DNA profiles from 10 ultrafiltered urines (retentates) in order to evaluate whether a formal genetic identification was technically feasible. No nuclear DNA profiles could be established from retentates, despite 34 PCR-cycles amplifications. Contrastingly, mitochondrial DNA (mtDNA) profiles were obtained for 9 out of the 10 retentates. Apart from some particularities, retentate mtDNA profiles were all distinct and matched mtDNA profiles of corresponding reference samples [08234].

**Differences in laboratory accuracy**

The main action of recombinant human erythropoietin (rHuEpo) is to increase the oxygen carrying capacity of the blood. To prevent a possible misuse of rHuEpo, this is tested in urine samples collected from athletes by World Anti-Doping Agency (WADA)-accredited laboratories. Recently the test has met serious critiques, and the aims of one study were to investigate the detection power of the test as well as the variability in the test power comparing the results of two WADA-accredited laboratories. Eight human subjects were studied for 7 weeks and treated with rHuEpo for 4 weeks with 2 weeks of “boosting” followed by 2 weeks of “maintenance” and a post period of 3 weeks. Urine samples were obtained during all periods. Laboratory A determined rHuEpo misuse in all subjects during the boosting period, whereas laboratory B found no misuse, with one sample to be negative, and
the remaining seven to be suspicious. The detection rates decreased throughout the maintenance and post period when total hemoglobin mass and exercise performance were elevated. During this period, laboratory A found only two of 24 samples to be positive and three to be suspicious, and laboratory B found no positive or suspicious samples. The study demonstrates a poor agreement in test results comparing two WADA-accredited laboratories. Moreover, after the initial rHuEpo boosting period the power to detect rHuEpo misuse during the maintenance and post periods appears minimal [08231].

**Hepcidin as a marker**

Inhibition of hepcidin expression by erythropoietic signals is of great physiological importance; however, the inhibitory pathways remain poorly understood. To investigate the direct effect of erythropoietin (Epo) and the contribution of putative mediators on hepcidin repression, healthy volunteers were injected with a single dose of Epo, either low (63 IU/kg, n=8) or high (400 IU/kg, n=6). Low-dose Epo provoked hepcidin down-modulation within 24 h; the effect was not immediate as hepcidin circadian variations were still present following injection. High-dose Epo induced no additional effect on the hepcidin response, that is hepcidin diurnal fluctuations were not abolished in spite of extremely high Epo levels. It was not found significant changes in putative mediators of hepcidin repression, such as transferrin saturation, soluble transferrin receptor, or growth differentiation factor 15. Furthermore, the potential hepcidin inhibitor, soluble hemojuvelin, was found unaltered by Epo stimulation. This finding was consistent with the absence of signs of iron deficiency observed at the level of skeletal muscle tissue. The data suggest that hepcidin repression by erythropoietic signals in humans may not be controlled directly by Epo, but mediated by a still undefined factor [13307].

The human hepcidin-25 hormone has a key role in iron regulation in blood. The clinical relevance of this hepatic about 2.8 kDa cysteine-rich peptide is rapidly increasing, since altered levels can be associated with inflammatory events and iron dysfunctions, such as hereditary hemochromatosis and iron overload. Moreover, hepcidin has also attracted the anti-doping field for its possible role as indirect marker of erythropoietin blood doping. Methods currently reported are based on immunoassays (ELISA and RIA), or various types of mass spectroscopy (MS)-based protocols, semi-quantitative or quantitative. Despite the great effort in optimizing robust and simple assays measuring hepcidin in real matrices, at present this challenge remains still an open issue. To explore the possibility to face hepcidin detection through the development of affinity-based biosensors, we set up a comparative study by surface plasmon resonance (SPR) technology. An immuno-based, on anti-hepcidin-25 IgG, and a biomimetic-based, on a synthetic peptide corresponding to the hepcidin-binding site on ferroportin (HBD), biosensors were developed. It was reported behaviors and analytical performances of the two systems, discussing limits and potentialities [13308].

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observed at the level of skeletal muscle tissue. Our data suggest that hepcidin repression by erythropoietic signals in humans may not be controlled directly by Epo, but mediated by a still undefined factor [13309].

MicroRNAs

Due to the complexity of detecting and differentiating recombinant EPO from its natural analog in human urine or blood, alternative indirect approaches have been subject of various recent studies. One of these elucidated the impact of EPO administration on circulating and/or renally eliminated microRNAs (miRNA) and their potential as long-term biomarkers for ESA doping. Following the intravenous or subcutaneous administration of Mircera (200 microg), plasma samples were collected for up to 27 days and analyzed for miRNA affected in a statistically significant manner. Among a variety of marker candidates, miR-144 was the most influenced parameter, which was significantly elevated 27 days post-administration of Mircera and thus possibly representing a valuable alternative marker for illicitly administered ESAs. These preliminary findings will require in-depth elucidation and validation but possess the potential for expanded complementary doping control assays. Hepcidin has been considered as another potential marker for EPO administration due to its decreased serum concentration following subcutaneous EPO injections. Whether the effect also prevails upon i.v. applications of 50 IU/kg bodyweight was subject of a study by Laine et al. in 2012. Despite significantly elevated (4 h post administration) and subsequently decreased (24 h post administration) serum hepcidin levels, the dynamics and variability of the marker essentially excluded its utility as indicator for doping control purposes [13012].

MicroRNAs (miRNAs) are small non-coding RNAs that regulate various biological processes. Cell-free miRNAs measured in blood plasma have emerged as specific and sensitive markers of physiological processes and disease. In this study, we investigated whether circulating miRNAs can serve as biomarkers for the detection of autologous blood transfusion, a major doping technique that is still undetectable. Plasma miRNA levels were analyzed using high-throughput quantitative real-time PCR. Plasma samples were obtained before and at several time points after autologous blood transfusion (blood bag storage time 42 days) in 10 healthy subjects and 10 controls without transfusion. Other serum markers of erythropoiesis were determined in the same samples. Our results revealed a distinct change in the pattern of circulating miRNAs. Ten miRNAs were upregulated in transfusion samples compared with control samples. Among these, miR-30b, miR-30c, and miR-26b increased significantly and showed a 3.9-, 4.0-, and 3.0-fold change, respectively. The origin of these miRNAs was related to pulmonary and liver tissues. Erythropoietin (EPO) concentration decreased after blood reinfusion. A combination of miRNAs and EPO measurement in a mathematical model enhanced the efficiency of autologous transfusion detection through miRNA analysis. Therefore, our results lay the foundation for the development of miRNAs as novel blood-based biomarkers to detect autologous transfusion [13320].

Further biomarkers could be derived from circulating miRNAs for the abuse of ESAs. On the basis of microarray results, a significant difference in the levels of miRNAs was measured in plasma after CERA injection, even for a longer period of time as the increase of the specific miR-144 lasted 27 days after CERA administration. Similarly, the same group described that autologous blood transfusion leads to an increase of selected circulating miRNAs in plasma of pulmonary and liver tissue origin compared to a non-transfused control group (personal communication) [13006].

Confounding factors for erythropoietin detection
The detection window for rhEPO is known to be relatively short and depends on the dosage and the method of administration: Whereas subcutaneous administration of rhEPO in clinical dosages (e.g. 50 IU/kg body weight) might be detected over several days, it has been demonstrated that intravenous administration and microdosing (i.e. 500 IU) are virtually undetectable while still producing a measurable erythropoietic effect. In order to blur the IEF picture, athletes nowadays use mixtures of different rhEPO, knowing that these will produce uncharacteristic patterns which will have difficulties in meeting the positivity criteria for positive rhEPO tests set by WADA. To overcome this limitation and other sampling-related problems such as bacterial degradation or exercise-associated proteinuria, which might interfere with IEF results, another method for the detection of EPO misuse, SDS/SAR-PAGE (sodium dodecyl sulfate/sarcosyl polyacrylamide gel electrophoresis) was developed. This method relies on the difference in molecular weight of the different types of EPO and is able to identify different rhEPO variants. With rhEPO being one of the commercially most successful drugs developed over recent decades, many biosimilars were marketed in different countries (epoietin alpha, delta, kappa, omega etc). As these biosimilars are also slightly different in their molecular mass (because they originate from different transfected cells), they are detectable by the same testing principles of SDS-PAGE. Because of low urine excretion Continuous Erythropoietin Receptor Activator (CERA) is harder to detect, however, glomerular permeability is increased with strenuous exercise. In addition CERA can be detected with ELISA. However, even substances with modified molecular structure such as darbepoietin (Aranesp®) or CERA (continuous erythropoietin receptor activator, Mircera®), developed to have a longer half-life in the organism, can be detected, as they usually contain a conventional epoietin backbone paired with another component to slow its excretion and prolong its action in the organism (i.e. methoxy polyethylene glycol for CERA). It has to be acknowledged that not all rhEPO variants will be detectable, as those manufactured by underground laboratories constitute an analytical challenge, these EPO forms being slightly different and changing from batch to batch. Conversely, when applying the WADA positivity criteria, these are often not met. Unfortunately, microdoses of rhEPO, sufficient to give a pharmacological effect can have a detection window as low as 12-18 h. Urine manipulation with protein degrading proteases might decrease this even further [13006].

Available erythropoietins

Peptidic erythropoiesis-stimulating agents
Currently available rhEpo preparations (epoetins) are produced in Epo complementary DNA (cDNA) transfected Chinese hamster ovary (CHO) or baby hamster kidney (BHK) cell cultures. The only therapeutic rhEpo engineered in human cells (epoetin-beta) is off market since the beginning of 2009. Because the patents of the originator epoetins have died, biosimilar products have been approved in many parts of the world. Furthermore, various copied CHO cell-derived rhEpos are available in countries without a regulated market. Endogenous Epo and the epoetins have an invariant sequence of 165 amino acids, but they differ in glycosylation. Compared with the epoetins, endogenous Epo isoforms are more acidic and smaller in size. Epo can be separated by isoelectric focusing (IEF) or electrophoresis of urine samples. After IEF, a double-blotting procedure is performed. The mutein darbepoetin-alfa migrates more in the acidic range than Epo on IEF. The WADA has established criteria to achieve harmonization in the performance of the test for epoetin and darbepoetin in urine. A recent detection problem has arisen with the addition of proteases by athletes to their urinary samples, which destroys the erythropoietic proteins. The adulteration of urine with proteases is a prohibited method, and techniques have been developed for the detection of their misuse. Another difficulty relates to the fact that once hemoglobin concentration has been raised in athletes by the administration of recombinant ESAs, only microdoses or less frequent injections of the drugs are needed to maintain hemoglobin at the
high level. In this situation, the window of detection of rhEpo in urine is only 12-18 hours, compared with about 3 days on regular dosing (50 U/kg body weight 3 times a week). Thus, although the detection of rhEpo in urine is effective if the injection frequency is high, this is certainly not the case when the injection rate is reduced to weekly injections. Because darbepoetin-alfa has a 3- to 4-fold longer half-life (24-26 hours) than the epoetins (6-8 hours), the window of detection of darbepoetin-alfa is prolonged to approximately 7 days. CERA has an even longer half-life of approximately 6 days. IEF of CERA yields bands in the less acidic area compared with native Epo. IEF for investigation of doping with CERA has also been applied to blood samples.47 In addition, CERA can be detected by ELISA. Of particular interest are also recombinant fusion proteins of Epo with the Fc region of human IgG because one of these was shown to stimulate erythropoiesis, when administered as an aerosol in a phase 1 trial. Epo mimetic peptides (EMPs) are synthetic cyclic peptides of about 20 amino acids. A potent pegylated EMP dimer (INN: peginesatide; Hematide) proved to stimulate erythropoiesis in experimental animals and in healthy male volunteers. Peginesatide is currently in phase 3 trials for the treatment of patients with chronic renal failure. Peginesatide can be detected by enzyme-linked immunoassay. In an alternative approach, EMPs have been constructed onto human IgG1-based scaffolds by recombinant DNA technology. The seminal compound, CNTO 528 (Centocor), produced a reticulocytosis and increased hemoglobin concentration on intravenous administration in a phase 1 study in healthy men. The follow-on product CNTO 530, a dimeric EMP fused to a human IgG4 Fc scaffold, has been shown to expand the pool of erythroid progenitors in vitro and in vivo [11116].

**Drugs activating the endogenous Epo gene**
The Epo enhancer is under the control of HIFs, heterodimeric proteins composed of two subunits. HIF-2 is the main factor inducing Epo gene expression. The C-terminus of HIF is composed of proline residues that are hydroxylated in the presence of oxygen. Prolyl hydroxylated HIF binds the von Hippel-Lindau tumor suppressor protein in complex with an E3-ligase and undergoes immediate proteasomal degradation. The transcriptional activity of the HIFs is suppressed by O2-dependent hydroxylation of an asparagine residue. The HIF hydroxylases contain Fe^{2+} and are inactivated by Fe^{2+} removal. However, iron chelators are not suited for stimulation of erythropoiesis in the long-term because iron is required for heme synthesis. HIF-dependent Epo gene expression is augmented by divalent transition metals, such as cobalt or nickel. It has been known that cobalt increases erythropoiesis in experimental animals. Cobalt binds to HIF thereby preventing the docking of von Hippel-Lindau tumor suppressor protein. Cobalt is a very potent inducer of Epo gene transcription. Indeed, the international Epo unit (IU) was originally defined as the dose eliciting the same erythropoiesis stimulating response in rats as 5 micromol of cobaltous chloride. However, the treatment of anemic uremic patients with cobalt (commonly administered as enteric-coated tablets, 30-150 mg daily) is no longer performed because of its toxicity. However, cobalt may be misused by athletes as a proper means to enhance Epo levels and Hb_{mass}. Cobalt is very potent, inexpensive, and not comprehended in the WADA’s “Prohibited List.” Furthermore, the HIF hydroxylases require alpha-ketoglutarate for their catalytic action. alpha-Ketoglutarate competitors (“HIF stabilizers”) are orally active compounds that stimulate Epo production and erythropoiesis. A number of chemically different HIF stabilizers has been identified. However, HIF stabilizers induce the expression of more than 200 genes apart from Epo genes, which may result in serious unwanted effects in athletes. On the other hand, it is probable that some of the HIF-activated genes encode proteins that may increase physical performance (e.g. glycolytic enzymes, glucose transporters, and angiogenic peptides) [11116].

**Other erythropoietic hormones**
Several hormones may stimulate the renal and/or hepatic production of Epo, including
prostanoids, thyroid hormone, angiotensin II, growth hormone (GH), and testosterone. The latter are of particular interest regards blood doping. A study in anemic uremic patients has shown that plasma Epo levels increase 6 hours after the start of GH infusion, with peak values reached after 96 hours. The fact that the rise in plasma Epo occurred earlier than the rise in insulin-like growth factor-1 (IGF-1) indicates that GH directly stimulates Epo production. IGF-1 was earlier shown to promote the growth of erythrocytic progenitors. The concentration of circulating IGF-1 correlates with hematocrit in uremic patients. Synthetic GH secretagogues (ghrelin mimetics), recombinant human GH, and recombinant human IGF-1 are available for therapeutic purposes. Anabolic-androgenic steroids also increase both the production of Epo and the proliferation of erythrocytic progenitors in the bone marrow [11116].

C.E.R.A., a continuous erythropoietin receptor activator

Methoxy polyethylene glycol-epoetin beta is a third-generation molecule of continuous erythropoietin receptor activator (CERA), incorporating a large polymer chain and has an elimination half-life in humans that is up to 6 times longer than darbepoetin alpha and up to 20 times longer than epoetin, making it possible for once in 2 weeks to once a month dosing strategy. The successful conversion of patients on epoetin or darbepoetin to CERA has been successfully demonstrated. The starting dose of CERA would be 125 microg if the patient had previously received <8000 U epoetin weekly or <40 microg darbepoetin weekly, or a dose of 200 microg if the patient had previously received 8000-16,000 units epoetin weekly or 40-80 microg darbepoetin weekly. It is administered either iv or sc. The iv route is recommended for patients receiving hemodialysis because it may be less immunogenic. CERA can be administered once in every 2 weeks or once monthly to patients whose hemoglobin has been stabilized by treatment with an EPO [12242].

Since the 90's, human erythropoietin is produced recombinantly and used clinically. There are various products from different suppliers, which differ primarily in their production but not in their half-life or effectiveness. 2001 genetically modified darbepoetin alpha was launched, which is characterized by an approximately three times longer half-life. A further extension of the half-life to 130 hours is achieved with the current continuous erythropoietin receptor activator (CERA), which therefore must be applied only once or twice a month. The indication for epoetin therapy is primarily for the symptomatic renal anemia and chemotherapy-associated anemia. Corrections of low hemoglobin levels in asymptomatic patients are not allowed. The generally recommended hemoglobin target range is 10-12 g/dL. Hb values > 13 g/dL should be avoided because they are associated with significant adverse effects and do not improve patient survival [11465].
erythropoiesis-stimulating agent (ESA) that has recently been linked with abuse in endurance sports. In order to combat this new form of doping, we examined an enzyme-linked immunosorbent assay (ELISA) designed to detect the presence of C.E.R.A. in serum samples. The performance of the assay was evaluated using a pilot excretion study that involved six subjects receiving C.E.R.A. Validation data demonstrated an excellent reproducibility and ensured the applicability of the assay for anti-doping purposes. To maximize the chances of detecting the drug in serum samples, we propose the use of this specific ELISA test as a high-throughput screening method, combined with a classic isoelectric focusing test as a confirmatory assay. This strategy should make C.E.R.A. abuse relatively easy to detect, thereby preventing the future use of this drug as a doping agent [09171].

Erythropoietin (EPO) has been misused in sports for many years due to its performance-enhancing effect. In the last decade, detection of abuse has been possible with isoelectric focusing (IEF) based on the different isoform profiles of endogenous and recombinant EPO. The release of new EPOs on the market, such as the recombinant erythropoietin epoetin delta (Dynepo™) and the chemically modified EPO, CERA (Mircera™) potentially represents analytical challenges to the fight against doping. One study set out to investigate the possibility of and the time window for detecting the administration of a single dose of Dynepo™ and CERA. Our results are in agreement with earlier findings that detection of Dynepo™ is best achieved by combining IEF with SDS-PAGE. Hematological parameters were monitored for possible effects due to the long half-life (130 hours) of CERA in blood. Interestingly, although several haematological parameters were significantly changed after the injection of CERA, the endogenous EPO signal was still present in all collected samples. Due to the long half-life and the large size of the CERA molecule (about 60 kDa), it was uncertain whether CERA would be excreted into urine in detectable amounts unless urine collection was preceded by strenuous physical exercise. It was found that CERA can be detected in urine without prior exercise in several, but not all, subjects. CERA is nevertheless best detected in serum with regard to both probability and length of detection, in addition to stability in matrix over time [11143].

Darpoietin/NESP

Erythropoietin (EPO) is a hormone that regulates red blood cell production. Recombinant human EPO (rHuEPO) and NESP (novel erythropoiesis stimulating protein) have been produced for therapeutic purposes and also to improve sports performance. The primary sequences of rHuEPO and NESP differ by just five amino acids. Due to the high homology, no antibodies that are able to discriminate between both molecules have been obtained until now. The aim of one work was to design synthetic peptides corresponding to the sequence that differs between EPO and NESP (87-90aa), that can then be used as immunogens to develop specific rabbit polyclonal antibodies for selectively detecting EPO and NESP. Three peptides were synthesized: EPO (81-95), NESP (81-95), and NESP (86-104), and these were coupled to KLH and OVA for immunization and screening purposes, respectively. The sera obtained were tested by ELISA on synthetic peptide-OVA conjugates and purified by immunoaffinity chromatography against the corresponding synthetic peptide. The specific purified antibodies were characterized by ELISA, SDS-PAGE, and isoelectric focusing, followed by western blot. Antisera raised against EPO (81-95) recognized rHuEPO but not NESP. In contrast, anti-NESP (84-106) sera gave a specific anti-NESP response only after immunoaffinity purification on a NESP (86-91) column. An efficient strategy for generating specific antibodies against EPO and NESP can be achieved by selecting suitable synthetic peptides. The antibodies obtained are able to differentiate between rHuEPO and NESP, and may be particularly useful for screening purposes in both therapeutic and antidoping contexts.
Darbepoetin alfa (DPO) or Novel Erythropoiesis Stimulating Protein (NESP), an analog of recombinant human erythropoietin (rhEPO), is abused as a blood doping agent along with the latter in human sports. One paper described a new method for unequivocal identification of DPO in human plasma. The analyte was extracted from plasma by immunoaffinity separation with anti-rhEPO antibodies, digested by trypsin followed by PNGase F, and analyzed by liquid chromatography coupled to tandem mass spectrometry. The deglycosylated tryptic peptide was employed in DPO identification using liquid chromatographic retention time and major product ions of the peptide. The limit of detection of this method for DPO was 0.1 ng/mL in plasma, and that of identification was 0.2 ng/mL. This method is definitive and devoid of false positive results, providing "mass fingerprints" for identification of DPO in human plasma samples. Although this method is not applicable to identification of rhEPO in human plasma because it cannot differentiate rhEPO from endogenous EPO, it is the first successful attempt towards establishing a reliable and selective method for definitive identification of exogenously administered EPOs in doping control analyses.

Darbepoetin alfa is a modified erythropoietin (EPO) molecule with a longer serum half-life than recombinant human erythropoietin (rhEPO). Because the detection period of rhEPO in urine is only 2-3 d after the last injection, blood algorithms have been developed in order to expand the detection window of rhEPO misuse. The main objectives were to establish the period of detection of darbepoetin alfa by isoelectric focusing (IEF) and examine the applicability of blood algorithms and individual variations in blood variables in an antidoping context. Six recreationally active males and six recreationally active females had 0.78 microg/kg per week of darbepoetin alfa administered for 3 weeks. Blood and urine samples were collected continuously during and after administration. Urine samples were analyzed by IEF and immunoblotting for darbepoetin alfa, and blood samples were analyzed for erythropoietic sensitive blood variables on a hematological analyzer. Darbepoetin alfa was detected in 8 of 12 samples at 10 d after the last injection. Ten subjects showed variations in hemoglobin concentration > 10 percent, whereas only three males and one female exceeded suggested upper hemoglobin concentration limits of 17.0 and 16.0 g/dL, respectively. Four subjects exceeded the 1:1000 ON- as well as the OFF-model cutoff limit. It was concluded that the large number of samples containing detectable amounts of darbepoetin alfa at 10 days into the washout period stipulate the possibility of a 7-days window of detection after administration, wherein a sample would be regarded as an adverse analytical finding. The marked variations in all examined blood parameters could be used for the targeting of urine samples. These preliminary findings open up for larger scale studies with more frequent urine sampling in the washout period on elite athletes.

Darbepoetin alpha (a hyperglycosylated rhEPO): It has five N-glycosylation sites as compared to three in the rhEPO. This is created by a process called "site mutagenesis" and confers higher negative charge and threefold longer half-life. This is based on the principle that increase in number of glycosylation sites may enhance its activity. This helps in giving once a week dosing strategy. The approved dosage in anemia of CKD is 0.45 mcg/kg iv or sc. Weekly monitoring of hemoglobin is suggested upon initiation of therapy and then to maintain hemoglobin levels <12 g/dL and to avoid increases of hemoglobin >1 g/dL over a 2-week period.

The current official direct recombinant erythropoietin (rHuEPO) detection anti-doping test based on 1D isoelectric focusing (IEF) of urinary proteins was performed to determine the detection window of darbepoetin-alpha when applying the positivity criteria established by the World Anti-Doping Agency (WADA). Following WADA's positivity criteria, the IEF based
The misuse of recombinant human erythropoietin (rhEPO) increases the proliferation/production of erythrocytes, which enhance oxygen transport capacities, and has grave consequences with respect to human health and fairness in sports. For sports drug testing, the current analytical methods for rhEPOs are mainly gel electrophoretic methods, such as isoelectric focusing-polyacrylamide gel electrophoresis. Mass spectrometry is fundamentally necessary for the reliable identification of rhEPOs in doping control. In this study, a high-sensitivity and high-throughput mass spectrometric qualitative detection method for darbepoetin alfa in human urine was established by a bottom-up approach. The novel method involves the immunopurification of human urine (10 mL), protease digestion with endoproteinase Glu-C (V8-protease) in an ammonium bicarbonate buffer (pH 7.8) and ultra-performance liquid chromatography using a charged surface hybrid C(18) column coupled with electrospray-ionisation high-sensitivity tandem mass spectrometry for improved selectivity of the target molecules. The specific fragment digested from darbepoetin alfa was (90)TLQLHVDKAVSGLRSLTTLLRALGAQKE(117) (V(11)). The lower limit of detection of urinary darbepoetin alfa was 1.2 pg/mL. The limit of detection for the confirmation analysis was estimated to be 5 pg/mL. The developed method allows high-throughput confirmation analysis, namely 6 h for sample preparation and an analytical run time of only 10 min per sample; this high-throughput method dramatically decreases the workload in the laboratory. Darbepoetin alfa could be identified in human urine collected after the intravenous administration of 15 microg darbepoetin alfa (n=3). This mass spectrometric method is an innovative and powerful tool for detecting darbepoetin alfa in human urine for doping control testing [13322].

Epoetin delta (Dynepo®)

A novel recombinant human erythropoietin (epoetin delta, Dynepo®) has been marketed in the European Union for the treatment of chronic kidney disease, cancer patients receiving chemotherapy, and so forth. Epoetin delta is engineered in cultures of the human fibrosarcoma cell line HT-1080 by homologous recombination and “gene activation." Unlike recombinant erythropoietins produced in other mammalian cells, epoetin delta is supposed to have a human-type glycosylation profile. However, the isoelectric focusing profile of epoetin delta differs from that of endogenous erythropoietin (both urinary and plasmatic). In one work, structural and quantitative analysis of the O- and N-glycans of epoetin delta was performed and compared with glycosylation from recombinant erythropoietin produced in Chinese hamster ovary cells. From the comparison, significant differences in the sialylation of O-glycans were found. Furthermore, the N-glycan analysis indicated a lower heterogeneity from epoetin delta when compared with its ovary cell homologue, being predominantly tetraantennary without N-acetyllactosamine repeats in the former. The sialic acid
characterization revealed the absence of N-glycolylneuraminic acid. The overall sugar profiles of both glycoproteins appeared to be significantly different and could be useful for maintaining pharmaceutical quality control, detecting the misuse of erythropoietin in sports, and establishing new avenues to link glycosylation with biological activity of glycoproteins [08232].

Aiming for the determination of detection windows concerning the administration of Dynepo or Mircera, haematological parameters as well as SDS-PAGE and IEF-PAGE were conducted on urine and serum samples of participants of an excretion study. Following a single subcutaneous injection of 75 microg (men) or 50 microg (women) of Mircera or 4000 IU of Dynepo, urine, EDTA blood, and serum were collected up to 21 days. Dynepo was identified in urine up to 4 days while Mircera was observed in urine in several cases for more than 6 days and in serum in most volunteers up to 14 days [12016].

Hematide®

Erythropoietin (EPO) and other erythropoiesis-stimulating agents possess a high misuse potential in elite sport due to their ability to increase the oxygen transport capacity, which plays a vital role in enhancing endurance performance. Currently, a new generation of EPO-mimetic peptides is under development from which peginesatide (also referred to as Hematide®), a pegylated homodimeric peptide of approximately 45 kDa with no structural relationship to erythropoietin, is the most advanced drug candidate undergoing phase-III clinical trials. Since preventive doping research aims at the development of detection methods before a drug receives clinical approval, a selective and sensitive assay has to be established knowing that conventional doping control assays for EPO will not succeed in detecting peginesatide. Thus, a pegylated EPO-mimetic peptide simulating the structure and properties of the lead drug candidate peginesatide was synthesised as a model compound for this new class of emerging drugs and characterised by means of gel electrophoresis, matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry, as well as liquid chromatography/electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS) after proteolytic digestion. Based on these results, a mass spectrometric detection method of the product in plasma was developed targeting a pentapeptide fragment after protein precipitation and subtilisin digestion. Its fitness for purpose was evaluated by the determination of selected method characteristics focusing particularly on specificity, recovery (about 60 %), and limit of detection (1 ng/mL) [13339].

Erythropoiesis-stimulating agents (ESAs) have frequently been confessed to be illicitly used in elite sports due to their endurance enhancing effects. Recently, peginesatide, the first representative of a new generation of ESAs, referred to as Erythropoietin (EPO)-mimetic peptides, obtained approval in the USA under the trade name Omontys(®) for the treatment of anaemic patients. Lacking sequence homology with EPO, it consists of a pegylated homodimeric peptide of approximately 45 kDa, and thus, specific approaches for the determination of peginesatide in blood were developed as conventional detection assays for EPO do not allow for the analysis of the EPO-mimetic peptides. However, as urine specimens are the most frequently provided doping control samples and pharmacokinetic studies conducted in rats and monkeys revealed the excretion of the pegylated peptide into urine, a detection method for peginesatide in urine would be desirable. A mass spectrometric assay in human urine was developed consisting of protein precipitation with acetonitrile followed by proteolytic digestion after the removal of the acetonitrile fraction under reduced pressure. Purification and concentration of the resulting proteotypic target peptide was accomplished by means of solid-phase extraction on strong cation-exchange resin prior to liquid chromatographic-tandem mass spectrometric analysis. Method validation was
performed for qualitative purposes and demonstrated specificity, precision, linearity as well as sufficient sensitivity (limit of detection: 0.5 ng/ml) while proof-of-concept for the applicability of the assay for the determination of peginesatide in authentic urine samples was obtained by analyzing animal in vivo specimens collected after a single i.v. administration of peginesatide over a period of 4 days [12259].

As recently reported, dried blood spot (DBS) analysis is an advantageous technique for doping control purposes due to the minimal invasive sample collection, the simple and economic manner, as well as the low susceptibility to manipulation. Its general applicability to the sports drug testing arena has been shown for analytes of various substance classes, all of which comprise exclusively low molecular mass compounds. The aim of one study was to investigate whether the technique of DBS analysis is applicable also to (pegylated) peptides with relevance for doping controls. As target analyte, peginesatide (Omontys, Hemitide), a recently approved pegylated erythropoietin-mimetic peptide of approximately 45 kDa, tested for the treatment of anaemia in patients with renal failure, was chosen, which has been prohibited in elite sports due to its assumed endurance enhancing effects. Therefore, a detection method for peginesatide employing DBS was developed based on extraction, proteolytic digestion and cation-exchange purification followed by liquid chromatography-tandem mass spectrometry analysis. Eventually, the assay was validated for qualitative purposes and proved to be specific, sensitive (limit of detection, 10 ng/mL) and precise (relative standard deviations below 18 %), demonstrating the general suitability of DBS analysis in sports drug testing also for (pegylated) peptides [12260].

Non-EPO-related erythropoiesis stimulating agents

With the market for anti-anemia drugs in 2012 estimated to be close to USD 10 billion in the USA alone, it is clear that there is a strong research incentive for pharmaceutical companies which has led to the development of new ESAs, unrelated to the conventional EPO. Several approaches have been proposed:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Mechanism of action</th>
<th>Stage of development</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPO mimetic peptides, peginesatide (Hemitide/Omontys)</td>
<td>Direct stimulation of erythropoiesis</td>
<td>Withdrawn from market in February 2013 after adverse reaction</td>
</tr>
<tr>
<td>EPO fusion proteins CNTO 528, 530 (Centocor)</td>
<td>Direct stimulation of erythropoiesis</td>
<td>Phase I clinical trials</td>
</tr>
<tr>
<td>EPO gene manipulation Transcription activation Gene transfer</td>
<td>Introduction of EPO-producing cells into human organism/induction of EPO gene transcription</td>
<td>Animal studies/experimental human study – proof of concept</td>
</tr>
<tr>
<td>HIF stabilizers/propyl hydroxylase inhibitors</td>
<td>Induction of the EPO production cascade by inhibiting HIF-1alpha degradation</td>
<td>Clinical trials</td>
</tr>
<tr>
<td>GATA inhibitors</td>
<td>Increase the transcription of the EPO gene and thereby EPO production</td>
<td>Clinical trials</td>
</tr>
</tbody>
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Most of the substances are still in clinical trials, but the past has shown that black market laboratories are quick in copying drugs before even official approval has been granted to the developing pharmaceutical companies. One drug which had US Food and Drug Administration (FDA) approval in the USA until recently is peginesatide (marketed as Omontys®), a peptide structurally unrelated to EPO but with erythropoietic abilities. After
hypersensitivity reactions in first-time users had resulted in three deaths, the drug was withdrawn from the market in February 2013. Fusion proteins are peptides resulting from the fusion of genes that initially coded for different proteins. The resulting peptides very often have properties from both of the genes they originated from. Fusion proteins are found in cancer cells, where they occur naturally as a result of cancer-associated mutation. In EPO research, fusion proteins showing erythropoietic capacity have been created and advanced to clinical trials. Some have been attached to immunoglobulin, which facilitates their administration (e.g. as an aerosol through a nasal spray). CNTO 530, an IgG4 fused peptide, increased erythropoietic activity after a single administration in mice. Unfortunately EPO gene manipulation has for many years been recognized as a potential undetectable doping method. There are several scientific reports on the successful transfer of the EPO gene via a viral vector into muscle or dermal cells. The technique demonstrated reliable increase in all erythropoietic markers. Hypoxia inducible factor (HIF) 1alpha is the upstream regulator of the EPO signal in the organism. Under conditions of normoxia, this protein is usually immediately degraded by prolyl hydroxylases after its synthesis. Hypoxia, however, inhibits prolyl hydroxylases and thereby stabilizes the HIF-1alpha protein which can then fulfill its action and induce the transcription of EPO. EPO will subsequently stimulate the erythropoietic cascade. Therapeutic anti-anemia strategies aim to inhibit the prolyl hydroxylases that induce the breakdown of HIF-1alpha. A multitude of substances have been developed in this context and are currently at various stages of clinical experimentation [13006].

Other than through prolyl hydroxylases and HIF-1alpha, EPO gene expression is also regulated through transcription factors that will negatively affect the transcription: So-called GATA proteins bind to a certain sequence on the EPO promoter gene and inhibit its transcription, thus negatively affecting the EPO cascade. The activity of these GATA binding proteins can be altered by GATA inhibitors, which have been shown to measurably affect erythropoiesis. As most of the substances mentioned above are not naturally occurring in the body, their detection with conventional methods is, in theory, not problematic. Furthermore, many of the drugs are aimed at long-term action to overcome the frequent injections necessary with conventional EPO, which would improve the quality of treatment for the patient, but also the detection window for anti-doping tests. Interestingly, the EPO gene transfer seems also to be detectable: Lasne et al. demonstrated in primates that the EPO originating from the transferred gene is different from the endogenous one, possibly because of the mild difference in cell organelles between the different types of cells. Whereas natural, endogenous EPO originates from kidney fibroblasts, the EPO genes are often transferred into muscle cells by using their cell organelles and thus creating a slight difference in glycosylation and molecular mass [13006].

**Synthetic peptide-based EPO receptor agonist**

The development of erythropoiesis-stimulating agents (ESA) for the treatment of anemia has been a highly active field over the past 2 decades, wherein 6 different rEPOs have been licensed worldwide and more than 90 biosimilar rEPOs or copies have become available in countries with low regulatory controls of pharmaceutical products. This frenzied rhythm is expected to continue because new generations of ESAs are expected in the near future, such as the synthetic peptide-based EPO receptor agonist Hematide®; the conjugated EPO-mimetic peptide Sestide®; hypoxia-inducible transcription factor stabilizers, such as FG-2216®; and modified cells that carry the human EPO gene or the EPO protein, such as EpoDure®. In parallel with increasing numbers of prescribed ESAs, some designer rEPOs have been created by black market laboratories to get around existing drug tests. All ESAs aim to improve oxygen transport by the metalloprotein hemoglobin. Consequently, measurement of hemoglobin concentration (HGB), one of the most common medical tests in a full blood count profile, has been used as a biomarker of blood doping. In the mid-1990s,
some sports federations introduced upper limits on HGB and hematocrit (HCT) levels, and athletes with values above these limits were temporarily suspended from competition in an attempt to limit the abuse of rEPO. Interestingly, such doping biomarkers are independent from the marketing of novel doping substances, and although the pharmaceutical industry continues to market new drugs every year, the biology of the human body is relatively stable for general physiological functions. The evolution of the human body takes at least several generations, and owing to this biological stability, a biomarker of doping such as hemoglobin measurement will remain sensitive to any past, present, or future ESA abuse. As a result, there is an ongoing paradigm shift in testing, from the direct identification of banned substances in the biological fluids of athletes to the detection of abnormalities in biomarkers that potentially indicate that doping has occurred. Although it is difficult to predict which of the new ESAs will be available during the 2016 Olympic Games in Rio de Janeiro and beyond, the biological characteristics of the athletes participating in these games will not differ from the biological characteristics of athletes that are competing today. Consequently, all of the doping biomarkers that have already been developed will remain applicable in the upcoming Olympic Games and for several decades into the future, whereas a toxicology test must be established for almost every newly marketed drug. For example, today’s biomarkers of blood doping are already sensitive to gene doping with the human EPO gene [11426].

EPO-Fc fusion protein

The neonatal Fc receptor (FcRn) has been under investigation for several years as a pharmaceutical drug target. Clinical studies have shown that fusion proteins consisting of human recombinant erythropoietin (rhEPO) and the Fc-part of IgG can be transported after pulmonary administration via FcRn across the airway epithelium to the blood stream. So far, no clinically approved pharmaceutical formulation of EPO-Fc is available. Since various forms of recombinant erythropoietins have been frequently misused by athletes as performance-enhancing agents, EPO-Fc might play a similar role in sports in the future. In order to investigate the detectability of EPO-Fc in human blood, different strategies were tested and developed. Only two of them fulfilled the necessary requirements regarding sensitivity and specificity. A rapid protocol useful for screening purposes first enriches EPO-Fc from human serum via high capacity protein A beads and subsequently detects EPO-Fc in the eluate with a commercial EPO ELISA kit. The limit of detection (LOD) of the method is about 5 pg (45 micromol) EPO-Fc and is independent of the serum volume used. For screening and/or confirmation purposes a second protocol was evaluated, which consists of a fast EPO immunopurification step followed by sodium dodecyl sulfate or sarcosyl polyacrylamide gel electrophoresis (SDS-PAGE, SAR-PAGE) and Western double-blotting with chemiluminescence detection – a method already established in routine EPO antidoping control. The latter strategy allows the detection of EPO-Fc in serum together with all other recombinant erythropoietins and with an identical LOD (5 pg/45 micromol) as for the rapid screening protocol [12245].

A long-acting erythropoietin

The Fc fusion technology has been introduced to generate long-acting antagonistic drugs such as Enbrel, Oencia and Amevive. Here, Genexine created a novel noncytolytic hybrid Fc (hyFc) as a carrier of agonistic protein drugs using naturally existing IgD and IgG4 Fcs without any mutation in the hyFc region. The erythropoietin (EPO) fused with hyFc exhibited little binding activity to FcyR and C1q molecules that are main mediators for death of target cells. The EPO-hyFc showed higher in vitro and in vivo bioactivities than EPOIgG1 Fc and highly glycosylated EPO (Aranesp). Phase I clinical trial with EPO-hyFc is currently undergoing in Korea [12261].
New erythropoietin-like drug principles

Among the class of ESAs banned in sports, erythropoietin (EPO) and respective mimetics such as Hematide (Peginesatide) represent the major challenges for doping control laboratories. As a medicinal success story developed over 140 plus years, EPO and its derivatives have been misused to increase performance which resulted in numerous cases of adverse analytical findings and doubt as to the honesty and validity of athletes and their sport victories. The traceability of EPO abuse has been a complex issue and the methodologies available to sports drug testing laboratories enabling the differentiation of recombinant human EPO from its naturally produced analogue are laborious and time-consuming. Moreover, findings of EPO in particular, have frequently been challenged by athletes. Consequently, much effort and research resources were invested in improving the analytical approaches by focusing on sample preparation alternatives as well as identifying and eliminating technical issues. A technical problem concerning lane streaking during isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) was identified to result from detergents present in commonly used application pieces. Already a brief contact of nitrile glove-protected fingertips with the electrolyte strips considerably affected the analytical result making an evaluation impossible. Thus, the use of latex-based gloves or (metal) forceps was recommended to circumvent the issue. Another reason for insufficient EPO analytical data (especially due to extensive smearing) was found to be the presence of therapeutic amounts of heparin in urine. Due to its polyanionic nature, heparin interacts with carrier ampholytes commonly employed in IEF-PAGE and thus interferes significantly with the focusing of EPO isoforms as demonstrated with urine samples collected after co-administration of Dynepo and heparin. Following immunoaffinity purification (which is routinely employed in several doping control analytical procedures), the interference of heparin was eliminated. Further, SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) analysis of EPO was not affected by heparin and would provide supporting information in case of inconclusive test results [12016].

Increasing the blood's capacity for oxygen transport by erythropoiesis-stimulating agents (ESAs) constitutes a prohibited procedure of performance enhancement according to the World Anti-Doping Agency (WADA). The advent of orally bio-available small-molecule ESAs such as hypoxia-inducible factor (HIF) stabilizers in the development of novel anti-anaemia therapies expands the list of potential ESA doping techniques. Here, the erythropoiesis-stimulating properties and doping relevance of experimental HIF-stabilizers, such as cobaltous chloride, 3,4-dihydroxybenzoic acid or GSK360A, amongst others, are discussed. The stage of clinical trials is reviewed for the anti-anaemia drug candidates FG-2216, FG-4592, GSK1278863, AKB-6548, and BAY85-3934. Currently available methods and strategies for the determination of selected HIF stabilizers in sports drug testing are based on liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). For the support of further analytical assay development, patents claiming distinct compounds for the use in HIF-mediated therapies are evaluated and exemplary molecular structures of HIF stabilizers presented. Moreover, data concerning the erythropoiesis-enhancing effects of the GATA inhibitors K7174 and K11706 as well as the lipidic small-molecule ESA PBI-1402 are elucidated the context of doping analysis [12258].

Darbepoetin, an analogue of rhEPO with a half-life of 18-24 h, as well as epoietin delta and continuous erythropoiesis receptor activator (CERA) with a half-life of about 6 days, are novel protein-based, stimulating erythropoiesis agents employed in blood doping. Both erythropoietin and darbepoetin bind to the EPO receptor inducing intracellular signalling via the same intracellular molecules as native EPO [12011].

Erythropoietin promotes the production of red blood cells. Recombinant human erythropoietin
is illicitly used to improve performance in endurance sports. Expression of the erythropoietin gene is negatively controlled by the transcription factor GATA-binding protein (GATA). Specific GATA inhibitors have recently been developed as novel drugs for the management of anemia. These drugs could, therefore, be illicitly used like recombinant human erythropoietin to improve performance in sports. To examine alterations in levels of plasma protein after administration of GATA inhibitors, proteomic analyses were conducted on mouse plasma samples treated with the potent GATA inhibitor K-11706. The analysis based on gel electrophoresis identified 41 protein spots differentially expressed when compared with normal plasma. Each spot was identified with liquid chromatography coupled to tandem mass spectrometry and 2 of them, fetuin-B and prothrombin, were verified by Western blotting. The results showed that the expression of fetuin-B in mice plasma was increased by K-11706, but not by recombinant human erythropoietin or hypoxia. These results suggest the potential of proteomic-based approaches as tools to identify biomarkers for the illegal use of novel drugs (e.g. GATA inhibitors). Also, fetuin-B could be a sensitive marker for the detection of abuse of GATA inhibitors [11433].

Recombinant human erythropoietin (rHuEPO) engineered in Chinese hamster ovary (CHO) cell cultures (Epoetin alfa and Epoetin beta) and its hyperglycosylated analogue Darbepoetin alfa are known to be misused by athletes. The drugs can be detected by isoelectric focusing (IEF) and immunoblotting of urine samples, because "EPO" is in reality a mixture of isoforms and the N-glycans of the recombinant products differ from those of the endogenous hormone. However, there is a plethora of novel erythropoiesis stimulating agents (ESAs). Since the originator Epoetins alfa and beta are no longer protected by patent in the European Union, rHuEPO biosimilars have entered the market. In addition, several companies in Asia, Africa and Latin America produce copied rHuEPOs for clinical purposes. While the amino acid sequence of all Epoetins is identical, the structure of their glycans differs depending on the mode of production. Some products contain more acidic and others more basic EPO isoforms. Epoetin delta is special, as it was engineered by homologous recombination in human fibrosarcoma cells (HT-1080), thus lacking N-glycolyneuraminic acid like native human EPO. ESAs under development include EPO fusion proteins, synthetic erythropoiesis stimulating protein (SEP) and peptidic (Hematide®, CNTO 528) as well as non-peptidic EPO mimetics. Furthermore, preclinical respectively clinical trials have been performed with small orally active drugs that stimulate endogenous EPO production by activating the EPO promoter ("GATA-inhibitors": diazepane derivatives) or enhancer ("HIF-stabilizers": 2-oxoglutarate analogues). The prohibited direct EPO gene transfer may become a problem in sports only in the future [10121].

Erythropoietin (EPO) is a glycoprotein that is mainly produced in the adult kidney, and it was initially highlighted for its action on the hematopoietic system. Moreover, EPO is also expressed in several non-hematopoietic tissues, where it plays a role in the protection from apoptosis and inflammation due to hypoxia, toxicity or injury. These protective effects are mainly known and studied in cardioprotection and neuroprotection but are also reported in retina degeneration, auditory injury and pancreatic-related diseases. The tissue protective effect of EPO is mainly mediated through the interaction with the heterodimeric receptor EPOR/betacR. Human recombinant EPO (HuREPO), which has been developed to treat anemia, is not adequate for tissue protection. The low affinity of the alternative receptor for EPO involves the injection of excessive concentration of erythropoiesis-stimulating agents (ESAs), implicating side effects due to the cross-talk with hematopoietic activity. For these reasons, EPO derivatives with less affinity for the EPO homodimeric receptor are under development. In this review, it was provided an overview of the erythroid and non-erythroid functions of EPO by detailing the molecular mechanisms activated by the binding of EPO to its receptors in different tissues [11466].
Adverse effects of erythropoietin

Adverse effects of recombinant human erythropoietin [12242]:
- flu-like symptoms: commonest side effect but subsides within 24 hours
- allergic and anaphylactic reactions
- seizures and hyperkalemia: rare
- hyperviscosity
- thrombosis: a meta-analysis involving nearly 10,000 cancer patients indicates that treatment with rhEPO increases the risk of thrombosis
- hypertension
- possibility of cancer progression: there is somewhat less convincing evidence that rhEPO enhances tumor progression
- pure red cell aplasia (mainly reported in patients with CKD): autoantibodies in the serum can neutralize both rhEPO and endogenous EPO. This was mainly observed in CKD patients, especially after sc injection. Its incidence after 2000 has reduced, especially with the iv formulations

Novel erythropoietin doping strategies

There are several theoretical ways of doping with erythropoietin as basis [12242]:

a. Synthetic erythropoiesis protein (SEP): SEP consists of a polypeptide chain similar to that of EPO and two covalently attached, branched polymer moieties that bear a total of eight negative charges. These polymers enhance the molecule’s stability by protecting it from proteolytic cleavage. It is also less immunogenic as it is synthesized chemically and has less contaminating antigens.

b. PO mimetics: They are small molecules capable of activating EPO-R in a way similar to EPO. On binding to the EPO-R, they cause the receptor to dimerize and activate multiple cellular signaling pathways. Subsequently, multiple genes are transcriptionally induced and mediate proliferative, antiapoptotic, and erythropoietic effects of EPO (e.g. Hematide).

c. Inhibition of hematopoietic cell phosphatase (HCP): An indigenous negative regulator of the EPO–EPO-R signaling cascade. Combination of EPO mimetics with HCP inhibitors could provide an oral substitute of endogenous EPO with equivalent potency.

d. EPO delivery by cell encapsulation: In this method, the modified cells are enclosed inside semi-permeable membrane polymers that isolate the encapsulated cells and thus prevent antigen recognition and immune rejection. Cell encapsulation as a form of immunoprotection has been shown to enhance erythropoiesis.

e. EPO gene doping: Gene doping is defined as the transfer of genetic material to improve athletic performance. In 2003, the IOC and the World Anti-Doping Authority incorporated gene doping into their list of prohibited practices. Two approaches are used here:
   i. In vivo gene transfer through intramuscular injection of a virus containing gene encoding EPO. Other gene delivery methods include plasmid DNA, liposomes, and protein-DNA conjugates, or direct injection of EPO gene into muscles.
   ii. Ex vivo gene transfer into cells that are subsequently transplanted into the recipient organism.

Detection rate
Hemoglobin concentration ([Hb]), reticulocyte percentage (retic%) and OFF(hr score) are well-implemented screening tools to determine potential recombinant human erythropoietin (rHuEpo) abuse in athletes. Recently, the International Cycling Union implemented the OFF(z score) and the Hb(z score) in their anti-doping testing programme. The aim of this study is to evaluate the sensitivity of these indirect screening methods. Twenty-four human subjects divided into three groups with eight subjects each (G1; G2 and G3) were injected with rHuEpo. G1 and G2 received rHuEpo for a 4-week period with 2 weeks of "boosting" followed by 2 weeks of "maintenance" and a wash-out period of 3 weeks. G3 received rHuEpo for a 10-week period (boost = 3 weeks; maintenance = 7 weeks; wash out = 1 week). Three, seven and eight of the 24 volunteers exceeded the cut-off limits for OFF(hr score), [Hb] and retic%, respectively. One subject from G1, nobody from G2, and seven subjects from G3 exceeded the cut-off limit for Hb(z score.) In total, ten subjects exceeded the cut-off limit for the OFF(z score); two subjects from G1, two subjects from G2 and six subjects from G3. In total, indirect screening methods were able to indicate rHuEpo injections in 58 percent of subjects. However, 42 percent of these rHuEpo-injected subjects were not detected. It should be emphasised that the test frequency in real world anti-doping is far less than the present study, and hence the detection rate will be lower [10122].

**MicroRNAs**

MicroRNAs (miRNAs) are small, non-protein coding transcripts involved in many cellular and physiological mechanisms. Recently, a new class of miRNA called 'circulating miRNAs' was found in cell-free body fluids such as plasma and urine. Circulating miRNAs have been shown to be very stable, specific, and sensitive biomarkers. In one paper, it was investigated whether circulating miRNAs can serve as biomarkers for erythropoiesis-stimulating agent abuse. To this end, we analyzed miRNA levels in plasma by miRNA microarrays and quantitative real-time polymerase chain reaction (PCR). Plasma samples are derived from a clinical study with healthy subjects injected with erythropoiesis-stimulating agent (C.E.R.A.). Based on microarray results, we observed a significant difference in the levels of miRNAs in plasma after C.E.R.A. injection. It was demonstrated that a specific miRNA, miR-144, exhibit a high increase that lasts 27 days after C.E.R.A. stimulation. Considering the fact that miR-144 is an essential erythropoiesis agent in different organi-3sm, these findings suggest the possibility of using miR-144 as a sensitive and informative biomarker to detect CERA. abuse [11464].

**Detection of misuse of erythropoietin in competitive sports and laboratory testing**

Two different analytical techniques, isoelectric focusing (IEF) and chemiluminescent immunoassay, were applied for quantification of EPO. Urine samples were treated with a stabilization mixture to chemically inactivate proteolytic enzymes and improve the electrophoretic signal of EPO, a method that might improve measurement of EPO. Novel indirect methods based on haematological and/or molecular derivatives profiling also theoretically show promise as screening tools, followed by analytical testing of those athletes who are suspected of doping. However, an indirect technique that utilizes multiple markers of enhanced erythropoiesis, combined with a confirmatory test (isoelectric patterning), is likely to be the most feasible protocol for detection of rhEPO. This protocol was approved by the IOC Medical Commission as the "ON model" and has been applied since the 2000 Sydney Summer Olympics. An already established method in routine EPO antidoping control is the sequential deglycosylation by exoglycosidase treatment (Reagent Array Analysis Method, RAAM) and subsequent sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), which has disclosed a structural difference between human endogenous and recombinant EPOs. A rapid protocol was introduced for screening purposes by enriching EPO-neonatal fetal receptor (Fc) from human serum and subsequently achieving detection of
EPO-Fc in the eluate with a commercial EPO ELISA kit. rhEPO and the Fc part of IgG can be transported after pulmonary administration via the Fc receptor and airway epithelium to the blood circulation. It was recently shown that a fast EPO immunopurification step combined with SDS-PAGE and Western double-blotting with chemiluminescence detection is capable of disclosing EPO-neonatal Fc receptor in serum together with all other recombinant EPOs. Transcriptomics, proteomics and metabolomics have been introduced in the last decade for the discovery of biomarkers as an indirect method for doping detection. However, the results, especially with regard to rhEPO, have not as yet led to a routine application in doping control, this mainly due to the complexity and inter-individual variability of human transcriptomes, proteomes and metabolomes [12011].

Administration of rhEPO was prohibited in 1990 by the International Olympic Committee (IOC). The detection of EPO abuse has been challenging for several reasons [12050]:

- availability of specialized dedicated laboratories with immense infrastructure
- screening in large numbers may be difficult as it requires highly trained technicians and standardization between laboratories
- it is difficult to discriminate between the endogenous EPO and recombinant exogenous hormone
- EPO has a relatively short half-life in serum (the half-life of rhEPO-a is 8.5 ± 2.4 hours when administered iv and 19.4 ± 10.7 hours when administered sc)
- EPO is undetectable in urine after 3-4 days of injection.

Direct method of erythropoietin detection relies on the physicochemical properties of the hormone. It is based on different carbohydrate components of recombinant and endogenous hormones, conferring different net electrical charges and thus distinguishable isoelectric points. This is the underlying principle of the only direct method of rhEPO detection that has been approved by the court of arbitration for sport. It uses electrophoretic techniques to separate the isoform profiles of recombinant and endogenous EPO in the urine according to their isoelectric points. Indirect methods of detecting misuse of erythropoietin are [12242]:

- the hematocrit
- the reticulocyte hematocrit (fractional volume of the reticulocyte pool in the bloodstream, which equals the product of the number of reticulocytes and their mean corpuscular volume: Ret × MCV Ret)
- macrocyte percentage
- serum soluble transferrin receptor (sTfR)

To use these indirect methods, two statistical models were used: “ON” model and “OFF” model. ON model detects current users and is indicative of accelerated erythropoiesis that occurs during rhEPO use (HE model uses hemoglobin and serum EPO and HES model uses hemoglobin, serum EPO concentration, and sTfR). OFF model is designed to differentiate between recent rhEPO users and non-users. This is consistent with down-regulation of erythropoiesis, which occurs following discontinuation of rhEPO (HR model uses hemoglobin and reticulocyte count and HRE model uses hemoglobin, reticulocytes, and serum EPO concentration). Many common hematological conditions and inter-individual genetic variations associated with extreme hematological profiles can also obscure the specificity of these indirect detection methods [12242].

A rapid and easy-to-use test kit, EPO WGA MAIIA, which can be used for distinguishing various endogenous human erythropoietins (hEPOs) and several recombinant hEPO and EPO analogues, has been evaluated. The test is based on chromatographic separation of the glycosylated isoforms of EPO using wheat germ agglutinin (WGA) and a sensitive immunoassay using anti-EPO carbon black nanostrings and image scanning for
quantification. All of the reactions take place along the porous layer of a lateral flow microcolumn containing WGA and anti-EPO zones. The presence of molecules resembling hEPOs, such as Mircera, was detected by the aberrant affinity interaction with the antibody zone on the strip. It was possible to distinguish nine recombinant hEPOs expressed in hamster and human cell lines, as well as Aranesp and Mircera, from endogenous urine hEPO. The required amount of EPO in the samples, a few picograms, is very low compared with other methods for EPO isoform identification. This EPO isoform determination method opens the possibility to monitor recombinant EPO therapy for clinical research and seems to be a valuable candidate to the arsenal of EPO doping control tests [12248].

Capillary electrophoresis electrospray-mass spectrometry was used to detect and characterize the great variety of O- and N-glycopeptide glycoforms of recombinant human erythropoietin (rhEPO) using an orthogonal accelerating time-of-flight mass spectrometer to obtain their exact molecular masses (CE-TOF-MS). rhEPO was digested with trypsin and Glu-C and analyzed by CE-TOF-MS to detect O(126), N(83), N(24)-N(38) and N(24) and N(38) glycopeptide glycoforms, respectively. Neuraminidase was first used to enhance the detection of the glycopeptides and detect all possible glycoforms contained in each glycosylation site. O(126) and N(83) glycopeptides were extensively characterized. Twelve sialoforms corresponding to 5 different glycoforms were detected in N(83), and for the first time, a sulfated sialoform of this glycopeptide was also detected. In the case of O(126), different sialoforms with different types of sialic acids (Neu5Gc and Neu5Ac) were detected and an estimation of the relative percentage of Neu5Gc versus Neu5Ac was also carried out for this glycopeptide. N(24) and N(38) glycosylation sites were also characterized by CE-TOF-MS after Glu-C digestion and these results permitted to rule out some glycan combinations for N(24)-N(38) glycopeptide glycoforms. The study provided a reliable glycopeptide map of rhEPO and may be regarded as an excellent starting point to analyze rhEPO glycopeptides in biological fluids and detect the use of this hormone in sports [12249].

Recombinant human erythropoietin (rHuEPO) is a 30-34 kDa glycoprotein banned by the racing authorities. For some years this molecule has been detected in race horses in USA and in Europe, and even in racing camels. Although direct methods to differentiate horse endogenous EPO and rhEPO have been developed either by LC-MS/MS or by isoelectric focusing (IEF) with double-blotting, the short confirmation time of such prohibited hormone in plasma remains a problem for horseracing doping control laboratories. In order to improve the rHuEPOs confirmation process in horse plasma or urine in terms of reliability and delay, a small anti-EPO monolith membrane contained in a disposable column (anti-EPO monolith column) has been successfully used and validated (n=10). This new sample preparation, combined with LC-FAIMS-MS/MS, has been performed on plasma and urine samples collected from one horse which received an Eprex® treatment during six consecutive days and a second one with a single injection of Aranesp®. This inventive technology allowed the possibility to confirm the presence of rHuEPO within one day with a limit of detection validated for both urine and plasma at 250 pg/mL by means of a disposable, ready to use immunoaffinity column. The lower limit of detection (LLOD) obtained for each matrix was 100 pg/mL. These results provide an important improvement for rHuEPO doping control in horseracing especially the possibility to confirm these banned molecules in both matrices, urine and plasma, with a confidence of two specific target peptides [12250].

Erythropoietin (EPO) is an important glycoprotein hormone. Recombinant human EPO (rhEPO) is an important therapeutic drug and can be also used as doping reagent in sports. The analysis of EPO glycoforms in pharmaceutical and sports areas greatly challenges analytical scientists from several aspects, among which sensitive detection and effective and facile sample preparation are two essential issues. Herein, we investigated new possibilities for these two aspects. Deep UV laser-induced fluorescence detection (deep UV-LIF) was
established to detect the intrinsic fluorescence of EPO while an immuno-magnetic beads-based extraction (IMBE) was developed to specifically extract EPO glycoforms. Combined with capillary zone electrophoresis (CZE), CZE-deep UV-LIF allows high resolution glycoform profiling with improved sensitivity. The detection sensitivity was improved by one order of magnitude as compared with UV absorbance detection. An additional advantage is that the original glycoform distribution can be completely preserved because no fluorescent labeling is needed. By combining IMBE with CZE-deep UV-LIF, the overall detection sensitivity was $1.5 \times 10^{-8}$ mol/L, which was enhanced by two orders of magnitude relative to conventional CZE with UV absorbance detection. It is applicable to the analysis of pharmaceutical preparations of EPO, but the sensitivity is insufficient for the anti-doping analysis of EPO in blood and urine. IMBE can be straightforward and effective for sample preparation. However, antibodies with high specificity were the key for application to urine samples because some urinary proteins can severely interfere the immuno-extraction [12251].

Human erythropoietin (hEPO), a hormone involved in the formation of red blood cells, is a 30 kDa glycoprotein with a high carbohydrate content. The production of recombinant hEPO has made possible its widespread therapeutic use and its banned use in competition sports. Methods to analyze EPO and other erythropoiesis stimulating agents (ESAs) are necessary for the characterization and quality control of these biopharmaceuticals and also for doping control. In this paper, high resolution separation methods, namely high performance liquid chromatography (HPLC) and capillary electrophoresis (CE), with special attention to CE-coupled mass spectrometry, are reviewed. The usefulness of these techniques when applied in different modes to separate the glycoprotein isoforms, aggregates or excipients are detailed. In addition, sample preparation methods that have been applied to ESA samples for subsequent determination by HPLC or CE, as well as the potential compatibility of other preparation methods, are discussed. Applications of the HPLC and CE methods regarding regulatory considerations for biopharmaceuticals analysis, with emphasis on biosimilars, and doping control are also included [12252].

Direct detection of doping with recombinant erythropoietins (rhEPO) is accomplished by isoelectric focusing (IEF) or sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE). In a recent publication, Lasne et al [Electrophoresis 2011; 32: 1444] showed that improper use of nitrile examination gloves during sample collection, sample preparation, and IEF-PAGE may lead to distorted or absent EPO IEF-profiles. In order to clarify which substances are responsible for this observation, a mass spectrometric study on water extractable compounds found in nitrile gloves was performed. Several substance classes were shown to be present, among them polyethylene glycols (PEG), anionic and nonionic surfactants, as well as alcohol ethoxylates and plasticizers. It could be demonstrated that alkylbenzenesulfonates, the main category of detectable anionic detergents, and among them sodium dodecylbenzenesulfonate (SDBS) and its homologs, are the prime reason for the interference of nitrile gloves with EPO IEF-PAGE [12253].

Erythropoietin (EPO) is a peptide hormone responsible for hypoxia-induced promotion of erythrocyte production. The possibility of enhancing oxygen transport through an increase of erythrocytes has led to EPO abuse in sports. Detection of exogenous EPO is most commonly done via isoelectric focusing (IEF) which is a method provided by the Technical Document TD2009EPO of the World Anti-Doping Agency (WADA). Before analysis, collected urine samples need to be concentrated 500- to 1000-fold, leading to high protein abundance in the retentates. Reduction of protein concentration through an immunoaffinity purification using ELISA wells has been successfully used prior to SDS-PAGE. This ELISA kit was used to purify samples using an IEF-compatible elution. The purification showed recovery ratios between 50 and 90 percent depending on substance and application volume. Application of immunopurified samples to IEF was shown to improve the quality of the gels by reducing
streaks and curvatures within the lanes and bands of the gel. The result was an increase of
quality for IEF gels [12254].

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quality for IEF gels [12255].

Dipstick test
Recently a novel technology, referred to as the 'EPO WGA MAIIA' test, has been developed
by Swedish researchers to discriminate between endogenous and recombinant human
erthropoietin. In contrast to existing electrophoretic methods that are used by antidoping
laboratories, this dipstick-based technique is simple and fast. Moreover it can be applied to
either blood or urine specimens. These characteristics could prove advantageous if the test
were adopted by antidoping authorities to determine blood doping in sport. It was evaluated
the sensitivity of EPO WGA MAIIA to detect the presence of recombinant human
erthropoietin (rhEPO) in some archived plasma specimens which had been collected from
healthy, active subjects either 72 h or 96 h after a 'microdose' intravenous injection of rhEPO.
Under these conditions the test had modest sensitivity to discriminate rhEPO, with only two
of nine subjects exceeding an arbitrary cut-off 3.09 standard deviations beyond the expected
population mean. Sensitivity was improved to five out of six subjects if positivity was
assessed according to the subject's own previous values rather than a population-based
threshold. It was conclude that, with further refinement, the dipstick test may supplement
existing antidoping tests [12256].

Transgene detection
The practice of doping threatens fair competition in sports. With the very recent reports on
successful gene therapies for several diseases, the likelihood for abuse of gene transfer
techniques in elite sports is rapidly increasing. It is therefore very important to develop valid
detection techniques for transgenic DNA (tDNA) with ultimate sensitivity and specificity. To
date, three slightly different procedures have been reported to reliably detect tDNA with
sufficiently high sensitivity. Two utilize a real-time PCR-based approach and one uses a
primer-internal, intron-spanning PCR approach (spiPCR). The specificity and sensitivity of
these techniques, however, is still a matter of debate. Based on spiPCR, here we present a
novel one-tube nested PCR approach that minimizes the chances for cross-contamination
and shows increased sensitivity compared to non-nested PCR techniques. To further reduce
the occurrence of false-positives based on cross-contamination, a multi-functional 19bp
extended erythropoietin standard (EPO) was cloned which can be easily differentiated from
transgenic EPO DNA (tEPO) and can be used as an internal or external positive control in
PCR-based applications. It was found that one-tube nested PCR is superior in terms of
sensitivity and specificity compared to conventional PCR, and shows similar sensitivity
compared to real-time based PCR assays. Although it did not reach sensitivity of spiPCR, the
one-tube nested PCR technique described here is less laborious, less expensive and much faster than spIPCR. This technique might therefore be useful as a pre-screening tool for gene doping in the future [12257].

**Effects outside the bone marrow**

Systemic administration of erythropoietin protects the myocardium from an ischemic insult and promotes beneficial remodeling. It was hypothesized that intracardiac injection of erythropoietin may exhibit cardioprotective potential with reduced systemic toxicity. In conclusion, one study demonstrated that intramyocardial erythropoietin injection, following myocardial infarction, reduced inflammation, enhanced angiogenesis and proliferation, improved myocardial functions, and did not lead to intramural thrombus formation [08233].

**Economy**

The European Commission have granted marketing authorizations to three companies for generic versions, or “biosimilar”*, of the hormone erythropoietin. Amgen, the world's largest biotechnology company by sales, started selling Epogen®, its recombinant version of this naturally occurring hormone, in 1989, producing it from cells into which the relevant gene had been engineered. The drug's ability to boost red blood-cell production has led to its widespread use to treat the anemia associated with some diseases. Epogen and its newer incarnation, Aranesp, earned nearly USD 7 billion for Amgen in 2006, and more than USD 3 billion for Johnson & Johnson, which has licensed the drug from Amgen and markets it in the United States, Europe and elsewhere. Now that the commission has allowed other companies to market their own version, revenues from Johnson & Johnson's estimated 250,000 European customers look set to erode. Sandoz, the generic-drugs branch of Novartis, had its version of the drug on the market in the United Kingdom and Germany, and it cost 25-30 percent less than Johnson & Johnson is charging [07128].

**Experimental**

Recent publications reflect the anti-doping authorities' concern about the use of altitude simulator systems as violating the spirit of sport criterion [Levine 2006; Loland and Murray 2007; Spriggs 2005]. The aim of one study was to determine whether intermittent hypoxic treatments could modify the hemoglobin, hematocrit, reticulocytes, and erythropoietic stimulation index (OFF-Hr Score) values after administration of rHuEPO-alpha. Although these hematological parameters are of secondary nature some international sport federations currently exclude athletes who show aberrant values of these parameters from competition. Ten young male Wistar rats were treated, three times a week for 2 weeks, with 500 IU of rHuEPO-alpha. After the treatment, the animals were randomly divided into two groups: normoxic and hypoxic. The normoxic group was maintained at 21 percent O$_2$ 24 h a day for 23 days. The hypoxic group was maintained 12 h at 21 percent O$_2$ and 12 h at 12 percent O$_2$ (~4,000 m) the same time period. After the rHuEPO-alpha treatment, the hypoxic group of animals had a faster recovery rate in the reticulocyte count, elevated concentrations of hemoglobin and hematocrit and a significant increase in the endogenous EPO levels when compared with the normoxic group of animals. These changes led to significant modifications in the OFF-Hr Score between the hypoxic and normoxic animals. Intermittent hypoxic treatments after rHuEPO administration can significantly modify the main hematological parameters tested by the anti-doping authorities. The results in an animal model suggest checking the described phenomena in humans in order to reach major conclusions [09166].

Athletes who abuse recombinant human erythropoietin (rhEPO) consider only the benefit to
performance and usually ignore the potential short and long-term liabilities. Elevated haematocrit and dehydration associated with intense exercise may reveal undetected cardiovascular risk, but the mechanisms underlying it remain to be fully explained. One study aimed to evaluate the cardiovascular effects of rhEPO in rats under chronic aerobic exercise. A ten week protocol was performed in four male Wistar rat groups. rhEPO in trained rats promoted erythrocyte count increase, hypertension, heart hypertrophy, sympathetic and serotonergic overactivation. The suddenly died rat's tissues presented brain with vascular congestion; left ventricular hypertrophy, together with a "cardiac-liver", suggesting the hypothesis of heart failure as cause of sudden death. In conclusion, rhEPO doping in rats under chronic exercise promotes not only the expected RBC count increment, suggesting hyperviscosity, but also other serious deleterious cardiovascular and thromboembolic modifications, including mortality risk, which might be known and assumed by all sports authorities, including athletes and their physicians [09167].

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Recombinant human erythropoietin (rHuEPO) engineered in Chinese hamster ovary (CHO) cell cultures (Epoetin alfa and Epoetin beta) and its hyperglycosylated analogue Darbepoetin alfa are known to be misused by athletes. The drugs can be detected by isoelectric focusing (IEF) and immunoblotting of urine samples, because "EPO" is in reality a mixture of isoforms and the N-glycans of the recombinant products differ from those of the endogenous hormone. However, there are a plethora of novel erythropoiesis stimulating agents (ESAs). Since the originator Epoetins alfa and beta are no longer protected by patent in the European Union, rHuEPO biosimilars have entered the market. In addition, several companies in Asia, Africa and Latin America produce copied rHuEPOs for clinical purposes. While the amino acid sequence of all Epoetins is identical, the structure of their glycans differs depending on the mode of production. Some products contain more acidic and others more basic EPO isoforms. Epoetin delta is special, as it was engineered by homologous recombination in human fibrosarcoma cells (HT-1080), thus lacking N-glycolyneuraminic acid like native human EPO. ESAs under development include EPO fusion proteins, synthetic erythropoiesis stimulating protein (SEP) and peptidic (Hematide, CNTO 528) as well as non-peptidic EPO mimetics. Furthermore, preclinical respectively clinical trials have been performed with small orally active drugs that stimulate endogenous EPO production by activating the EPO promoter ("GATA-inhibitors": diazepane derivatives) or enhancer ("HIF-stabilizers": 2-oxoglutarate analogues) [09169].
Blood substitutes, in general

Historically, blood loss from critical surgery or trauma has been treated with either volume-replacing fluids or transfusions. Progress in chemical and biotechnological research has allowed the development of a novel approach to this issue, in the form of temporary oxygen carriers, widely known as “blood substitutes.” The blood substitutes currently available are chiefly polymerized hemoglobin solutions or hemoglobin-based oxygen carriers (HBOCs) and perfluorocarbons (PFCs). Alternatives include artificial RBCs, in which hemoglobin and supporting enzyme systems are encapsulated into liposomes. Major clinical advantages of these substitutes include sterilization of viral and bacterial contaminants, room temperature storage, longer shelf life, and absence of RBC antigens [10353].

Perfluorocarbon emulsions

PFCs are inert, water-insoluble, synthetic aromatic or aliphatic compounds, consisting primarily of carbon and of fluorine substitutions for most hydrogen atoms. PFCs are substantially clear and colorless liquid emulsions that are heterogeneous in molecular weight, surface area, electronic charge, and viscosity; their high content of electron-dense fluorine atoms results in little intramolecular interaction and low surface tension, making such substances excellent solvents for gases, especially oxygen and carbon dioxide. Some of these molecules can dissolve 100 times more oxygen than plasma. PFCs are naturally hydrophobic and need to be emulsified to be injected intravenously. Recently, sophisticated technology has allowed the generation of stable emulsions that contain tiny particles with a median diameter < 0.2 μm and that display a very low molecular weight (about 500 Da). Considerable effort has focused on ensuring the long-term stability of ready-to-use, concentrated PFCs. Since PFCs dissolve rather than bind oxygen, their capacity to serve as a blood substitute is determined principally by the pO₂ gradients in the lung and at the target tissue. Therefore, their oxygen transport properties differ substantially from those of whole blood and, especially, from those of RBCs. Erythrocytes exhibit a sigmoidal oxygen dissociation curve, whereas PFCs are characterized by a linear relationship between pO₂ and oxygen content. At a conventional ambient pO₂ of 135 mm Hg, the oxygen content of 900 ml/L perfluorocarbon is less than 50 ml/L, whereas an optimal oxygen content of 160 ml/L, which is still lower than that of whole blood in normal conditions, can be achieved only by a pO₂ greater than 500 mm Hg. In practice, at a conventional alveolar pO₂ of 135 mm Hg, PFCs will not be able to provide sufficient oxygenation to peripheral tissues. Owing to this limitation, optimization of the oxygen transport capacity can be achieved only through a substantially raised arterial pO₂ (i.e. ventilation with 100 % oxygen), which appears unsuitable and most unlikely outside a controlled hospital setting. Moreover, prolonged oxygenation may lead to oxidative stress and tissue damage and may trigger potentially adverse genetic effects. Owing to their small size, PFCs efficiently perfuse the microcirculation where erythrocytes may not flow. In tiny capillaries, PFCs produce the greatest benefit, as they increase local oxygen delivery much more efficiently than would be expected from the increase in oxygen content in larger arteries. In addition, as gases are in the dissolved state within PFCs, the pO₂ in the microcirculation may be considerably increased, thereby promoting an extremely efficient oxygen delivery to peripheral tissues. PFCs undergo a rapid and atypical metabolism. After initial uptake by the mononuclear phagocytic system, the emulsion particles are rapidly degraded, and the PFCs re-enter the blood stream mainly bound to blood lipids; they are finally excreted by the lungs via exhalation. The half-life of PFCs depends on the chemical composition and appears strongly dose dependent, ranging from 2 to 6 h. The first demonstration of the clinical efficacy of PFCs was provided in mice in 1966. Since the mid-1980s, improvements in both oxygen
capacity and emulsion properties of PFCs have led to the development of second-generation PFC-based oxygen carriers; two PFC products are being tested in phase III clinical trials. A delayed and transitory “flu-like” syndrome was occasionally described; symptoms consisted mainly of back pain, malaise, flushing, and a transient fever lasting several hours. As PFCs are cleared by cells of the reticulo-endothelial system, and the febrile response was blocked by ibuprofen or dexamethasone, it is conceivable that this syndrome was elicited by the release of inflammatory cytokines, namely, interleukin 6. An additional threat is the effect of PFCs on platelet function. Aggregation by collagen, ADP, or arachidonic acid appears substantially inhibited in a dose-dependent fashion in ex vivo porcine platelets following infusion of perflubron emulsion at a dose of 3 ml/kg. Given some elite athletes’ innate inclination to experiment with novel doping strategies, it is speculated that some have used artificial oxygen carriers, but no clearly positive cases have been detected so far. There are several issues regarding efficacy and clinical safety of blood substitute administration in athletes. Scientific data concerning the performance benefits are inconclusive. Although the use of PFCs may theoretically produce an athletic advantage, the biochemical characteristics (linear relationship between dissolved oxygen and pO₂), the form of administration (intravenous), the need for high oxygenation, the short half-life (up to 6 h) and the side effects still hinder the diffusion of this rather atypical form of blood doping. Despite these clear limitations, international sporting federations have been commendably proactive in adding this category of compounds to their banned substance lists [10353].

Efaproxiral

Efaproxiral is an analog of the cholesterol drug bezafibrate developed for the treatment of depression, traumatic brain injury, ischemia, stroke, myocardial infarction, diabetes, hypoxia, sickle cell disease, hypercholesterolemia and as a radio sensitizer. The chemical is a propanoic acid in the class of amphipathic carboxylic acids. Most propanoic acid produced is consumed as a preservative for both animal feed and food for human consumption. One use for efaproxiral is to increase the efficacy of certain chemotherapy drugs which have reduced efficacy against hypoxic tumours, and can thus be made more effective by increased offloading of oxygen into the tumour tissues. No benefit was seen for efaproxiral in phase III clinical trials. The increased oxygenation of tissues could theoretically also produce enhanced exercise capacity in feline, rat and canine models for approximately 100 min. immediately after a high dosage 45 min. intravenous infusion. This has led World Anti-Doping Agency to categorize efaproxiral under a prohibited method to artificially enhance the uptake, transport or delivery of oxygen. There is no existing evidence that efaproxiral can effectively enhance performance in humans. Efaproxiral can be absorbed via transdermal, rectal, inhalation and gastrointestinal routes, though not at plasma concentrations great enough to alter the oxygen-haemoglobin dissociation curve. Efaproxiral is explicitly excluded from the 2012 World Anti-Doping Agency list of Prohibited Substances and is explicitly included in the Prohibited Methods section M1 as a forbidden procedure to alter the oxygen-haemoglobin dissociation curve in order to allosterically modify hemoglobin. Efaproxiral (2-[4-[[3,5-dimethylanilino]carbonyl][methyl]phenoxyl]-2-methylpropionic acid, formerly referred to as RSR13) is thus prohibited in sports according to the World Anti-Doping Agency (WADA). The drug as well as structurally related compounds and a stable isotope-labeled derivative have been synthesized to elucidate the fragmentation pathway of efaproxiral, using electrospray ionization (ESI) and tandem mass spectrometry by employing a novel linear ion trap–orbitrap hybrid mass spectrometer – in positive and negative ionization modes. The elimination of 2-methyl acrylic acid (-86 u) has been identified as a major fragmentation process in both charge states. Negative ionization and collision-induced dissociation (CID) caused an additional release of carbon dioxide (-44 u), and positive
ionization the loss of formic acid (\(-46 \text{ u}\)). Efaproxiral was incorporated into an existing screening procedure for doping controls using solid-phase extraction (SPE) followed by liquid chromatography-tandem mass spectrometry, enabling a limit of detection of 2.5 ng/mL and interday precisions ranging from 7.9 to 13.0 percent [06162].

**Ex vivo erythrocyte generation**

The generation of RBC from haematopoietic cells or human induced pluripotent stem cells can now be considered as realistic and can be accomplished on an industrial basis. Understandably this is a most favourable achievement for healthcare systems and patients in order to face the increasing demand for a wider availability of blood, but is also a serious threat for sport medicine, since it reasonably offers the possibility of effective blood doping. The techniques that have been described so far can be used to obtain very large quantities of RBC, yielding as much as a 130-fold increase over the original quantity of progenitor cells and, thereby, producing nearly 560 units of RBC per umbilical cord donation (assuming 5x10⁶ CD34+ cells per donation). Umbilical cord blood is an optimal source for medical and ethical reasons, although it is also viable for blood doping, since the RBC that can be obtained are safe and highly controlled after selection of proper antigenic determinants. The procedure can even be implemented using bone marrow-derived hematopoietic cells or fibroblasts from the recipient. However, more realistically, the use of these biotechnological approaches in the doping context could become plausible only after the development of methods based on the use of hematopoietic stem cells derived from peripheral blood [12234].

The manufacture of RBC could overcome several problems of the procedures currently used in the doping context for blood collection and storage. Long storage is not required and the production can be tailored according to the athlete's needs. Moreover, the RBC are fresh and thereby more functional with regards to gas exchange than those maintained in blood bags, even when properly stored. The amount of RBC administered intravenously can be regulated by appropriate dilution to avoid anomalous laboratory test results even with proteomic studies and the biological characteristics and behaviour of these "new" RBC do not differ from those of their "natural" counterparts. To the best of our knowledge, the only indices that could be used in the anti-doping context are shape and dimensions, which seem to be slightly greater for manufactured RBC. In conclusion, the scientific as well as the sport medicine communities should be aware of the risk that these novel approaches to the generation of RBC could have with regards to blood doping and should cooperate closely to increase knowledge about RBC manufacturing and to develop appropriate tests to identify their unfair use in sports [12234].

**Biological engineering from progenitor cells**

The production of biomaterial particles with similar (or even identical) chemical and physical properties as those of mature, functional RBC has been reported, so we have already alerted the scientific community as well as anti-doping authorities on the possible, surreptitious use of these bioengineering techniques for unfair and illicit purposes in sports. The generation of RBC from haematopoietic cells or human induced pluripotent stem cells can now be considered as realistic and can be accomplished on an industrial basis. Understandably this is a most favourable achievement for healthcare systems and patients in order to face the increasing demand for a wider availability of blood, but is also a serious threat for sport medicine, since it reasonably offers the possibility of effective blood doping. The techniques that have been described so far can be used to obtain very large quantities of RBC, yielding as much as a 130-fold increase over the original quantity of progenitor cells and, thereby,
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**Irradiation of red blood cells**

Gamma irradiation of red blood cell (RBC) concentrates is routinely used to prevent transfusion-associated graft-versus-host disease. So far, the effects of ionizing radiation on RBC structure and function and especially the proteome are not fully understood. RBC concentrates were irradiated with 30 Gy and stored for 1 or 15 days at 4 ± 2°C. Following cell lysis and hemoglobin depletion, 2D-DIGE was used to examine the changes of the cytosolic RBC proteome. Significantly altered spots were analyzed using bottom-up proteomic approaches and selected marker proteins validated by western blotting. Gamma irradiation was found to enhance conventional RBC storage lesions. Following 15 days of postirradiation storage, the abundances of a total of 27 spots were significantly altered and 3 out of 13 identified proteins were selected and validated as potential marker proteins for the assessment of irradiation-induced cytosolic RBC lesions. Gamma irradiation and subsequent ex vivo storage according to the Council of Europe guidelines were found to affect RBC protein structures. The validated marker proteins can serve as a basis for the development of a screening assay to monitor the quality of irradiated RBC concentrates during ex vivo storage [13305].

**Enhancement of oxygen transfer**

2,3 DPG

Rapid growth during adolescence caused by metabolic changes and their metabolic response to anaerobic and aerobic exercise differs considerably from that in adults and this is especially true in the responses to stresses, such as altitude exposure. However, there is little information on the suitability of exercise training at altitude for young athletes. Six male
Korean adolescent alpine skiers (13-17 years), with a skiing career of 3-5 years, participated in the study. All subjects were exposed to an altitude of 2700 m (8858 ft) for 5 weeks and altitude exposure consisted of 6 d/week of training (4-5 h/d), with living quarters at 2100 m. The 5 week of ski training at altitude were maintained at the same level (the same number of slalom and giant slalom skiing trials) as at sea level. There was a significant increase in oxygen transport capacity, despite decreased erythropoietin (EPO) production (-31 %) after altitude training. Red blood cell (RBC), hemoglobin (Hb), hematocrit (Hct), and 2,3 DPG concentrations increased significantly during altitude exposure and after return to sea level. The results indicate that applying altitude training in adolescent skiers may improve their endurance performance. However, EPO production during altitude training needs to be evaluated in larger future studies [12263].

Hypoxia-inducible factor

Another alternative to stimulate erythropoiesis is represented by low molecular mass compounds acting as prolylhydroxylase inhibitors. Among these, some so-called hypoxia-inducible factor (HIF) stabilizers have advanced to phase-II clinical trials and demonstrated their capability to increase EPO serum levels and, consequently, elevated hematocrit values. With the disclosure of some lead drug candidates, model substances have been used to establish LC-MS/MS-based detection methods to allow the implementation of this class of prohibited compounds into routine doping controls. Supported by distinct dissociation pathways (e.g. nominal loss of 10 Da), both targeted and nontargeted detection strategies were developed and detection limits between 0.6-10 ng/mL and 300-1000 ng/mL, respectively, were accomplished [12017].

Hemoglobin-based oxygen carriers (HBOCs)

Hemoglobin-based oxygen carriers (HBOCs) are blood substitutes, synthesized by polymerizing hemoglobin, which are being developed and investigated as alternatives to blood for medical purposes. However, due to their ability to increase the oxygen carrying capacity when taken by healthy individuals, HBOCs have been used as a doping agent among endurance athletes and are included in the World Anti-Doping Agency’s Prohibited List. To maintain the fairness of competitions and continue the battle against doping, it is essential to be able to detect HBOCs if present in an athlete's blood. To achieve this goal, it is necessary to differentiate HBOCs from the native hemoglobin and to do so in a cost and time effective manner. It was developed a rapid capillary zone electrophoresis (CZE), UV absorbance, method capable of detecting HBOCs, in whole blood samples, at levels below those considered necessary to provide a performance enhancement. The approach to the analysis for HBOCs utilizes the whole blood sample, not just the plasma, and does not require the use of immunoprecipitants to ensure accurate analysis. By lysing the red blood cells and using centrifugal filtration, followed by our CZE separation, we are able to effectively distinguish between native hemoglobin and HBOCs. Through this method, we have been able to reliably detect concentrations of HBOCs at the equivalent of 5.5 g/L, the equivalent to a 3.5 percent increase in blood hemoglobin concentration for an athlete [12265].

Artificial oxygen carriers (AOCs) can be abused by the athletes to improve their aerobic capacity. The AOCs produce a performance enhancing effect, especially in endurance sports. This article presents a method for the rapid screening of hemoglobin-based oxygen carriers (HBOCs) in blood samples. Common screening tests to reveal HBOC misuse by
athletes are based on colorimetric detection since HBOC use causes discoloration of the plasma. In this communication we are presenting a different approach for HBOC detection using an hematological analyzer capable of measuring hemoglobin by two methods: a standard cyanmethemoglobin colorimetric method to calculate the amount of total hemoglobin (HGBtot) and a flow cytometric optical method to calculate the amount of hemoglobin within the red blood cells (HGBcell). Thanks to this dual contemporary hemoglobin measurement, the HGB delta value (corresponding to free HGB) is automatically calculated by subtraction of HGBcell from HGBtot and can be used as a fast screening index of HBOC abuse. It was tested the effectiveness of this approach using 68 normal blood samples with different basal HGB values fortified with three different HBOCs at varying concentrations. It was evaluated the performance of the method by calculating the correlation between HGBcell and HGBtot values in normal samples. Finally we used a simple statistical approach to calculate a reliable HGB delta cut-off value (0.35 g/dL) as a limit of decision to discriminate between a clear negative sample and a suspect sample to submit to a confirmation analysis [09173].

Blood doping involves the use of products that enhance the uptake, transport, or delivery of oxygen to the blood. One approach uses artificial oxygen carriers, known as hemoglobin-based oxygen carriers (HBOCs). This study describes an analytical strategy based on CE for detecting intact HBOCs in plasma samples collected for doping control. On-capillary detection was performed by UV/Vis at 415 nm, which offered detection selectivity for hemoproteins (such as hemoglobin and HBOCs). On-line ESI-MS detection with a TOF analyzer was further used to provide accurate masses on CE peaks and to confirm the presence of HBOCs. An immunodepletion sample preparation step was mandatory prior to analysis, in order to remove most abundant proteins that interfered with CE separation and altered the ESI process. This analytical method was successfully applied to plasma samples enriched with Oxyglobin, a commercially available HBOC used for veterinary purposes. Detection limits of 0.20 and 0.45 g/dL were achieved in plasma for CE-UV/Vis at 415 nm and CE-ESI-TOF/MS, respectively [10127].

Artificial oxygen carriers (AOCs) can be abused by the athletes to improve their aerobic capacity. The AOCs produce a performance enhancing effect, especially in endurance sports. This article presents a method for the rapid screening of hemoglobin-based oxygen carriers (HBOCs) in blood samples. Common screening tests to reveal HBOC misuse by athletes are based on colorimetric detection since HBOC use causes discoloration of the plasma. In this communication we are presenting a different approach for HBOC detection using an hematological analyzer capable of measuring hemoglobin by two methods: a standard cyanmethemoglobin colorimetric method to calculate the amount of total hemoglobin (HGBtot) and a flow cytometric optical method to calculate the amount of hemoglobin within the red blood cells (HGBcell). Thanks to this dual contemporary hemoglobin measurement, the HGB delta value (corresponding to free HGB) is automatically calculated by subtraction of HGBcell from HGBtot and can be used as a fast screening index of HBOC abuse. We tested the effectiveness of this approach using 68 normal blood samples with different basal HGB values fortified with three different HBOCs at varying concentrations. It was evaluated the performance of the method by calculating the correlation between HGBcell and HGBtot values in normal samples. Finally we used a simple statistical approach to calculate a reliable HGB delta cut-off value (0.35 g/dL) as a limit of decision to discriminate between a clear negative sample and a suspect sample to submit to a confirmation analysis [10128].

Hemoglobin is naturally suited to bind, carry, and deliver oxygen when encased by the RBC membrane; once removed, it becomes dysfunctional and rapidly dissociates. The administration of free hemoglobin to humans is unsuitable; dimers of about 32 kDa produced
by dissociation are cleared by the kidney where they may accumulate, generate renal obstruction and oxidant injury or necrosis, and trigger renal failure. Therefore, hemoglobin must be stabilized before it can be safely infused. HBOCs are intra- and/or inter-molecularly "engineered" human or animal hemoglobins, optimized for oxygen delivery and longer intravascular circulation. Several approaches have been attempted to stabilize and modify the hemoglobin molecule: polymerization of human (PolyHeme®) or bovine (HBOC-201) hemoglobin with glutaraldehyde or raffinose (Hemolink®), pyridoxylation, conjugation with polyethylene or maleimide-polyethylene glycol (MP4), and the cross-linking of the subunits by diaspirein (HemAssist) and oxidized mono/di/tri/poly saccharides. Most of the synthetic compounds exhibit low oxygen affinity, high cooperativity, an enhanced Bohr effect, and a slower rate of autoxidation of the heme iron, which finally results in more efficient oxygen delivery. The presence of 2,3-diphosphoglycerate within erythrocytes maintains the normal affinity of hemoglobin for oxygen. As erythrocyte-free hemoglobin loses this interaction, unmodified human HBOC solutions have a very high oxygen affinity which compromises their function. Chemical methods developed to overcome this problem have resulted in carriers that effectively release oxygen at the physiological pO₂ of peripheral tissues. A common feature of all HBOCs is their resistance to disaggregation when dissolved in infusion media, which contrasts to the native hemoglobin property of natural dissociation under non-physiologic conditions. First-generation HBOCs were developed principally to serve as oxygen carriers and as a substitute for erythrocytes in situations of acute and clinically threatening blood depletion, such as peri-operative use. Depending on the type of modification used to stabilize the protein, second-generation HBOCs were considered to be useful in other clinical settings, such as enhancement of radiation therapy and nitric oxide scavenging. Third-generation HBOCs, based on microencapsulation of hemoglobin and RBC enzymes either in liposomes or in biodegradable nanocapsules, appear so far to be the most promising products and are currently under evaluation in phase II and III clinical trials. The plasma half-life ranges from 12 to 48 h for cross-linked and surface-linked hemoglobin, respectively. There are several concerns about the therapeutic and unfair use of HBOCs. Phase II clinical trials and biological studies suggest that resuscitation with HBOCs, in lieu of stored RBCs, attenuates the systemic inflammatory response invoked in the pathogenesis of multi-organ failure. Specifically, HBOCs obviate stored RBC-provoked neutrophil priming, endothelial activation, and systemic release of interleukins 6, 8, and 10. Some HBOCs are in clinical phase III trials, but no product has yet achieved market as free hemoglobin and many forms of modified hemoglobins display strong vasoconstrictor reactivity due to the scavenging of endogenous nitric oxide, a powerful vasoactive agent released from endothelial cells. Therefore, administration of HBOCs may generate microvascular permeability and failure, causing systemic and pulmonary hypertension. Additional threatening side effects include gastrointestinal dysfunction characterized by increased tone of the intestinal sphincters, marked flatulence and meteorism, renal toxicity, and alteration of some biochemical and hematologic parameters, including increase in liver enzymes and alteration of platelet function. If one of the HBOCs becomes available, red (hemoglobin-colored) plasma or urine would make it readily detectable. However, HBOCs recently have been included within the International Olympic Committee and the World Anti-Doping Agency lists of substances and methods prohibited in sports [10353].

The development of artificial oxygen carriers has attracted considerable recent interest because of the increasing cost of collecting and processing blood, public concerns about the safety of blood products, complications from blood transfusions, military requirements for increased volumes of blood during military conflicts, and a decrease in the number of new donors. To overcome these problems, perfluorocarbon-based oxygen carriers as well as acellular- and cellular-type, hemoglobin-based oxygen carriers have been developed for use as artificial oxygen carriers. Despite their extensive evaluation, including formulation and pharmacology, they have not been extensively used in clinical settings. One of the reasons
for this is that their pharmacokinetics have not been well characterized. Artificial oxygen carriers require not only an acceptable level of physicochemical activity, but also clinical efficacy, as reflected by their retention in the circulation, and the absence of measurable accumulation in the body, if unexpected adverse effects are to be avoided. In one review, the pharmacokinetic properties of artificial oxygen carriers are discussed, with a focus on recent developments of the research related to the pharmacokinetic properties a cellular type of hemoglobin-based oxygen carrier [11145].

Modified stromal-free hemoglobins have been investigated as oxygen carriers since 1940. Cell-free Hb maintains its ability to transport oxygen. However, Hb tetramers rapidly breakdown into dimers and monomers, which diffuse freely into the renal tubules and are cleared by the kidney. To overcome these limitations, modification methods were developed in the last few decades, such as purification, conjugation, crosslinkage and polymerization. Crosslinked and polymerized stromal-free Hb are capable of carrying oxygen to tissues and organs, as well as achieving blood plasma expansion. Hemopure (glutaraldehyde polymerized bovine Hb in Ringer lactate) is an example. Polyhemoglobin consists of stromal-free Hb crosslinked with a bifunctional or polyfunctional crosslinking agent, yielding a water-soluble Hb product. It has a very high oxygen affinity and can transport and supply oxygen to vital tissues and organs, maintaining intravascular oncotic pressure. Nanobiotechnology has been used for the preparation of polyhemoglobin by assembling Hb molecules into soluble nanodimension complexes. Liposome-encapsulated Hbs are artificial red cells, in which Hb and supporting enzyme systems are encapsulated into liposomes. They present several advantages, such as decreasing the toxicity of free Hb, increasing its circulation time and permitting co-encapsulation of Hb protectants and other compounds. It must be stressed that HBOCs may be misused in endurance sports in order to improve the maximal oxygen capacity (one of the limiting factors of endurance performance), and not owing to their plasma expander properties. Since 2003, HBOCs are banned in sport, as enhancement of oxygen transfer was prohibited by the IOC. Analysis of HBOCs can be performed by two screening procedures (direct visual screening of plasma discoloration and electrophoretic technique). Two complementary methods may confirm the presence of exogenous Hb in plasma: a SEC-HPLC with photodiode array detection, which in tandem with the electrophoretic screening technique, provides evidence of the presence of HBOCs; and an HPLC analysis of enzymatic digests (peptide segments arising from the tryptic digestion of bovine HBOC), with detection by ESI ion trap MS. Concerning Hemopure, besides the SEC-HLPC, a method was described for its detection in athletes’ blood samples; after a visual screening of plasma samples (red coloration), cell-free human Hb is analyzed with the Advia 120 hematology system, followed by an HPLC separation, in order to distinguish between human Hb and Hemopure. Regarding the presence of HBOCs in urine, some authors advocate that urinary concentrations are too low and variable to be considered of any value in developing an effective test for HBOC [11146].

Whereas many tissues and organs of the human body such as joints or cardiac valves can be replaced by artificial substitutes, medical science is still on the quest for a suitable blood replacement that features all qualities of the simple red blood cell to carry oxygen. So far, the greatest advances have been made in the development of HBOCs which, in contrast to other approaches such as perfluorocarbons, rely on hemoglobin molecules of animal origin or from genetic engineering. A HBOC (Oxyglobin®) was approved for veterinary use in certain countries 15 years ago. Such substances are understood to have entered the world of sports already with the aim of improving oxygen delivery to tissues and have allegedly been used by athletes in an attempt to boost performance. However, the definite proof of the efficiency of these substances to improve performance is still lacking and the few studies showing a positive effect have been criticized. It has been speculated that the oxygen transport effects are impaired by a significant vasoactive action of the HBOCs related to NO scavenging.
properties of free hemoglobin, which interferes with oxygen delivery. With the advancement of medical science it can nevertheless be anticipated that sooner or later a viable HBOC that is able to improve oxygen delivery and thereby performance in humans will be available. The detection of HBOC is straightforward and relies on electrophoresis as a screening procedure and size exclusion–high-performance liquid chromatography (SEC-HPLC) [13006].

Hemoglobin-based oxygen carriers (HBOCs) are thought to have an adverse risk:benefit profile when compared to that of transfusing stored red blood cells (RBCs). However, there are clinical circumstances when RBC transfusion is not an option (e.g. patient refusal, unavailability owing to issues of compatibility or remote location). For these circumstances assessment of the risks of an HBOC should be compared to the risks of untransfused acute anemia. In this article we compare the risk of allowing a patient with severe anemia to have a further small decrease in hemoglobin (Hb) concentration to the risk of infusing an HBOC. It was conclude that at Hb concentrations less than 6 g/dL, the risk of a further decrease in Hb concentration greatly exceeds the risk of HBOC infusion. Thus, it was suggested that there may be a place for use of HBOCs when RBC transfusion is not an option [13349].

The development of oxygen (O₂)-carrying blood substitutes has evolved from the goal of replicating blood O₂ transport properties to that of preserving microvascular and organ function, reducing the inherent or potential toxicity of the material used to carry O₂, and treating pathologies initiated by anemia and hypoxia. Furthermore, the emphasis has shifted from blood replacement fluid to "O₂ therapeutics" that restore tissue oxygenation to specific tissues regions. One review covered the different alternatives, potential and limitations of hemoglobin-based O₂ carriers (HBOCs) and perfluorocarbon-based O₂ carriers (PFCOCs), with emphasis on the physiologic conditions disturbed in the situation that they will be used. It describes how concepts learned from plasma expanders without O₂-carrying capacity can be applied to maintain O₂ delivery and summarizes the microvascular responses due to HBOCs and PFCOCs. The review also presented alternative applications of HBOCs and PFCOCs namely: How HBOC O₂ affinity can be engineered to target O₂ delivery to hypoxic tissues; and how the high gas solubility of PFCOCs provides new opportunities for carrying, dissolving, and delivering gases with biological activity. It is concluded that the development of current blood substitutes has amplified their applications horizon by devising therapeutic functions for O₂ carriers requiring limited O₂ delivery capacity restoration. Conversely, full, blood-like O₂-carrying capacity reestablishment awaits the control of O₂-carrier toxicity [13350].

The worldwide blood shortage has generated a significant demand for alternatives to whole blood and packed red blood cells for use in transfusion therapy. One such alternative involves the use of acellular recombinant hemoglobin (Hb) as an oxygen carrier. Large amounts of recombinant human Hb can be expressed and purified from transgenic Escherichia coli. The physiological suitability of this material can be enhanced using protein-engineering strategies to address specific efficacy and toxicity issues. Mutagenesis of Hb can
- adjust dioxigen affinity over a 100-fold range
- reduce nitric oxide (NO) scavenging over 30-fold without compromising dioxigen binding
- slow the rate of autooxidation
- slow the rate of hemin loss
- impede subunit dissociation
- diminish irreversible subunit denaturation

Recombinant Hb production is potentially unlimited and readily subjected to current good manufacturing practices, but may be restricted by cost. Acellular Hb-based O₂ carriers have
superior shelf-life compared to red blood cells, are universally compatible, and provide an alternative for patients for whom no other alternative blood products are available or acceptable. Remaining objectives include increasing Hb stability, mitigating iron-catalyzed and iron-centered oxidative reactivity, lowering the rate of hemin loss, and lowering the costs of expression and purification. Although many mutations and chemical modifications have been proposed to address these issues, the precise ensemble of mutations has not yet been identified. Future studies are aimed at selecting various combinations of mutations that can reduce NO scavenging, autooxidation, oxidative degradation, and denaturation without compromising O₂ delivery, and then investigating their suitability and safety in vivo [13351].

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**Allosteric modulators of hemoglobin**

Allosteric ("different shape," from allos = other and steric = solid or space) activators or inhibitors of proteins are substrates designed to alter the affinity of a protein for a ligand or an enzyme for its substrate. Allosteric modulators usually bind non-covalently to the enzyme at regulatory sites that are distant from the catalytic or active sites. The improvement of oxygen delivery to hypoxic tissues by a decrease in the oxygen affinity of hemoglobin has been a major aim in recent years, because this may reduce the consequences of anemia and/or improve tissue oxygenation in cases of decreased blood perfusion. The spectrum of clinical applications of allosteric modulators is rather broad. Since these synthetic compounds increase tumor pO₂ and reduce the tumor hypoxic fraction, they have been developed to maximize the effectiveness of radiation therapy. They also may be useful in acute ischemic disorders, such as acute coronary syndrome and brain ischemia, as they improve myocardial oxidative metabolism and contractile function in models of myocardial ischemia and increase brain pO₂, thus reducing neuronal cell death following cerebral ischemia. Although these modulators has not been tested in humans for sport performance enhancement, its biological effects make it a potentially effective performance-enhancing agent for endurance athletes. Nearly one-third of patients undergoing therapy experience significant side effects, including headache, nausea, mucosal irritation, hypoxemia, allergic reaction, and transient renal dysfunction characterized by increased levels of serum creatinine [10353].

**New products**

Nearly 14 million units of packed red blood cells are transfused in the United States each year. According to the U.S. Department of Health and Human Services, in 1999, 6 percent of hospitals reported a shortage of blood, resulting in the cancellation or postponement of surgical procedures. The many limitations and risks of transfusions of packed red blood cells in critically ill patients have facilitated interest in developing alternative agents for oxygen delivery. Over the past few decades, safe and effective substitutes have been in development. However, no currently approved agent provides both oxygen transport and volume in place of packed red blood cells. Oxygen therapeutic products have several
advantages compared with packed red blood cells, including a prolonged shelf-life, lack of a cross-matching requirement, and minimal infectious risks or concerns about immunogenicity. Hemoglobin-based oxygen carriers and perfluorocarbons are being developed. Two products are undergoing clinical trials. Polyheme is undergoing a phase III study in trauma patients, and Hemopure is being evaluated in a phase II study in patients undergoing cardiopulmonary bypass surgery. A third product (Hemolink) was being evaluated in a phase III study in patients undergoing coronary artery bypass grafting surgery; however, the trial was suspended. In addition, several other hemoglobin-based oxygen carriers are in the preclinical stages. Oxygen therapeutics have several potential clinical applications in the management of perioperative blood loss, trauma, acute normovolemic hemodilution, traumatic brain injury, and blood requirements in patients who refuse or have contraindications to transfusions of red blood cells [06163].

Laboratory techniques

Hemoglobin-based oxygen carriers (HBOCs) are blood substitutes based on hemoglobin of either bovine or human origin and they can potentially be misused in elite sports to improve endurance performance. Recently, three methods have been proposed in doping control analysis to allow HBOCs screening and identification by application of electrophoresis, size-exclusion chromatography coupled with HPLC and LC coupled with tandem mass spectrometry (LC/MSMS). In view of the Athens 2004 Olympic Games, modifications were introduced in order to increase the specificity of these methods. The sample preparation protocols of the electrophoretic and SEC-HPLC methods were modified with the introduction of sequential ultra filtration steps to remove all heme containing material below 100 kDa, thus leaving only HBOCs material for analysis. Furthermore, a modification of the LC/MSMS methodology was introduced to allow full scan MS-MS spectra of peptide segments arising from the tryptic digestion of bovine HBOCs. These relatively simple methodological modifications have major impact, as far as time and cost effectiveness is concerned in doping control procedures, because they provide a useful tool in order to identify which suspect samples from the initial visual screening are due to hemolysis and exclude them from further analysis [06164].

HBOCs such as Hemopure and Oxyglobin (approximate average mol weight=250 kDa) consist of inter- and intramolecularly covalently crosslinked bovine hemoglobin, and they have been prohibited from use in sports due to the potential increase in blood oxygen transport capacity that they afford. Owing to their xenobiotic nature, several different LC-MS/MS approaches have been established to determine their presence in human serum. Chemical stabilization of the hemoglobin subunits using crosslinking agents resulted in an N-terminal derivatization of methionines of beta chains, which have been identified as potential targets in analytical approaches employing proteasomal degradation of serum with endoproteinase Glu-C. Alternative procedures have exploited the differences in the primary structures of bovine and human hemoglobins, as well as the presence of crosslinks in hemoglobin molecules. Due to a sequence homology of only 85 percent, several proteotypical peptides uniquely derived from bovine hemoglobin upon trypsin digestion are detectable using LC-MS/MS approaches and provide evidence for the presence of HBOCs at concentration levels of 2 mg/mL. Additionally, crosslinks introduced at lysines of hemoglobins impede the activity of trypsin at the respective residues and considerably change the ratios of tryptic peptides obtained from bovine hemoglobin and Hemopure. The latter information in particular will be useful for differentiating between human hemoglobin and its crosslinked analogs (e.g. PolyHeme) if these are launched by pharmaceutical companies [07050].

Hemoglobin-based oxygen carriers (HBOCs) are blood substitutes based on hemoglobin of
either bovine or human origin and they can potentially be misused in elite sports to improve endurance performance. Recently, three methods have been proposed in doping control analysis to allow HBOCs screening and identification by application of electrophoresis, size-exclusion chromatography coupled with HPLC and LC coupled with tandem mass spectrometry (LC/MSMS). In view of the Athens 2004 Olympic Games, modifications were introduced in order to increase the specificity of these methods. The sample preparation protocols of the electrophoretic and SEC-HPLC methods were modified with the introduction of sequential ultrafiltration steps to remove all heme containing material below 100 kDa, thus leaving only HBOCs material for analysis. Furthermore, a modification of the LC/MSMS methodology was introduced to allow full scan MS-MS spectra of peptide segments arising from the tryptic digestion of bovine HBOCs. These relatively simple methodological modifications have major impact, as far as time and cost effectiveness is concerned in doping control procedures, because they provide a useful tool in order to identify which suspect samples from the initial visual screening are due to hemolysis and exclude them from further analysis [07129].

**Hemopure®**

Hemoglobin-based oxygen carriers (HBOCs) such as are touted as a tenable substitute for red blood cells and therefore potential doping agents, although the mechanisms of oxygen transport of HBOCs are incompletely understood. It was investigated whether infusion of Hemopure increased maximal oxygen uptake (VO_{2max}) and endurance performance in healthy subjects. Twelve male subjects performed two 4-minute submaximal exercise bouts equivalent to 60 percent and 75 percent of VO_{2max} on a cycle ergometer, followed by a ramped incremental protocol to elicit VO_{2max}. A crossover design tested the effect of infusing either 30 g (6 subjects) or 45 g (6 subjects) of Hemopure versus a placebo. Under our study conditions, Hemopure did not increase VO_{2} (2max) nor endurance performance. However, the infusion of Hemopure caused a decrease in heart rate of approximately 10 bpm and an average increase in mean (approximately 7 mmHg) and diastolic blood pressure (approximately 8 mmHg) at submaximal and maximal exercise intensities. Infusion of Hemopure did not bestow the same physiological advantages generally associated with infusion of red blood cells. It is conceivable that under exercise conditions, the hypertensive effects of Hemopure counter the performance-enhancing effect of improved blood oxygen carrying capacity [07130].

**Laboratory technique**

A specific, sensitive and rapid analytical procedure based on capillary electrophoresis with UV/Vis detection at 405 and 415 nm was developed and validated to detect human haemoglobin and haemoglobin-based oxygen carriers (Hemopure®, Oxyglobin® and Polyheme®) in blood samples collected for doping control. The electrophoretic separation, based on capillary dynamic coating, was achieved in less than 10 min. The effects of capillary temperature, injection conditions and initial ramping were investigated. The optimum separation voltage was 25 kV with a capillary temperature of 20 °C, initial ramping of 1 kV/s and an injection pressure of 0.5 psi for 10 s. The removal of haptoglobin using anti-human haptoglobin antibody prior to the analysis was mandatory to increase the specificity of the analysis. Sufficient resolution between endogenous haemoglobin variants and the three haemoglobin-based oxygen carriers here investigated was obtained, thus allowing discrimination between a normal haemolysed sample and a sample in which Oxyglobin®, Hemopure® or Polyheme® is present. Good repeatability of migration times (CV % less than 1), peak resolution and adequate sensitivity (limit of detection: 2.5 mg/mL) was obtained [11463].
**Hematide/Peginesatide**

In addition to recombinant EPO and its derivatives, EPO-mimetic agents have been under development for several years with Hematide/Peginesatide being the first representative that received FDA approval (March 2012). Due to its ability to stimulate erythropoiesis it has been considered a banned substance by WADA for several years; however, its dissimilar structure compared to EPO does not allow its detection in blood or urine employing conventional EPO tests. Consequently, complementary methods were required and established on three different platforms: ELISA, SDS-PAGE, and LC-MS/MS. The ELISA utilized the sandwich-approach with a capture antibody directed against the PEG moiety and an antibody recognizing the homodimeric peptide residue allowing for colorimetric qualitative and quantitative determination with an LOD of 0.5 ng/ml in serum and plasma.\[80\] Since purely immunological detection assays require a second, independent confirmatory assay, the option of SDS-PAGE followed by western blotting was exploited, demonstrating comparable sensitivity as the ELISA-based assay. Authentic administration study plasma samples were obtained from a clinical study where healthy individuals received Peginesatide at 50 microg/kg bodyweight intravenously. Sample collection was conducted up to 28 days and both assays enabled the detection of the injected EPO-mimetic drug up to 10 days. Using LC-MS/MS, the detection of peginesatide was accomplished in serum and plasma following a simple protein precipitation and subsequent enzymatic hydrolysis of the peptidic moiety. Due to the presence of various non-natural amino acids, subtilizing yielded the desired diagnostic (proteotypical) target peptide, which was detected down to 1 ng/ml in spiked plasma and serum specimens. Considering pharmacokinetic data on peginesatide, plasma concentrations up to 500 ng/ml are expected when therapeutic dosages (e.g. 50 microg/kg bodyweight) are administered. Hence, the utility of alternative minimal-invasive sample collection strategies such as DBS sampling was evaluated and found to provide a conceivable matrix allowing for detection limits of 10 ng/ml by means of LC-MS/MS [13012].

**Hypoxia-inducible factor (HIF) stabilizers**

Another alternative to stimulate erythropoiesis is represented by low molecular mass compounds acting as prolylhydroxylase inhibitors. Among these, some so-called hypoxia-inducible factor (HIF) stabilizers have advanced to phase-II clinical trials and demonstrated their capability to increase EPO serum levels and, consequently, elevated hematocrit values. With the disclosure of some lead drug candidates, model substances were used to establish LC-MS/MS-based detection methods to allow the implementation of this class of prohibited compounds into routine doping controls. Supported by distinct dissociation pathways (e.g. nominal loss of 10 Da), both targeted and non-targeted detection strategies were developed and detection limits between 0.6-10 ng/ml and 300-1000 ng/mL, respectively, were accomplished [13012].

**Cobalt salt as erythropoietic agent**

Unfair athletes seek ways to stimulate erythropoiesis, because the mass of haemoglobin is a critical factor in aerobic sports. Here, the potential misuse of cobalt deserves special attention. Cobalt ions (Co²⁺) stabilize the hypoxia-inducible transcription factors (HIFs) that increase the expression of the erythropoietin (Epo) gene. Co²⁺ is orally active, easy to obtain, and inexpensive. However, its intake can bear risks to health. To elaborate this issue, a review of the pertinent literature was retrieved by a search with the keywords “anaemia”, “cobalt”, “cobalt chloride”, “erythropoiesis”, “erythropoietin”, “Epo”, “side-effects” and “treatment”, amongst others. In earlier years, cobalt chloride was administered at daily doses of 25 to 300 mg for use as an anti-anaemic agent. Co²⁺ therapy proved effective in
stimulating erythropoiesis in both non-renal and renal anaemia, yet there were also serious medical adverse effects. The intake of inorganic cobalt can cause severe organ damage, concerning primarily the gastrointestinal tract, the thyroid, the heart and the sensory systems. These insights should keep athletes off taking Co²⁺ to stimulate erythropoiesis [13321].

**Phthalates**

One idea was to identify markers that indirectly allude to the autologous transfusion. After withdrawal, freshly donated blood requires an initial processing and storage for a certain amount of time, which mostly occurs in plastic bags. The softening substances of these plastic blood bags or so-called plasticizers are phthalates (di-(2-ethylhexyl)phthalate (DEHP)) and their derivates. A considerable amount of the phthalates will diffuse from the plastic bag into the stored blood during long-term storage and will be transfused with the blood. Monfort et al. demonstrated that phthalates are metabolized in the human body to distinct molecules (mono-(2-ethylhexyl)phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP), and mono-(2-ethyl-5-oxohexyl)phthalate (MEOHP)) and can be detected in urine for a short time after transfusion using LCMS methods. The measurement of phthalates is the first approach using urine as a matrix, which is collected commonly at anti-doping controls. This may also include using phthalate analyses for homologous blood transfusion for which the direct test requires the collection of blood samples. Blood samples are now collected in a much higher percentage than in the past but not in all subjects. On the other hand, as phthalates are ubiquitous and present in many products of daily use, testing for phthalates will very likely not be a suitable stand-alone detection method for blood transfusion. Therefore, several validation studies have been conducted in order to identify threshold values for the different metabolites in athletes, taking into account the normal daily exposure to plasticizers. Recently, two further metabolites were described as possible markers (mono-(2-ethyl-5-carboxypentyl)phthalate (5cx-MEPP) and mono-(2-carboxymethylhexyl)phthalate (2cx-MMHP)) for blood transfusions measured by ultraperformance liquid chromatography tandem mass spectrometry together with the molecules MEHP, MEHHP, and MEOHP. The use of urine for the detection of blood transfusions with phthalates seems promising as all urine samples submitted to doping analysis in any accredited laboratory could provide corroborating evidence in subjects potentially using blood transfusion, e.g., based on ABP data. Phthalate data could also be applied together with other indirect markers in a multifactor approach [13006].

Elevated concentrations of phthalates and respective metabolites in urine have further been suggested as indicators for illicit blood transfusions in sport. In a follow-up study, it was reported on an LC-MS/MS methodology allowing the quantification of the main five metabolites of one of the most common plasticizers di-(2-ethylhexyl)phthalate (DEHP). Using enzymatic hydrolysis followed by LLE, target analytes were separated on a C-18 analytical column (2.1 x 100 mm, particle size 1.7 microm) and determined in positive and negative ESI with MRM detection. The accuracy of the analysis was supported by three isotopically labelled internal standards, and quantification limits between 1.2 and 2.6 ng/mL were accomplished. Applying the method to reference groups consisting of 30 control individuals and 464 athletes from the doping control pool, threshold concentrations were suggested for each analyte (ca. 160–340 ng/mL), above which suspicion of blood transfusion was mentioned to be indicated [13009].

**Granulocyte colony-stimulating factor (G-CSF)**
The use of growth factors in sports is restricted under the terms of the World Anti-Doping Code (WADC). While the beneficial effects of erythropoietin (EPO) on erythropoiesis and therefore its performance-enhancing properties have been well documented and established for decades, the aim of one study was to elucidate the relevance of the cytokine G-CSF in a doping control context, particularly concerning its influence on selected blood parameters representing central aspects of the Athlete Biological Passport. For that purpose, the effect of repeated subcutaneous granulocyte colony-stimulating factor (G-CSF) injections in therapeutic dosages (10 µg/kg/d) on white blood cells, erythrocytes, hemoglobin, hematocrit and percent reticulocytes was analyzed by using commonly employed fluorescence flow cytometry-based approaches. A total of 20 people were tested (14 male, 6 female) and both white blood cell count and reticulocyte percentages were found to significantly increase following a 5-day treatment with G-CSF. Simultaneously, all other volume-dependent parameters (red blood cell count, hemoglobin, hematocrit) slightly but significantly decreased. Due to the relevance of these measurands for the validity of blood tests for doping controls and the anecdotal evidence of G-CSF being potentially misused by elite athletes, G-CSF analyses might be indicated in case of unusually altered blood profiles [12239].

Other blood products

Actovegin

In recent years, the use of Actovegin® (calf blood hemodialysate) in sports medicine has caused a lot of controversy in many sports disciplines. Although it is unlikely for this deproteinised substance to have oxygen-enhancing capacity, there is an anecdotal belief that Actovegin® can increase an athlete’s performance. Actovegin® is produced by Nycomed Austria GmbH and has been used by doctors across Europe, China and Russia for about 60 years. Nevertheless, very little is known regarding the effects of Actovegin on muscle injuries. One article reviewed the current evidence on Actovegin®, its legal status with sports governing bodies and its potential role in sport injuries. It was also reported experience with this drug in treating muscle injuries. In a pilot study, players in the Actovegin treatment group were able to return to play 8 days earlier (95% confidence interval -1.2 to -14.8) compared to physiotherapy alone, which was a significant difference. No adverse reactions were recorded in any of the participants [11299].

Calf-derived deproteinised haemodialysate, Actovegin (Nycomed Austria), is a component in calves’ blood that gained attention when its use by the Australian rugby teams was reported to improve endurance and recovery from injuries. Although the medical research suggests some evidence (i.e. ‘potential’) of efficacy in the treatment of soft tissue injuries, the Australian Sports Anti-Doping Authority (ASADA) said that Actovegin was not then on the WADA's list of prohibited substances. Thus its use as a substance when restricted to intramuscular injections is therefore not prohibited. However, it is illegal as a method when injected into a vein. Actovegin was initially listed on the banned list by the International Olympic Committee (IOC) in December 2000 because of the concerns about its abuse in cycling. It was, however, removed from the IOC banned list in February 2001 pending further research. Actovegin is not a prohibited substance in WADA’s most current lists in 2012 although these lists do have an inclusion statement not present in previous versions: the prohibition of any growth factor(s) affecting muscle, tendon or ligament protein synthesis/degradation, vascularisation, energy utilisation, regenerative capacity or fibre type switching. This inclusion statement has, however, created more uncertainty over other therapeutic substances, besides Actovegin, used in sports medicine settings. So far, the
lawful challenge of using Actovegin as a tendon healing substance has not been made, although this legal issue is evolving at the time of writing. According to the latest 2012 WADA prohibited list, Actovegin is not prohibited in any sports. However, to complicate matters, WADA has issued specific guidance on Actovegin on its website that, according to section M2 of the WADA code, the volume of intravenous injection of any non-prohibited substance must not exceed 50 mL with a simple syringe, and further serial injections must be at least six hours apart. This means that under the latest (2012) interpretation of the Code, Actovegin cannot be administered by intravenous infusion or single intravenous injection with a volume exceeding 50 mL. Detection of doping of Actovegin would depend on catching athletes in the act of performing the banned method (not adhering to the rule of an intravenous injection of less than 50 mL or an intramuscular injection of Actovegin) and not simply detecting Actovegin in the athlete’s body [12015].

There is much speculation that Actovegin® is ergogenic, but no scientific work has been published in this field. Eight participants (mean age, height and mass of 24 years, 176 cm and 80.1 kg, respectively) completed 3 exhaustive arm crank ergometry tests. Following Baseline testing 2 further tests were performed 2 h following the injection of either 40 mL of Actovegin® or a saline placebo. Peak power (Wpeak), peak physiological responses, concentrations of blood glucose and lactate, exercise efficiency (%), VO₂ gain (mL/W), and the respiratory compensation point (RCP) were determined. Values of mean bias were calculated to further explore quantitative differences between trials. Strong trends for variations in Wpeak and RCP were evident; likely meaningful effects existed between the Baseline and both injection trials, but only a trivial effect was noted between placebo and Actovegin®. Concentrations of blood lactate and glucose changed across time, but did not differ between the 3 trials. The data suggests the Actovegin® is not ergogenic and did not influence functional capacity in the context of the exhaustive, upper-body test employed [12271].

**Intravenous fluid use in athletes**

Time allowing, euhydration can be achieved in the vast majority of individuals by drinking and eating normal beverages and meals. Traditionally, the use of intravenous (IV) administration of fluids has thus been reserved for the treatment of clinical dehydration and in the setting of presumed heat illness in the collapsed athlete. Treatment of exercise-associated hyponatremia with hypertonic IV infusion to correct plasma sodium levels is also a standard and accepted use of IV fluid infusions. In clinical practice, the use of IV fluids is more widespread, in both the prevention and treatment of exercise-related conditions. The use of IV fluid for prehydration is difficult to quantify. Anecdotally, IV hydration is common among some teams. A recent survey of the National Football League teams revealed that 24 of 32 of the teams utilized IV infusion of fluids for prehydration in at least some otherwise healthy individuals. The most common reason cited was the prevention of muscle cramps (23 of 24 teams). Of teams that administered IV fluids prior to competing, there was an average of 5 to 7 athletes per team receiving IV infusions. Timing of IV and the type of fluids utilized prior to competition had not been studied regarding exercise performance. In the National Football League, an average of 1.5 L of normal saline was administered approximately 2.5 hours prior to competition. With modern sports’ premium on winning, it only follows that individuals and teams look for performance advantages. This mind-set has led to an expanded role for IV hydration outside the context of medical emergencies to include prevention and rapid treatment of dehydration and exercise-associated muscle cramps (EAMC). Important to the competitive athlete is prevention of dehydration as it is linked to a decline in athletic performance. Among elite triathletes, muscle cramping is one of the most common reasons cited for not finishing. Intravenous (IV) prehydration and rehydration has been proposed as
an ergogenic aid to achieve euhydration more effectively and efficiently. PubMed database
was searched in November 2011 for all English-language articles related to IV utilization in
sport using the keywords intravenous, fluid requirements, rehydration, hydration, athlete,
sport, exercise, volume expansion, and performance. Limited evidence exists for
prehydration with IV fluids. Although anecdotal evidence does exist, at this time there are no
high-level studies confirming that IV prehydration prevents dehydration or the onset of
exercise-associated muscle cramps. Currently, there are no published studies describing IV
fluid use during the course of an event, at intermission, or after the event as an ergogenic
aid. It was concluded that convincing research to support IV fluid administration prior to
competition for performance enhancement, dehydration prevention, or muscle cramp
prevention does not exist. Current studies do not support the use of IV fluids for rehydration
when an athlete can tolerate oral fluids. IV fluids and plasma binders are not allowed in
World Anti-Doping Agency-governed competitions. Routine IV therapy cannot be
recommended as best practice for the majority of athletes [12272].

Discussion on dangerous dehydration in sports

Pre- and postexercise body weight measurements are the most common means to estimate
overall water loss but are condition specific. More elaborate indices of dehydration can be
measured using blood and urine samples, but these tests are often impractical or unavailable
to most athletes. Sweat composition is another parameter that is difficult to directly measure.
Furthermore, sweat composition is highly variable, as body weight, genetics, heat
acclimatization, and metabolic efficiency all influence the sweat rate and composition.
Studies utilizing labeled deuterium oxide (D$_2$O) fluid show that during exercise D$_2$O appears
in sweat 10 minutes after oral ingestion. A follow-up study compared ingestion of labeled oral
fluid versus a combination of oral and IV labeled D$_2$O. After dehydration, subjects were
rehydrated and exercised until D$_2$O was measured in sweat. There were no differences in the
groups despite an apparent increase in plasma volume afforded by IV rehydration. It appears
that 1 to 2 percent body weight loss is well tolerated by the exercising athlete. Dehydration,
defined as greater than 2 percent loss of body weight, can negatively affect performance. In
highly trained endurance athletes, plasma volume and sodium serum concentration were
preserved despite a 5 percent body weight loss. In Ironman triathletes, dehydration to 5
percent body weight loss did not correlate with occurrence of medical complications. As
degree of dehydration increases, more physiological detrimental effects may occur. This
finding is more pronounced in the endurance athlete performing in hot and humid conditions.
Progressive dehydration can lead to diminished cognitive function and may, in severe cases
(>12 %), cause death [12272].

Oral versus intravenous fluids

Safety of the athlete is always a primary concern of the treating physician, and avoiding harm
is imperative. Comparing IV versus oral hydration methodologies reveals several differences.
First, IV hydration has increased risks relative to oral hydration. Even though severe
complications are relatively low, one must consider infection, bleeding, soft tissue infiltration,
and air embolism as potential complications that are nonexistent with oral hydration. Second,
physiologically, IV hydration bypasses the oropharyngeal reflex and the associated cascade
digestive enzymes and hormones. The potential consequences of these differences are
poorly understood in the context of athletic performance. In addition to the safety of IV
rehydration, the practitioner must consider legal and ethical questions. According to World
Anti-Doping Agency regulations, the routine practice of administering IV fluids (>50 mL per 6
hours) to healthy individuals is prohibited. The code states the following in section M2,
numeral : “Intravenous infusions and/or injections of more than 50 mL per 6 hour period are
prohibited except for those legitimately received in the course of hospital admissions or clinical investigations” [12272].

Exertional muscle cramp prevention

Exercise-associated muscle cramps (EAMC) are a major problem during competition for many athletes, including team, individual, and endurance athletes. Some controversy exists regarding the etiology of EAMC; the cause is likely multifactorial. Recent evidence suggests the etiology of EAMC is related to muscle fatigue and neuronal excitability. Several studies have shown no correlation between hydration status or electrolyte concentrations with EAMC. However, there may be a subset of muscle cramping that is associated with a loss of both body fluid and sodium. These individuals are the so-called heavy sweaters. Although only anecdotal evidence does exist, at this time there are no high-level studies confirming IV prehydration prevents EAMC. Further studies need to be performed assessing the efficacy of IV fluids in the prevention of muscle cramping. There are no published studies on the treatment of EAMC with IV fluids during competition. However, current best practice recommendations are to replace fluid and salt losses, eliminating potential muscle cramping. For a large majority of individuals, this can be accomplished by oral intake of fluid and electrolytes. There are beneficial anecdotal reports of EAMC treatment in elite and professional-level athletes with IV hydration during the course of an event [12272].

Preexercise fluid requirements

Prior to exercise, it is highly recommended to address an athlete’s fluid status. There are multiple published guidelines regarding prehydration. The American College of Sports Medicine has published a position statement including specific recommendations for preexercise hydration. Its recommendations include the following:

- hydration should begin hours prior to exercise, especially if known deficits are present
- fluids should be consumed at a slow, steady rate, with 5 to 7 mL/kg taken 4 hours prior to exercise
- if urine output is insufficient, the color is dark, or the specific gravity is greater than 1.020, it is recommended to consume an additional 3 to 5 mL/kg 2 hours prior to exercise.

Per oral fluid absorption rate is influenced by the osmolality of the fluid. In one study the concentration of sodium (0, 20, 50 mEq) was varied to determine if absorption was based on the sodium ion. Sodium concentration did not produce significant changes in the rate of absorption but was primarily dependent on carbohydrate concentration. Subjects ingested 1100 mL per hour of drink, with an average absorption rate of 550 mL per hour. This represented 50 percent of the ingested fluids per hour. The sweat rate was also measured during this time and was 634 mL per hour. Therefore, absorbed fluid represented 86 percent of fluid loss through sweat. Again, there were no differences among varying sodium concentrations [12272].

IV volume expanders

Hyperhydration through the use of plasma volume expanders may be accomplished via oral or IV routes. Glycerol is the primary agent for oral hyperhydration. Glycerol hyperhydration and rehydration may benefit endurance athletes performing in hot and humid environmental conditions. Glycerol hyperhydration allows an excess of 1.5 L of hydration prior to an event. Current recommendations for endurance athletes intending to hyperhydrate with glycerol are
1.2 g/kg in 26 mL/kg of fluid over a period of 60 minutes, ending 30 minutes prior to exercise. Side effects from glycerol include nausea, gastrointestinal discomfort, and light-headedness, which sometimes preclude its utilization. There are IV formulations with comparable solutes for plasma volume expansion. The infusion of 6 percent dextran solution (403 ± 21 mL) on stroke volume during submaximal exercise resulted in an 11 percent increase in stroke volume in the untrained men but no increase in endurance-trained men. Infusion of dextran above 400 mL did not result in a further increase in stroke volume. In a follow-up study, maximal oxygen uptake and exercise time to fatigue were measured after untrained men were infused with either 200 to 300 mL or 500 to 600 mL of 6 percent dextran. The elevation of plasma volume by 200 to 300 mL via dextran infusion resulted in 15 percent increase in stroke volume, 4 percent increase in VO₂max, and an increase in the exercise time to fatigue. In contrast, further expansion of plasma volume to 500 to 600 mL resulted in excessive hemodilution and return to baseline VO₂max and exercise time to exhaustion. IV infusion of 6% dextran was also studied in elite endurance-trained cyclists chosen to reflect high baseline blood volumes via training adaptations. Infusion of 6 percent dextran increased blood volume (547 ± 61 mL). No change in maximal oxygen intake or endurance performance (time to exhaustion at 95% VO₂max) was found. Highly trained athletes may already be at the optimum blood volume, which is at or near the limits of their diastolic reserve capacity. Hypervolemia can be induced through either training adaptations or acute volume expansion with 6 percent dextran in euvoletic subjects. Both hypervolemic states showed improved cycling time trial performance, but no difference was seen between the hyperhydrated states. Albumin has been investigated for the purpose of volume expansion. Albumin infusion increased plasma volume 13 percent over the saline infusion. There were lower heart rates in the albumin infusion group at 25 minutes and 40 minutes. No differences were found in performance time, final heart rate, or rectal temperature between the 2 group [12272].

Rehydration

If rapid recovery from dehydration is desired, one should ingest 1.5 L of fluid for each kilogram of body weight lost. Replacing 150 percent of body weight loss over 60 minutes has been tolerated without complications. Even though the majority of cases of dehydration can be treated via oral consumption, IV treatment of severe dehydration (>7 % body weight loss), exertional heat illness, nausea, emesis, or diarrhea, and in those who cannot ingest oral fluids for other reasons, is clinically indicated. Otherwise, as the American College of Sports Medicine consensus guidelines state, “IV fluids do not provide an advantage over drinking oral fluids and electrolytes.” Limited evidence exists for prehydration with IV fluids. At the time of this publication, only 1 clinical trial has been published comparing the exercise performance of a group prehydrated with rapidly infused IV (normal saline) and euhydrated (oral) subjects. Ten moderately fit first responders wearing chemical protective clothing exercised on a treadmill. Hyperhydration occurred prior to exercise on the treadmill. No significant differences between the 2 groups were found for maximum heart rate, maximum core temperature, and exercise duration [12272].

The effect of an IV infusion during exercise has been studied. Although impractical in the competing athlete, the effect of an IV saline infusion versus no infusion during cycling has been compared. Nine males exercised to exhaustion at 84 ± 3 percent of maximum O₂ consumption on a cycle ergometer while receiving no fluids or an infusion of 0.9 percent normal saline (mean volume, 1280 ± 107 mL). Measured variables included core and skin temperatures, time to exhaustion, blood samples, and expired gases (breath by breath). Recorded measurements included hydrogen ion concentration, blood lactate level, peak O₂ uptake, carbon dioxide production, and end-tidal partial pressure of carbon dioxide. A decreased core temperature (38.5 ± 0.2 versus 39.0 ± 0.2) and heart rate (186.0 ± 5.1
versus 194.1 ± 3.9) was found for the IV group, but this did not translate to improved performance as measured by endurance time. No other significant differences were found between groups. The effects of a saline infusion during exercise on a cycle ergometer at 60 percent of maximum aerobic power for 50 minutes in warm and cool environments have been investigated. The control group received no infusion, and measured outcomes included forearm blood flow, heart rate, and esophageal temperature. Restoration of plasma volume via IV infusion decreased heart rate by 6 beats per minute at the 50-minute mark in the warm environment. No difference was found in the cold environment. Importantly, no performance data was reported. Similarly, markers of cardiovascular drift that included stroke volume, heart rate, and rectal temperature have been studied in 10 endurance-trained athletes who cycled in a 22°C room for 2 hours. The study group received an IV infusion, and a control group received no fluid replacement. There was no oral fluid replacement group in the study. No difference between the groups was demonstrated at 1 hour of exercise. However, after 2 hours of exercise, the rectal temperature was 0.6°C higher in the group not receiving IV infusion. Also, stroke volume and cardiac output were 11 to 16 percent lower in the control group versus the IV infusion group [12272].

Fluid hydration during an event is critical for preventing the unwanted effects of dehydration. Currently, there are no published studies describing IV fluid use during the course of an event, at intermission, or after the event as an ergogenic aid. In these settings, there is a perceived benefit for the convenience and speed of fluid replacement. The speed of replacing plasma volume by IV infusion is primarily a consequence of bypassing gastric emptying, which is volume dependent. Large volumes of IV fluid can be given over a short period. An 18G peripheral IV can provide 50 to 60 mL per minute of IV fluid by gravity. No high-level studies have determined the ideal rate for IV fluids hydration in healthy subjects [12272].

Athletes frequently need to rapidly replace fluid volumes between training sessions or events when there is limited time (2-a-day practices or stacked training sessions). Some athletes have turned to IV hydration for its speed of fluid administration. Studies evaluating the benefits of IV hydration for repeated performances include a dehydration phase via exercise, a rehydration time, and a second exercise phase. The hydration routes in acclimated and nonacclimated highly trained cyclists were studied by dehydrating to 4 percent body weight loss. Rehydration occurred to 2 percent body weight loss with 0.45 percent oral or IV fluid. A control group received no fluids. Subjects then cycled at 70 percent VO2peak to exhaustion in 37°C. No difference was found in exercise time to exhaustion. Plasma volume was better restored during rehydration with IV fluids at preexercise and 5 minutes of exercise. At 15 minutes, there was no difference between IV and oral rehydration. The same study design evaluated perceptual responses to exhaustion after IV or oral rehydration. Perceived exertion, thirst, and thermal sensations were measured. Central perceived exertion was lower in the oral rehydration group at 5 and 15 minutes. Overall perceived exertion was lower in the oral rehydration group at 15 minutes. Thermal sensation was higher in IV group at 15 minutes. Thirst ratings were lower at 0, 5, and 15 minutes with oral rehydration. Thus, all measures were more favorable in the oral rehydration group. In a similar study, 8 males were dehydrated via a 75-minute phase to 1.7 to 1.8 L of fluid loss. The negative fluid balance was replaced in 20 minutes by either IV of 0.45% normal saline or oral rehydration. The participants then completed a heat tolerance (treadmill speed of 2.4 m per second and an incline grade of 2.3 %) at 37°C and 45 percent relative humidity. More rapid restoration of plasma volume was accomplished in the IV treatment group with no advantages over oral rehydration in physiological strain, heat tolerance, ratings of perceived effort, or thermal sensations. Sensation of thirst was lower in the oral rehydration group. Performance (heat tolerance time) was not significantly improved in the IV group. The effect of IV hydration on recovery following marathon running has also been studied. IV fluids were administered
immediately postmarathon to assess time to recovery in 66 participants. The experimental group received 2.5 L of 2.5 percent glucose/0.45 percent sodium chloride solution, whereas the placebo group received 100 mL of 0.9% sodium chloride solution. No significant differences between the groups were found for time to recovery, number of days with pain, number of days with stiffness, sleep disturbance, fatigue, rectal temperature, and loss of appetite. The current data thus suggest that IV rehydration is faster than oral. There may be physiological benefits of decreased heart rate and norepinephrine in athletes rehydrated via IV route, but the clinical significance is not known. Oral rehydration does decrease thirst better than IV rehydration and may decrease perceived exertion [12272].

Plasma volume expanders

The use of plasma volume expanders (PVE), such as dextran (DEX) and hydroxyethyl starch (HES), is prohibited in sports. DEX is a naturally occurring glucose polymer, whereas HES is synthetically produced from amylopectin starch by substitution with hydroxyethyl groups. In doping control, the commonly applied enzymatic and colorimetric screening methods are lacking adequate specificity for DEX and HES. Also, gas chromatographic-mass spectrometric (GC-MS) screening methods have specificity issues with DEX. In addition, due to the nature of the target compounds, time-consuming derivatisation steps are required in GC-MS. Based on the high molecular weight of carbohydrate polymers excreted in urine after administration of DEX and HES, a screening method was developed involving size exclusion chromatography (SEC) combined with time-of-flight mass spectrometry (TOFMS). By using solely a SEC guard column as an analytical column allowed sufficient chromatographic resolution in a minimal amount of time and with reasonable repeatability (average RSD of 10 %). Detector response was linear throughout the measurement range for both analytes. Limits of detection were 100 and 250 microg/mL for DEX and HES, respectively. Ion suppression was found to be 52 percent at maximum. In-source collision-induced dissociation (ISCID) was used to produce characteristic fragments at a mass accuracy better than 2 mDa. The specificity of the SEC-ISCID-TOFMS method was demonstrated with 120 PVE negative doping control samples analyzed in parallel with a routine GC-MS screening method. In addition, seven urine samples from diabetic athletes, causing interpretation problems in routine GC-MS, showed here a definitely negative profile [13340].

Plasma volume expanders comprise a heterogeneous group of substances used in medicine that are intravenously administered in cases of great blood loss owing to surgery or medical emergency. These substances, however, can also be used to artificially enhance performance of healthy athletes in sport activities, and to mask the presence of others substances. These practices are considered doping, and are therefore prohibited by the International Olympic Committee and the World Antidoping Agency. Consequently, drug testing procedures are essential. One article provided an overview of plasma volume expanders, assembling pertinent data such as chemical characteristics, physiological aspects, adverse effects, doping and analytical detection methods, which are currently dispersed in the literature [11147].

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Hypovolemia from a range of etiologies can lead to severe morbidity and mortality unless blood volume and tissue perfusion are restored. The treatment of hypovolemia has included the improvement and restoration of blood volume loss by the intravenous infusion of plasma expanding therapeutic agents. These have included crystalloid and/or colloid solutions, and a brisk controversy as to which modality is better has engaged therapeutics for the past 30 years. In addition, those favoring either modality have debated which crystalloid, and which colloid, is better. This area was given a dramatic turn a decade ago when a Cochrane meta-analysis concluded that albumin, a historically important plasma expander, resulted in increased mortality when administered to critically ill patients. Although subsequently modified by other studies, the Cochrane meta-analysis has served to generate an ongoing interest in the safety of plasma expanders. One review assessed the safety of these therapies from the viewpoint of the heterogeneous range of clinical indications for which they are used [11149].

Plasma volume expanders comprise a heterogeneous group of substances used in medicine that are intravenously administered in cases of great blood loss owing to surgery or medical emergency. These substances, however, can also be used to artificially enhance performance of healthy athletes in sport activities, and to mask the presence of others substances. These practices are considered doping, and are therefore prohibited by the International Olympic Committee and the World Antidoping Agency. Consequently, drug testing procedures are essential. The present work provides an overview of plasma volume expanders, assembling pertinent data such as chemical characteristics, physiological aspects, adverse effects, doping and analytical detection methods, which are currently dispersed in the literature. Crystalloid solutions are primarily extravascular space expanders, since 1 h after infusion, approximately three-quarters have already moved out from capillary blood into the interstitial space. However, they are the least expensive and most readily available of the plasma volume expanders. The second important group is represented by colloid solutions. These homogeneous noncrystalline solutions contain high-molecular weight (MW) substances, such as proteins, large glucose polymers or blood derivatives, which do not settle out under the influence of gravity. Colloid solutions are intravascular space expanders, since colloid molecules cannot pass easily through the endothelium; hence, they exert an oncotnic pressure, which attracts extravascular fluid into the circulation. The resulting expansion is at least equivalent to the infused volume. In both groups, fluid administration initially results in expansion of the intravascular compartment. The basic difference is the extent and persistence of effects, which depend on whether or not there is a free transfer of molecules across the vascular endothelium. Hemoglobin-based oxygen carriers (HBOCs) are described in the colloid group, since they consist of high-MW substances that pull
interstitial fluid into the intravascular space due to their relevant oncotic pressure. These HBOCs combine oxygen transport with volume-expanding properties. Other oxygen carriers, such as perfluocarbons, are not been included in this group, since they are not considered plasma expanders. The third group of PVEs includes osmotic diuretics, which are low-MW substances that are freely filtered at the glomerulus, being only slightly reabsorbed at the renal tubules. The additional osmotically active particles in the glomerular filtrate prevent water reabsorption and promote diuresis. Increased osmotic pressure in plasma draws water from tissues into the vascular space, expanding plasma volume. Osmotic diuretics are mainly used to reduce cerebral edema and intra-ocular pressure and to preserve kidney [11146].

Dextran and hydroxyethyl starch

Dextrans are branched polysaccharides, made up of glucose units, mainly in alpha-1,6 linkage, in predominantly linear macromolecules. Preparation consists of Leuconostoc bacteria acting on sucrose, acid hydrolysis and subsequent ethanol fractionation, leading to the final dextran products. Currently, there are two commercially available types: Dextran 40 and Dextran 70; they do not transmit hepatitis virus and are inexpensive. Renal elimination depends on molecular weight. Dextran molecules of less than 50-55 kDa are freely filtered at the renal glomerulus, while elimination of molecules of greater than 55 kDa may take several days. Dextrans prevent hypovolemia, exert thromboprophylactic activity and improve microcirculation. However, severe adverse effects may occur, such as anaphylactic or anaphylactoid reactions, renal impairment in patients with risk factors, as well as biochemical alterations. Blood volume expansion in athletes by dextran was studied in 1994 (Utah research), evidencing enhancing effects. Therefore, since January 2000, the use of dextran by athletes has been prohibited by the IOC and WADA. An initial screening for dextran by Benedict’s reaction and TLC is performed by the same procedure as described for HES. A characteristic pattern of bands, different from the HES profile, may indicate the presence of dextran in urine. Confirmation must be made by chromatographic/MS techniques. A commonly used identification method for urinary dextran consists of qualitative analysis of the partially methylated alditol acetates obtained by degradation and subsequent derivatization. However, this method is unable to perform the required quantitation by partially methylated alditol acetates derivatization procedures. The dextran polymer is enzymatically hydrolyzed by alpha-1,6-glucosidase (dextranase), generating isomaltose subunits. Subsequent acetylation allows the chromatographic separation of different disaccharides, such as lactose, saccharose and isomaltose, as well as the identification and quantification of the analyte in human urine [11146].

A rapid screening procedure based on liquid chromatography-electrospray ionization-tandem mass spectrometry with in-source collision-induced dissociation was developed for detection of the polysaccharide-based plasma volume expanders dextran and hydroxyethyl starch (HES) in human urine. The in-source fragmentation strategy of the approach represented a valuable tool in the analysis of the polysaccharide-based compounds, allowing the use of tandem mass spectrometry. After direct injection of urine specimens, analytes were chromatographically separated on a monolithic reverse-phase column and detected via multiple reaction monitoring of diagnostic ions at detection limits of 10 microg/mL for HES and 30 microg/mL for dextran. Validation was performed regarding the parameters specificity, linearity, precision (8-18 %) and accuracy (77-105 %) and the method was applied to the investigation of approximately 400 doping control samples and seven dextran and two hydroxyethyl starch post-administration samples. The approach demonstrated its capability as a rapid screening tool for the detection of dextran and hydroxyethyl starch and represents an alternative to existing screening procedures since time consuming hydrolysis or derivatization steps were omitted [08235].
Hydroxyethyl starch (HES)

The polysaccharide hydroxyethyl starch (HES) consists of d-glucose units joined by alpha-1,4 linkage, in a branching structure, in which the number of a-1,6 branch points can vary. Hydroxyethyl groups are attached to carbons 2, 3 or 6 of the glucose units. HES solutions are artificial colloids derived from corn starch amylopectin. They are used for treatment and prevention of hypovolemia, being among the most frequently used PVEs in the medical field. HES prevents dehydration and enhances microcirculation. However, it has been associated with unwanted side effects, such as pyrexia, anaphylactoid reactions and effects on coagulation. HES is removed from the circulation predominantly by renal excretion. The first phase occurs almost immediately upon administration, because polymers with a MW less than 59 kDa are rapidly eliminated by glomerular filtration. The second phase is more prolonged, due to the enzymatic degradation of higher molecular weight HES molecules. The hydroxyethyl groups remain intact and attached to the glucose units. Volume expansion is seen for approximately 24 h, with 29 to 38 percent of the colloid still available intravascularly after this period. The most common form of HES is hetastarch HES 450/0.7, which means it has an average MW in kDa/average molar substitution that represents the average number of hydroxyethyl residues per glucose unit. Hydroxyethyl starch has been exploited by the athletic community, owing to blood volume increase, preventing dehydration and enhancing microcirculation, control of hematocrit value and, mainly, masking blood doping by rEPO. Therefore, HES has been prohibited since 2000 by the IOC, and more recently by the WADA. An initial screening has been described, based on acid hydrolysis of HES in urine and detection of the resulting glucose and glucose derivatives by Benedict’s reaction. Following, hydrolysates from suspect urine samples are submitted to TLC and a characteristic pattern of bands may suggest the presence of HES. Such samples are sent to chromatographic/MS techniques for confirmation. Other polysaccharides may also present suspect results by Benedict’s reaction. The TLC procedure may suggest possible polysaccharides. Several procedures based on LC-(ESI)-MS/MS with in-source collision-induced dissociation and direct injection of urine (for HES and dextran). Voluven is a third-generation HES. It must be mentioned that there has been renewed interest in the clinical applicability of HES. High doses of first- and second-generation HES were demonstrated to be associated with several adverse effects, mainly on renal function and coagulation. Major developments have been achieved over recent years concerning safety, and the newest generation presents significantly improved properties in comparison with earlier products. The third-generation of HES, the tetrastarches, enhance degradation and minimize retention in the circulation and tissues. Moreover, extensive clinical studies in major surgery showed that very high doses of Voluven produced no adverse effects on coagulation [11146].

The artificial colloid hydroxyethyl starch (HES) is among the most frequently used plasma volume expanders in the medical field. However, in 1998, its misuse by the athletic community was officially reported and since 2000, HES is prohibited by the International Olympic Committee (IOC). Therefore, several methods enabling the detection of HES in urine were developed, most based on gas chromatography-mass spectrometry (GC-MS). In one work, a simple and low-cost screening method, intended to reduce the number of samples to be analysed by GC-MS, was developed. The method is based on the acid hydrolysis of HES and detection of the resulting glucose and hydroxyethyl glucose derivatives by Benedict’s reaction (reduction of copper sulfate to brick-red cuprous oxide by glucose and/or derivatives). Samples considered suspect were submitted to GC-MS analysis for identification of HES. The method was successfully applied for screening of HES in 2627 urine samples from 1346 Brazilian soccer players and 1281 athletes from the Pan-American Games (Rio de Janeiro, 2007); 71 (2.7 %) samples, considered suspect, were submitted to GC-MS, but no positive results were found. Moreover, a thin layer chromatography (TLC) method was adapted for visualisation of the characteristic band pattern of HES hydrolysis.
In one contribution it was tested the possibility to use microwave irradiation for the screening and confirmation pre-treatment steps of hydroxyethylstarch, with the aim to speed up gas chromatography-mass spectrometric procedures. Acid hydrolysis and derivatization processes were conducted in a temperature-controlled single beam microwave oven for organic synthesis. The kinetics of hydroxyethylstarch chemical hydrolysis and derivatization were investigated at different microwave power, incubation temperature and incubation time. The best hydrolysis conditions were found at a microwave power value of 1200 W (T 100°C) with an incubation time of 2 min; whereas the best derivatization conditions were found at a microwave power value of 1020 W (T 100°C) with an incubation time of 5 min. The effectiveness of this approach was evaluated by gas chromatography-mass spectrometry analyzing more than 20 different pools of blank urine samples spiked with hydroxyethylstarch at a concentration of 1 mg/mL. The results showed that the effect of microwave irradiation on the chemical hydrolysis process was very remarkable: the total sample preparation time can be shortened by 58 min compared to the reference method (2 min instead of 60 min). In addition to this, the time necessary for the derivatization process can also be drastically shortened with respect to the reference procedure (5 min instead of 30 min). The repeatability of the hydrolysis and derivatization recoveries, the limit of detection and the matrix interferences were comparable to the reference method accredited under the ISO 17025 guidelines and presently followed by the accredited sports anti-doping laboratory of Rome [10494].

The morphological and structural properties of basic types of starch used as excipients in solid drug form technology with reference to its bioadhesive properties with fully polymer biodegradation. The production technology and application of: carboxymethyl starch (CMS), pregelled starch, hydroxyethyl starch (HES), hydroxypropyl starch (HPS) and the share of modified starch in technology of selected food products is provided. It was focused on hydroxyethyl starch, which is used not only as blood and plasma substitution but also as drug camouflage agent in sports competition. In papers were presented based on literature data actually used types of starch and their chemical modifications products in drug forms and food technology [09174].

Hydroxyethyl starch (HES) is a widely used plasma substitute for correcting perioperative hypovolemia. HES preparations are defined by concentration, molar substitution, mean molecular weight, the C(2)/C(6) ratio of substitution, the solvent, and the origin. The possible unwanted side effects of HES are anaphylactic reactions, alterations of hemostasis resulting in increased bleeding, kidney dysfunction, accumulation, and pruritus. In view of the potential side effects, it is crucial to distinguish among the different HES preparations; all HES preparations are not the same. The first generation of HES preparation showing a high mean molecular weight (>450 kD) and a high molar substitution (>0.7) was associated with negative effects with regard to coagulation, organ function, and accumulation. One review focused on whether modern (third generation), more rapidly degradable HES preparations with a lower mean molecular weight (130 kD) and a lower molar substitution (<0.5) are safer and have fewer side effects. Several studies demonstrated that such modern HES preparations appear to be safe with regard to hemostasis, kidney function, itching, and accumulation. Modern HES preparations are dissolved in balanced, plasma-adapted solutions that no longer contain unphysiological amounts of sodium and chloride and are thus suitable for correcting hypovolemia [09175].

High doses of first- and second-generation HES were associated with adverse effects on renal function, coagulation, and tissue storage, thereby limiting their clinical applicability. Newer HES products have lower molar substitution and in vivo molecular weight, resulting in
more rapid metabolism and clearance. In this review article, the differences between HES generations are highlighted, with particular emphasis on the improved safety profile of the third generation products. These improvements have been achieved with no loss of efficacy, and they contradict the assumption that efficacy of HES solutions is directly linked to plasma concentration. The impact of source material on structure and pharmacokinetics is highlighted, and the role of the carrier solution is critically assessed [09176].

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Glucose solution

Glucose solutions, given by intravenous infusion, exert volume effects that are governed by the amount of fluid administered and also by the metabolism of glucose. They can be used with or without saline. Glucose solutions are frequently used to hydrate patients with acute disease or after surgery. Hypertonic glucose solutions are given to provide glucose as a metabolic substrate. Although glucose is not considered an illegal substance, the WADA decided that glucose infusions are illegal, unless they are used as a legitimate medical treatment in case of dangerous hypoglycemia during competition. An interesting reagent-free method using mid-infrared attenuated total reflection (ATR) for analysis of glucose (and also albumin, ALB), owing to the significant mid-infrared absorption bands has been reported. The calibration model of glucose in urine has been established, using ATR mid-infrared spectroscopy combined with partial least-squares regression analysis [11146].

Saline solution

A 0.9 percent sodium chloride isotonic solution remains in the circulation only briefly. Approximately 20-30 min after the infusion, only 20-25 percent remains in the intravascular compartment. Therefore, large volumes need to be infused to result in effective plasma volume expansion; in such cases, however, hyperchloremic metabolic acidosis may result. A balanced electrolyte solution with glucose is likely to maintain close-to-normal plasma osmolarity and energy metabolism [11146].

Ringer lactate (or acetate) solution

This solution has become widely accepted for fluid replacement because it contains electrolytes in plasma concentrations. It consists of a balanced electrolyte composition, containing potassium, sodium, calcium, chloride and lactate (acetate). Besides, coagulation
problems do not occur and solutions are low-cost. However, large quantities involve risks and complications, such as reduction of the osmotic pressure, superhydration, edema, hyponatremia and pulmonary dysfunction from water [11146].

**Hypertonic crystalloid solution**

The high osmolarity of these solutions rapidly mobilizes extravascular fluid into the circulation. However, irritation to veins and hypertension may result. Hypertonic saline solutions are solutions with high levels of sodium (e.g. 7.5 %). Plasma expansion peak occurs in 5 min, but the effect duration is brief. The main advantages are the small volume required and low cost [11146].

**Albumin**

Albumin is the major protein of human plasma, with a molecular weight of approximately 69 kDa. It makes up 60 percent of total plasma protein, being responsible for approximately 80 percent of its osmotic pressure. ALB is highly soluble in water, and bears a strong negative charge. Preparations of human ALB (pooled from human donors' plasma) have been used in medical and surgical emergencies. In general, infusion of 100 mL of 25 percent albumin expands the plasma volume by 450 mL. Controversy regarding risks and benefits compared with crystalloids has continued to date. Adverse effects associated with administration of ALB have been closely scrutinized and debated, and a recent study showed no difference in mortality between intensive care unit patients receiving ALB and those receiving saline as resuscitation fluid. Abnormal elevation of urinary albumin is a sign of kidney damage, but it may also be a long-term consequence of performance-enhancing substances intake. It is well known that exercise induced albuminuria is a common consequence of physical activity, being transient. Therefore, when urinary ALB levels exceed the established range of albuminuria due to exercise, this may be a sign of misuse. Detection of ALB in physiological fluids has been performed by various methods, including electrophoresis (serum protein fractionation) or dye binding with spectrophotometric detection. In urine, it is commonly determined by various quantitative immunochemical methods, such as immunonephelometry, immunoturbidimetry and radioimmunoassay or by semi-quantitative dipsticks. Recent studies have revealed that the ALB molecule is not a clearly defined entity in urine. Modified ALB forms, such as degraded and fragmented species of ALB, truncated forms and ALB bound to ligands present in urine have been identified [11146].

**Plasma protein fraction**

Plasma protein fraction is produced by partially fractionating a large plasma pool. It contains ALB plus several globulins fractions, closely resembling plasma in its constitution. Plasma protein fractions are effective PVEs that can be handled as pharmaceutical products. They are hepatitis-virus free but, being human-derived colloids, present disadvantages such as risks of infections and elevated cost [11146].

**Plasma**

Fresh frozen plasma has been considered most useful, since it contains nearly all the clotting factors in normal concentration, excepting platelets. However, risk of viral hepatitis is the same as for whole blood. Plasma as a PVE must be stored in a deep-freeze unit and requires special handling before administration [11146].

**Gelatins**
Gelatins are heterogeneous mixtures of polypeptides, containing large amounts of proline and hydroxyproline residues. They are usually prepared by hydrolysis of bovine collagen. Further modifications produce different types of gelatins: succinylated gelatin (Gelofusine®); urea-linked gelatin (Haemacel® and polygeline) and oxypolygelatin. The expansion capacity of gelatins is limited (1 mL corresponds to an expansion of 0.8 mL) owing to their short intravascular life. They may, however, affect coagulation or provoke anaphylactoid reactions. Since gelatins are rapidly removed by glomerular filtration, 70-90 percent are found in the urine within a few hours. In certain circumstances, however, gelatin preparations can be adequately used in patients with impaired kidney function. Gelatin-derived preparations are prohibited as intravenous infusions. Apparently, such infusions are not a preferred means of doping. No reports on gelatin misuse have been found. Owing to the large amounts of hydroxyproline in gelatin and its absence in normal plasma and urine, the determination of this amino acid enables measurement of the content of modified gelatin in these physiological fluids by any standard amino acid analyzer method. A specific quantitative gas-liquid chromatographic (GLC) assay of hydroxyproline was also developed. Moreover, gelatin-derived polypeptides can be detected by the biuret method. In case of hydroxyproline-free gelatin, determination can be made by reversed phase-LC and detection at 220 nm [11146].

**alpha-Keratose**

An interesting compound, alpha-keratose, is a protein derived from keratins, which are complex proteins found in skin, hair, nail, feathers and similar tissues. The preferred source for keratose preparations is human hair, which is abundant, renewable, nontoxic, non-antigenic and inexpensive. However, it presents disadvantages such as aggregation of red blood cells, among others. New approaches to developing keratin-based high viscosity plasma expanders have been studied, leading to improved compositions. A homogeneous liquid plasma expander composition, consisting of alpha-keratose and an electrolyte solution, has been described by [11146].

**Combined solutions (hypertonic crystalloid/colloid)**

Owing to the brief plasma expansion of hypertonic crystalloids, the colloids HES or dextran can be added to increase expansion duration. Hypertonic saline dextran requires only small volumes to rapidly elevate blood pressure and is capable of prolonged maintenance of volemic expansion. It was introduced in 1985, but its take-up has been slow, mainly due to concerns about complications and lack of human trials. However, it became apparent that hypertonic saline dextran may be an efficient means of correcting hypotension and a trend towards increased survival has also been suggested [11146].

**Pullulan**

Pullulan is a fungal non-ionic exopolysaccharide produced from starch by Aureobasidium pullulans. It consists of alpha-1,6 linked maltotriose residues and its molecular weight ranges from thousands to millions daltons. A. Pullulan is easily soluble in hot and cold water, producing clean and viscous solutions. It is blood compatible, biodegradable, nontoxic, non-immunogenic, nonmutagenic and noncarcinogenic, and is therefore being explored for medical application, including use as a potential plasma-blood substitute [11146].

**Levan**
Levan, a branched polymer of d-fructose, has great potential as a functional biopolymer in the pharmaceutical and chemical industries, depending on the molecular weight. To date, microbial levans are produced from a sucrose-based substrate by enzymatic reaction using a variety of microorganisms, including the recently investigated Bacillus subtilis. Levans with small molecular weights are nontoxic and non-antigenic, being slowly eliminated from the body when injected into the bloodstream. Therefore, levan with a MW below 100 kDa has potential as a PVE [11146].

**Acetyl starch**

An interesting alternative to the PVEs used to date is the synthetic colloid acetyl starch (ACS). Efficient preparation yields polysaccharide based products suitable for pharmaceutical and biomedical applications, especially as PVEs. ACS avoids accumulation of persisting macromolecules and presents an excellent physiological compatibility. It is as effective as HES, with identical plasma concentrations during the first 6 h after infusion. In contrast to the biphasic elimination of an HES solution, an equivalent ACS solution is eliminated monophasically. Although the initial phase of glomerular filtration is similar for both (immediate elimination of molecules <50,000 Da), HES undergoes a further prolonged phase of enzymatic degradation, while ACS is rapidly degraded to acetic acid and glucose, which are excreted renally. Significant amounts of HES are still detectable after 24 h, but ACS is nearly completely metabolized. Apparently, ACS is not a preferred PVE for doping purposes. No reports on ACS misuse were found. Acetyl starch can be determined using the method based on MALDI-TOF-MS. Moreover, a rapid LC method for quantitation in blood samples has been outlined, using a size-exclusion chromatography (SEC) system, with a refractive index detector [11146].

**Polyvinylpyrrolidone**

This compound was developed in Germany during World War II. However, it is no longer acceptable as a PVE since it remains indefinitely in organs and tissues, resulting in storage diseases [11146].

**Mannitol**

Mannitol, a six-carbon sugar alcohol, is used as an osmotic diuretic; it is applied by intravenous infusion to preserve renal function and to reduce raised intracranial and intraocular pressure, as well as to promote the excretion of poisons and toxic wastes. Adverse reactions may occur, such as pulmonary congestion, heart failure, electrolyte imbalance, headache, convulsions and others. The potential misuse of mannitol in sports is due to its urine-diluting effect, which lowers the concentration of banned substances in urine, and a possible bodyweight decrease. To detect a doping offence, urinary mannitol concentrations after a prohibited intravenous administration must be differentiated from mannitol resulting from a permitted oral intake. A GC-MS analytical method is described based on the peracetyl derivatives of the analytes. All possible urinary hexitols, including mannitol, can be rapidly separated and identified. The method was applied to an excretion study to investigate urinary mannitol concentrations following oral intake. Preliminary results, with regard to a threshold level for mannitol that could be utilized in doping control, were promising [11146].

**Glycerol**

Glycerol is prohibited as an ergogenic aid by the World Anti-Doping Agency (WADA) due to the potential for its plasma expansion properties to have masking effects. However, the
scientific basis of the inclusion of glycerol as a "masking agent" remains inconclusive. The purpose of this study was to determine the effects of a hyperhydrating supplement containing glycerol on doping-relevant blood parameters. Nine trained males ingested a hyperhydrating mixture twice per day for 7 days containing 1.0 g/kg body mass (BM) of glycerol, 10 g of creatine and 75 g of glucose. Blood samples were collected and total hemoglobin (Hb) mass determined using the optimized carbon monoxide (CO) rebreathing method pre- and post-supplementation. BM and total body water (TBW) increased significantly following supplementation by 1.1 ± 1.2 and 1.0 ± 1.2 L, respectively. This hyperhydration did not significantly alter plasma volume or any of the doping-relevant blood parameters (e.g. hematocrit, Hb, reticulocytes and total Hb-mass) even when glycerol was clearly detectable in urine samples. In conclusion, this study shows that supplementation with hyperhydrating solution containing glycerol for 7 days does not significantly alter doping-relevant blood parameters [12338].

Glycerol ingestion creates an osmotic drive that enhances fluid retention. The major practical applications for athletes are to either hyperhydrate before exercise so that they have more fluid to be lost as sweat during subsequent performance, thereby delaying the progression of dehydration from becoming physiologically significant, or improve both the rate of rehydration and total fluid retention following exercise. Recently we showed that rehydration may be improved further by combining glycerol with intravenous fluids. Improvements in endurance time, time trial performance and total power and work output have been seen during exercise following glycerol-induced hyperhydration or rehydration. Another recent trial showed that the increased body weight associated with the extra fluid does not inadvertently affect running economy. Concerns that the haemodilution associated with the fluid retention in the vascular space may be sufficient to mask illegal doping practices by athletes led the World Anti-Doping Agency (WADA) to add glycerol to its list of prohibited substances in 2010. Recent evidence suggests that doses of > 0.032 ± 0.010 g/kg lean body mass (much lower than those required for rehydration) will result in urinary excretion that may be detectable, so athletes under the WADA jurisdiction should be cautious to limit their inadvertent glycerol intake [12273].

Glycerol is a 3-carbon sugar alcohol that provides the backbone of triglycerides and is naturally found in foods as a component of dietary fats. However, its various physical and chemical properties are valuable in food technology: glycerol is added to manufactured foods and drinks as an emulsifier, humectant, sweetener, filler and thickener. Its viscosity also makes it useful as a component of lotions and creams, explaining its common availability for purchase in purified form under the name of glycerine. Although it has been suggested as a gluconeogenic precursor that could provide a substrate for exercise, the ingestion of glycerol by athletes is best known for its role as an osmolyte. When ingested or released following lipolysis, glycerol contributes to the osmotic pressure of body fluids until it is slowly metabolised. When consumed simultaneously with a substantial volume of fluid, there is a temporary retention of this fluid and expansion of body fluid compartments. Effective protocols for glycerol hyperhydration are 1-1.5 g/kg glycerol with an intake of 25-35 ml/kg of fluid. Such a protocol typically achieves a fluid expansion or retention of approximately 600-1000 ml above a fluid bolus alone by a reduction in urinary volume. Several challenging situations commonly arise in sport in which athletes have made use of these strategies to promote better hydration status. These include hyperhydration before exercise in hot environments where a large fluid deficit will otherwise accumulate, and reduction of diuresis associated with aggressive rehydration (eg, following weight-making practices in sport or after dehydrating exercise). General guidelines for fluid intake in exercise typically discount the value of hyperhydration or fluid overloading strategies. Indeed, there are occasional side-effects of glycerol use including nausea, gut discomfort and headaches from increased intracranial pressure. However, more focused reviews of glycerol hyperhydration in specific
situations in sport, including a meta-analysis of glycerol hyperhydration before exercise in hot environments, have reported evidence of enhanced fluid balance and endurance performance compared with the intake of large volumes of water alone or no hyperhydration. There has been less focus, but some support, for the addition of glycerol to rehydration beverages to promote the rapid reversal of dehydration and assist the performance of subsequent exercise. Indeed, there anecdotal reports that some athletes add glycerol to rehydration beverages for practical reasons; for example, to reduce overnight diuresis and its interruption to sleep patterns when completing dehydrating exercise sessions late in the day. The apparent contradictions in the literature may be due to the specificity of the situations in which glycerol hyper/re-hydration is beneficial. The major problem related to the use of glycerol by athletes, however, arises because of recent changes to antidoping codes. The 2011 World Anti-Doping Agency (WADA) list of substances prohibited at all times includes the category of Diuretics and other Masking agents (section 5). Plasma expanders further qualified in this section are: "e.g. glycerol, intravenous administration of albumin, dextran, hydroxethyl starch and mannitol." How the normal dietary intake of glycerol is distinguished from specific intake to aid fluid balance is unclear [11150].

The three-carbon sugar alcohol glycerol is used for hyperhydration and due to its ability to be easily and cleanly metabolized, for providing energy. Higher doses may cause headache, blurred vision and possible additional stress on the cardiovascular system. Administration of glycerol to endurance athletes promotes increased fluid retention and improved performance, principally under hot and humid conditions. Glycerol is considered relevant for sports drug testing, owing to the properties described above. Ingestion of large quantities causes glycerol to appear in urine, since it is rapidly reabsorbed by the proximal and distal tubules, causing minimal diuresis. Glycerol was formerly considered to be a diuretic by the US Olympic Committee, being banned because of a detrimental effect of dehydration and dilution of prohibited substances in urine. In 1997, the US Olympic Committee removed the ban on glycerol (owing to the minimal diuresis) and approved the use of glycerol ingestion. However, in the WADA 2010 Prohibited List, it appears again as a banned substance. A quantitative analytical method is described for glycerol, using a GC-isotope-dilution MS-based approach. Due to the natural occurrence of glycerol trace amounts in human urine, a range of normal levels in elite athletes’ urine, was initially determined. The analytical methods described above for the different groups of PVEs are, in their majority, based on chromatographic/MS techniques. They are accurate, specific and sensitive, being the most used methods for the identification of misused substances. The other detection procedures, including simple screening methods, may be used for clinical purposes, and also in order to precociously detect the presence of urine samples that are potentially positive for PVEs [11146].

The administration of glycerol to endurance athletes results in an increased fluid retention and improved performance, particularly under hot and humid conditions. Consequently, glycerol is considered relevant for sports drug testing and methods for its detection in urine specimens are required. A major issue in this regard is the natural occurrence of trace amounts of glycerol in human urine, which necessitates a quantitative analysis and the determination of normal urinary glycerol levels under various sporting conditions. A quantitative method was established using a gas chromatography/isotope-dilution mass spectrometry-based approach that was validated with regard to lower limit of detection (0.3 microg/mL), lower limit of quantification (0.9 microg/mL), specificity, linearity (1.0-98.0 microg/mL), intraday and interday precision (<20 % at 2.4, 24.1 and 48.2 microg/mL) as well as accuracy (92-110 %). Sample aliquots of 20 microL were enriched with five-fold deuterated glycerol, dried and derivatised using N-methyl-trimethylsilyl trifluoroacetamide before analysis. The established method was applied to a total of 1039 doping control samples covering various sport disciplines (349 endurance samples, 286 strength sport samples, 325 game sport samples and 79 other samples) in- and out-of-competition, which
provided quantitative information about the glycerol content commonly observed in elite athletes' urine samples. About 85 percent of all specimens yielded glycerol concentrations < 20.0 microg/mL and few reached values up to 132.6 microg/mL. One further sample, however, was found to contain 2690 microg/mL, which might indicate the misuse of glycerol, but no threshold for urinary glycerol concentrations has been established yet due to the lack of substantial data. Based on the results obtained from the studied reference population, a threshold for glycerol levels in urine set at 200 microg/mL is suggested, which provides a tool to doping control laboratories to test for the misuse of this agent in elite and amateur sport [08236].

Studies have shown that beverages containing glycerol can enhance and maintain hydration status and may improve endurance exercise performance by attenuating adverse physiological changes associated with dehydration. Improvements to performance include increased endurance time to exhaustion by up to 24 percent, or a 5 percent increase in power or work. However, some studies have found no performance benefits during either prolonged exercise or specific skill and agility tests. In studies that have shown benefits, the improvements have been associated with thermoregulatory and cardiovascular changes. These include increased plasma volume and sweat rates, as well as reduced core temperature and ratings of perceived exertion. In a very small number of subjects, glycerol consumption has been associated with side-effects including nausea, gastrointestinal discomfort, dizziness, and headaches. In summary, while glycerol and fluid ingestion results in hyperhydration, the documented benefits to exercise performance remain inconsistent [09177].

The authors determined, through a meta-analytic approach, whether glycerol-induced hyperhydration (GIH) enhances fluid retention and increases endurance performance (EP) significantly more than water-induced hyperhydration (WIH). Collectively, studies administered 24 mL of fluid/kg body weight (BW) with 1.1 g glycerol/kg BW, and hyperhydration was measured 136 min after its onset. Compared with WIH, GIH increased fluid retention by 7.7 mL/kg BW (pooled effect size, PES: 1.64, n=14). The use of GIH was associated with an improvement in EP of 2.6 percent (n=4). Unarguably, GIH significantly enhances fluid retention better than WIH. Because of the dearth of data, the effect of GIH on EP must be further investigated before more definitive conclusions can be drawn as to its ergogenic property [07131].

Due to its considerable polarity but modest proton affinity, glycerol analysis is difficult to be combined with most other routine doping control detection assays; however, since it was categorized as S5 substance in 2010, several studies particularly concerning its variability were conducted. In 2011, it was investigated the urinary excretion of ingested glycerol at rest and the influence of 1 g/kg of body weight on blood parameters such as hemoglobin and hematocrit. The urinary glycerol levels increased from baseline values (11 ± 16 microg/mL) to over 50 000 microg/mL accompanied by a modest though statistically significant plasma expansion. In another study, the correlation of glycerol ingestion and thus increased plasma glycerol with significantly increased renal elimination of glycerol was demonstrated. It was shown that glycerol administration higher than 0.1 g/kg of bodyweight resulted in urinary excretion exceeding the commonly observed urinary amounts of glycerol, hence, allowing a differentiation of legitimate hyperhydration with glycerol from its illicit use as a masking agent [13012].

Glycerol is prohibited as a masking agent by the World Anti-Doping Agency and a urinary threshold has recently been recommended. However, little is known about urinary glycerol excretion after exercise, when exogenous glycerol is metabolized increasingly and endogenous glycerol levels are elevated. The purpose of the placebo-controlled cross-over
study was to determine the effects of pre-exercise glycerol administration on glycerol metabolism, urinary excretion, and selected blood parameters. After administration of glycerol (1.0 g/kg body weight (BW) + 25 mL fluid/kg BW) or placebo (25 mL fluid/kg), 14 cyclists exercised 90 min at 60 percent VO_{2\max}. Samples were taken at 0 h (before administration), 2.5 h (before exercise), 4 h (after exercise) and 6.5 h and additional urine samples were collected until 24 h. Exercise increased endogenous plasma glycerol (0.51 ± 0.21 mmol/L) but peak concentrations were much higher in glycerol (2.5 h: 15.6 ± 7.8 mmol/L). Urinary glycerol increased rapidly (58 ± 71 microg/ml after 2.5 h) and was significantly higher than in placebo until 13.6 ± 0.9 h. In comparison with placebo administration, glycerol caused significantly greater changes in plasma volume and haemoglobin concentrations after 2.5 h. BW and urine production were significantly different between placebo and glycerol after 2.5 h and post-exercise. Despite exercise-induced increases in endogenous glycerol in the control group, urinary excretion remained well below the previously recommended threshold. In addition, exercise-related glycerol degradation did not appear to negatively affect the detection of exogenously administered glycerol [13353].

The use of glycerol in combination with excess fluid can be used to increase total body water. Because glycerol hyperhydration may also be misused to mask the effects of blood doping on doping-relevant parameters, namely haemoglobin and haematocrit, glycerol has been prohibited by the World Anti-Doping Agency since 2010. In order to test this rationale, the purpose of this meta-analysis was to quantify the effects of glycerol hyperhydration on plasma volume, haemoglobin, and haematocrit in comparison to administration of fluid only. Following a literature search, a total of seven studies was included and meta-analyses were performed separately for the effects on plasma volume (5 studies, total n=54) and on haemoglobin (6 studies, n=52) and haematocrit (6 studies, n=52). The meta-analysis revealed that the increase in plasma volume was 3 percent larger after glycerol administration when compared to fluid only. Reductions in haemoglobin were 0.2 g/dL larger and there was no difference in the changes in haematocrit between glycerol and fluid administration. In comparison with other plasma-volume expanding agents, glycerol hyperhydration has a very limited potential in increasing plasma volume and altering doping-relevant blood parameters [13354].

Glycerol is an endogenous substance that is on the World Anti-Doping Agency's list of prohibited threshold substances due to its potential use as a plasma volume expansion agent. The WADA has set the threshold for urine glycerol, including measurement uncertainty, at 1.3 mg/mL. Glycerol in circulation largely comes from metabolism of triglycerides in order to meet energy requirements and when the renal threshold is eclipsed, glycerol is excreted into urine. In part due to ethnic differences in postprandial triglyceride concentrations, we investigated urine glycerol concentrations in a population of elite athletes competing in North America and compared the results to those of athletes competing in Europe. 959 urine samples from elite athletes competing in North America collected for anti-doping purposes were analyzed for urine glycerol concentrations by a gas chromatography mass-spectrometry method. Samples were divided into groups according to: Timing (in- or out-of-competition), Class (strength, game, or endurance sports) and Gender. 333 (35 %) samples had undetectable amounts of glycerol (<1 microg/mL). 861 (90 %) of the samples had glycerol concentrations ≤20 microg/mL. The highest glycerol concentration observed was 652 microg/mL. Analysis of the data finds the effects of each category to be statistically significant. The largest estimate of the 99.9th percentile, from the in-competition, female, strength athlete samples, was 1813 microg/mL with a 95 % confidence range from 774 to 4251 μg/mL. This suggests a conservative threshold of 4.3 mg/mL, which would result in a reasonable detection window for urine samples collected in-competition for all genders and sport classes [13355].
Polyethylene glycol

Previous studies have found that increasing plasma viscosity as whole blood viscosity decrease has beneficial effects in microvascular hemodynamics. As the heart couples with systemic vascular network, changes in plasma and blood viscosity during hemodilution determine vascular pressure drop and flow rate, which influence cardiac function. One study aimed to investigate how changes in plasma viscosity affect on cardiac function during acute isovolemic hemodilution. Plasma viscosity was modulated by hemodilution of 40 percent of blood volume with three different plasma expanders (PEs). Dextran 2000 kDa (Dx2M, 6.3 cP) and dextran 70 kDa (Dx70, 3.0 cP) were used as high and moderate viscogenic PEs, respectively. Polyethylene glycol conjugated with human serum albumin (PEG-HSA, 2.2 cP) was used as low viscogenic PE. The cardiac function was assessed using a miniaturized pressure-volume conductance catheter. After hemodilution, pressure dropped to 84 percent, 79 percent, and 78 percent of baseline for Dx2M, Dx70 and PEG-HSA, respectively. Cardiac output markedly increased for Dx2M and PEG-HSA. Dx2M significantly produced higher stroke work relative to baseline and compared to Dx70. It was concluded that acute hemodilution with PEG-HSA without increasing plasma viscosity provided beneficial effects on cardiac function compared to Dx70, and similar to those measured with Dx2M. Potentially negative effects of increasing peripheral vascular resistance due to the increase in plasma viscosity were prevented [10359].

Newer plasma expanders

A variety of PVEs are constantly being modified or improved, aiming to obtain more efficient compounds and to avoid adverse effects evidenced in the past. For example, Voluven, a third-generation HES, has been improved owing to severe problems presented by the older generations. Research activities for development or improvement of new generation drugs will continue. Many interesting modified or new compounds have been developed, but several are still being submitted to animal or human trials. In the future they will probably undergo advanced clinical trials and soon after will be approved for clinical use. Among these compounds are: some polymerized Hb preparations; Hb vesicles; PEGconjugated albumin (a new-generation PVE that provides, according to preclinical studies, excellent microvascular and systemic recovery from hemorrhagic shock and extreme hemodilution); another albumin-based oxygen-carrying plasma expander, PEG-conjugated recombinant human serum ALB (HSA) incorporating the synthetic iron-porphyrin; many plant derived carbohydrates, such as alginates, inulin, carboxymethyl starch, pullulan and carboxymethylated Konjac glucomannan, a carboxymethylated polymer of mannose and glucose; and several other conjugated products. All of these are considered advisable as potential plasma expanders. Furthermore, using nanotechnology, nanoparticles (artificial red blood cells) are also being prepared for biomedical use, as oxygen carriers. In addition to the most used PVEs described, a few novel plasma expanders must be mentioned. Two new starches, carboxymethyl starch and carboxymethylated HES have been reported as presenting advantages over HES, with regard to their volume expansion effect and pharmacokinetic characteristics: although being considered as promising colloids, no precise statements were found about their potential use as PVEs. Another new-generation PVE, polyethylene glycol (PEG)-conjugated albumin, may provide, according to preclinical studies, the most extended and complete microvascular and systemic recovery from hemorrhagic shock, extreme hemodilution and endotoxemia. Moreover, it postpones the need of reestablishing intrinsic blood oxygen-carrying capacity to Hb concentrations lower than those associated with accepted transfusion triggers. Two other polysaccharides, alginate and inulin, have also been evaluated, being considered potential plasma expanders. Alginites are, a family of polysaccharides (mixture of manuronic and guluronic acid compounds) extracted from algae, are used extensively in biotechnology (e.g. agar). Produced by brown seaweed, they result in a high viscosity solution at a comparatively low concentration, with an extremely low oncotic
pressure. By mixing with a conventional plasma expander, such as Dextran 70, a specific viscosity may be obtained. Inulin, extracted from plant sources, may be crosslinked with different substances, resulting in compounds with high colloid osmotic pressure, strong expansion efficacy and no organ dysfunction, being therefore considered advisable as a potential plasma expander [11146].

**Intravenous rehydration**

Fluid is typically administered via intravenous (IV) infusion to athletes who develop clinical symptoms of heat illness, based on the perception that dehydration is a primary factor contributing to the condition. However, other athletes also voluntarily rehydrate with IV fluid as opposed to, or in conjunction with, oral rehydration. The voluntary use of IV fluids to accelerate rehydration in dehydrated, though otherwise healthy athletes, has recently been banned by the World Anti-Doping Agency. However, the technique remains appealing to many athletes. Given that it now violates the Anti-Doping Code, it is important to determine whether potential benefits of using this technique outweigh the risks involved. Several studies have shown that rehydration is more rapid with IV fluid. However, the benefits are generally transient and only small differences to markers of hydration status are seen when comparing IV and oral rehydration. Furthermore, several studies have shown improvements in cardiovascular function and thermoregulation with IV fluid, while others have indicated that oral fluid is superior. Subsequent exercise performance has not been improved to a greater extent with one technique over the other. The paucity of definitive findings is probably related to the small number of studies investigating these variables and the vast differences in the designs of studies that have been conducted. The major limitation of IV rehydration is that it bypasses oropharyngeal stimulation, which has an influence on factors such as thirst sensation, antidiuretic hormone (arginine vasopressin) release, cutaneous vasodilation and mean arterial pressure [10130].

**Hyponatremia**

The case histories were presented of four athletes taking part in a 95-mile ultra-endurance foot race in Scotland who were hospitalised after developing exercise-associated hyponatraemia and rhabdomyolysis. Exercise-associated hyponatraemia is relatively uncommon in temperate climates. Risk factors disposing to this disorder are discussed. Exercise-associated hyponatraemia is thought to be due to overconsumption of hypotonic fluid with other associated pathophysiology including an inability to suppress fully antidiuretic hormone during exercise or to mobilise adequate sodium from osmotically inactive internal stores. Non-specific symptoms make this disorder difficult to diagnose on site without the assistance of serum sodium measurement, but any delay in treatment of patients with encephalopathy can prove fatal. Mainstays of treatment include fluid restriction, hypertonic saline, loop diuretics and mannitol [09178].
CREATED HYPOXIA

The intermittent hypobaric hypoxia induced by specific facilities such as tents and rooms to simulate altitudes of up to 5500 m is an effective artificial technique to boost erythropoiesis, and is even more effective than natural exposure to the hypoxic environment of high altitudes (2800 m). This technique increases erythropoietin serum concentrations in trained athletes, although the main hematological parameters do not significantly differ from those of similarly trained athletes exposed to natural hypobaric hypoxia [06005].

In 1973, Sir Roger Bannister said that no clear proof of benefit of altitude training had emerged during a panel discussion on this topic, published in Br J Sports med. To date, most altitude training research is oriented towards individual endurance athletes, while the potential benefits for team sports remain largely unexplored. Hence, the safety and equality aspects of competitive football matches held above 2500 m have been passionately debated for over two decades. In 1993 this debate was invigorated when Brazil lost its first qualification game for a World championship in the stadium of La Paz (Bolivia), located at an altitude of 3600 m. Undoubtedly, the altered environment at altitude had a significant impact on players physical performances, and some athletes were better able to cope with the change in altitude than others, especially those who were better acclimated. Recently, the fact that Argentina suffered their worst loss in 60 years, a sound defeat of 6-1 against host Bolivia in a South Africa World Cup qualifier, clearly demonstrates that playing international games at altitude is a major challenge. Despite the apparent lack of strong scientific evidence, it is striking to observe that altitude-training centres have been established around the globe, and are now offering team sport players the opportunity to train under sport-specific hypoxic conditions. Forty years after the publication of the initial altitude training issue major advances have been made from a performance and mechanistic perspective. The three main points are [13324]:

- the current level of evidence for the efficacy of hypoxic methods to improve exercise performance at moderate or high altitude (acclimatisation) is well established. However, the benefits of using a “living high-training low”, “living high-training high” and “living low-training high” altitude training intervention or a combination of those methods to improve team sport-related physical performance on return to sea level are not as definitive
- training camps as short as 2 weeks can increase haemoglobin mass substantially in a range of professional team sport players, while limited data currently exists regarding the time course of non-haematological adaptations
- it is undeniable that no single recommendation is likely suitable for all players in a team, or across all team sports, requiring the development of optimised interventions at the individual player level.

Different ways of altitude training

In 2007, it was presented the main altitude/hypoxic training methods used by elite athletes: “live high – train high” (LHTH) and “live high – train low” (LHTL); sleeping at altitude to gain the haematological adaptations (increased erythrocyte volume) but training at sea level to maximise performance (maintenance of sea-level training intensity and oxygen flux). The LHTL method can be accomplished through a number of methods and devices:
natural/terrestrial altitude, nitrogen dilution, oxygen filtration and supplemental oxygen.

Another method is the “live low – train high” (LLTH) method including intermittent hypoxic exposure at rest (IHE) or during intermittent hypoxic training sessions (IHT). Noteworthy, all supporting references were conducted with endurance elite athletes (i.e., cyclists, triathletes, cross-country skiers, runners, swimmers, kayakers and rowers) and there is an extensive literature relative to LHTH as well as LHTL. However, there is a lack of evidence for the applicability of these methods in team-sport athletes. In recent times, media reports have provided us with coverage of some high-profile clubs or national squads in various team-sport disciplines undertaking fitness programmes at altitude during the early preseason or in preparation of a major competition. Despite the evident observation that athletes from different team sports and from all around the world are using altitude training more than ever before, it is stunning to note that to date there are only two hypoxic training studies that have been conducted with team-sport players. Therefore, there is an urgent need for mechanistic as well as applied studies investigating team-sport performance changes following hypoxic training in a sport-specific population before solid evidence-based recommendations can be definitely formulated. In parallel, over the last few years, an increasing interest for the practical application of altitude training in team sports – mainly in football but also in the rugby union or the Australian football league – was noted due to several reasons. First, there were controversies regarding the possibility of playing international football matches above 2500 m in the mountainous regions of South America, while guaranteeing players health and safety. Second, the FIFA 2010 Senior (South Africa) and 2011 U-20 (Columbia) World Cups held at altitude have highlighted the need for the squads to achieve optimal acclimatisation. Third, the development of new hypoxic devices (e.g., mobile inflatable hypoxic marquees) and methods (repeated sprints in hypoxia). This increased interest was translated by the organisation of international symposia on the topic by the leading sports organisation; for example, FIFA, symposium on playing football at altitude; International Olympic Committee, consensus statement on thermoregulatory and altitude challenges for all high-level athletes [13325].

Nomenclature

The intrinsic differences between factors underlying endurance (e.g., maximal aerobic speed, economy) and team-sport performance (e.g., repeated-sprint ability) as well as the constraints in the respective competition calendars explain why the aims and contents of the hypoxic methods and their periodisation in the yearly programme are largely different between individual and team sports. As such, we believe that the current nomenclature is probably not appropriate anymore for exploring the new boundaries of contemporary hypoxic methods offered to team-sport athletes. In 2010, we proposed to slightly modify the nomenclature by introducing the possibility of combining different hypoxic methods. New approaches include “IHE during interval-training” (IHT = IHT + IHE) and “live high-train low and high” (LHTLH = LHTL + IHT). Since more information became available on enhanced glycolysis and buffering capacity with IHT, it is discussed also the potential benefits of these hypoxic methods for anaerobic performance. Unfortunately, there is to date no expert consensus on how we should name the different hypoxic methods. IHT that should refer to interval training in hypoxia is also used for continuous low-intensity (<70 % VO2max) long duration (>30 min) exercise in hypoxia. Recently, it was also proposed a new hypoxic method (RSH, repeated sprint training in hypoxia) presumably based on different mechanisms than IHT. The time has come to update the current nomenclature since each method is likely based predominantly on different mechanisms; for example, increased oxidative capacity (CHT), buffering capacity (IHT) or compensatory fiber-selective vasodilatation (RSH). It is therefore suggested to divide the LLTH method in four subsets; that is, IHE, CHT (continuous >30 min low intensity training in hypoxia), IHT (interval-training in...
hypoxia) and RSH. Another point that deserves attention is the nature of altitude stress. With mounting evidence suggesting that hypobaric hypoxia induces different physiological adaptations than normobaric hypoxia, it is therefore believed that it is paramount to systematically report the method lowering ambient oxygen partial pressure [13325].

**Physiological mechanisms**

Altitude training has been used regularly for the past five decades by elite endurance athletes, with the goal of improving performance at sea level. The dominant paradigm is that the improved performance at sea level is due primarily to an accelerated erythropoietic response due to the reduced oxygen available at altitude, leading to an increase in red cell mass, maximal oxygen uptake, and competitive performance. Blood doping and exogenous use of erythropoietin demonstrate the unequivocal performance benefits of more red blood cells to an athlete, but it is perhaps revealing that long-term residence at high altitude does not increase hemoglobin concentration in Tibetans and Ethiopians compared with the polycythemia commonly observed in Andeans. This review also explores evidence of factors other than accelerated erythropoiesis that can contribute to improved athletic performance at sea level after living and/or training in natural or artificial hypoxia. It was described a range of studies that have demonstrated performance improvements after various forms of altitude exposures despite no increase in red cell mass. In addition, the multifactor cascade of responses induced by hypoxia includes angiogenesis, glucose transport, glycolysis, and pH regulation, each of which may partially explain improved endurance performance independent of a larger number of red blood cells. Specific beneficial non-hematological factors include improved muscle efficiency probably at a mitochondrial level, greater muscle buffering, and the ability to tolerate lactic acid production. Future research should examine both hematological and non-hematological mechanisms of adaptation to hypoxia that might enhance the performance of elite athletes at sea level [07137].

The purpose of hypoxic environments or other ergogenic aids that produce similar results is to increase aerobic exercise performance as demonstrated by improved performance in events such as distance running and cycling (e.g, le Tour de France). The mechanism by which hypoxic environments exert their effect is via changes in the partial pressure of oxygen, which leads to an increase in the body's hematocrit level. The decreased partial pressure of oxygen in the pulmonary capillaries results in a decrease in the percent oxyhemoglobin saturation. These changes increase the drive to breathe, thus increasing ventilation. The body senses these changes in partial pressure and increases the production of red blood cells (RBCs) as a result of the stimulus from the hormone, erythropoietin, which is secreted by the kidneys. The RBCs are rich in the oxygen-carrying protein, hemoglobin, which binds oxygen during transportation in the cardiorespiratory system. Thus, an increase in red blood cell mass (RBCM), increasing how much space in the blood is occupied by RBC, is the most important adaptation to occur through the use of a hypoxic environment. The greater the RBCM, the greater the oxygen-carrying capacity of the blood and, therefore, the aerobic potential of the system. RBCM may increase by about 9 percent during altitude training, which can translate into improvements in run performance of about 13.4 ± 10.0 s over 5000 m [07133].

**Cellular mechanisms**

The effects of concurrent hypoxic/endurance training on mitochondrial respiration in permeabilized fibers in trained athletes were investigated. Eighteen endurance athletes were divided into two training groups: normoxic (Nor, n=8) and hypoxic (H, n=10). Three weeks
(W1-W3) of endurance training (5 sessions of 1 h to 1 h and 30 min per week) were completed. All training sessions were performed under normoxic or hypoxic conditions (approximately 3,000 m) for Nor and H group, respectively, at the same relative intensity. Before and after the training period, an incremental test to exhaustion in normoxia was performed, muscle biopsy samples were taken from the vastus lateralis, and mitochondrial respiration in permeabilized fibers was measured. Peak power output (PPO) increased by 7.2 and 6.6 percent for Nor and H, respectively, whereas maximal \( \text{O}_2 \) uptake (\( \text{VO}_2\text{max} \)) remained unchanged for Nor and H, respectively, between pretraining (W0) and posttraining (W4). Maximal ADP-stimulated mitochondrial respiration significantly increased for glutamate + malate and significantly decreased for palmitate + malate in the H group. In contrast, no significant differences were found for the Nor group. The findings demonstrate that a 3-week training period increased the PPO at sea level without any changes in \( \text{VO}_2\text{max} \) and that a 3-week hypoxic exercise training seems to alter the intrinsic properties of mitochondrial function, i.e. substrate preference [07138].

Training at high altitude

High altitude training has become a mainstay in endurance sports, with live high-train low as the current protocol of choice. Athletes either live or sleep in artificial or natural hypoxic conditions with the aim to increase serum erythropoietin concentrations, which are thought to improve maximum oxygen uptake and thus exercise performance. Changes, however, are not very striking and only apparent in so-called responders, who are not a well-defined group and may be as little as 50 percent of the trained study population. Whereas some studies show minor improvement, others report no change or even worsening. Furthermore, the mechanisms behind the proposed beneficial changes remain obscure and are far from being proven. There is an evident lack of sufficiently powered randomized, double-blinded studies, with training protocols that are identical for all groups and groups that are indeed comparable. Several studies discriminate between responders and non-responders, without clearly assessing the characteristics of the so-called responders. Until this has been done, it remains unclear if such a group really exists and how these subjects are characterized. This, however, would be of immense value, so protocols could be tailored to athletes' needs. Taken together, the current literature on natural or artificial hypoxia somewhat documents improved performance at high but not low altitude [12266].

Altitude training is a well-established “natural” and legal technique to improve endurance performance at sea level. At higher altitudes, the relative oxygen content of the air is diminished; after a sufficient period of time, the body responds with a complex series of biological and metabolic changes. An understanding of the metabolic adjustment to altitude requires a review of human acclimatization and adaptation. The term acclimatization is suited to relatively short exposures to altitude, such as short periods in training camps, while adaptation refers to changes occurring over generations under constant exposure. Some, but not all, of the main features of acclimatization and adaptation are similar. Acclimatization is basically heterogeneous, depending upon the type of environmental stress to which the athlete is exposed. Passive stresses, such as altitude and climate, are persistent and substantially uniform over time, whereas active ones like training regimen and diet are more variable and possibly mutable. Finally, compliance to excessive stress is highly sensitive to individual characteristics. The duration of exposure to passive stress modifies the nature and resiliency of changes following stress removal. After exposure to moderate altitude, some initial changes occur in response to the changed conditions; as the exposure duration increases, the repertoire of changes expands and stabilizes. Therefore, the longer the body is in a fully acclimatized condition, the more habitual the changes become. Upon exposure to a passive stress, the body undergoes a hierarchy of responsive changes and eventually
becomes fully acclimatized to the point that the changes become constant as well as permanent while residing in the environment. Full acclimatization is compromised by shorter periods of exposure; generally, the shorter the time spent at altitude, the less dramatic is the acclimatization and the changes that do occur are quite transient [10363].

To determine the time course of hemoglobin mass (Hb$_{mass}$) to natural altitude training, Hb$_{mass}$, erythropoietin (EPO), reticulocytes, ferritin and soluble transferrin receptor (sTfR) were measured in 13 elite cyclists during, and 10 days after, 3 weeks of sea level (n=5) or altitude (n=8, 2760 m) training. Mean Hb$_{mass}$, with a typical error of approximately 2 percent, increased during the first 11 days at altitude (2.9 ± 2.0 %) and was 3.5 ± 2.5 percent higher than baseline after 19 days. The concentration of EPO increased 64 ± 19 percent after 2 nights at altitude but was not different from baseline after 12 nights. Hb$_{mass}$ and [EPO] did not increase in sea level. Reticulocytes (%) were slightly elevated in altitude at days 5 and 12, sTfR was elevated at day 12, but both returned to baseline by day 20. Hb$_{mass}$ and [EPO] decreased on descent to sea level while ferritin increased. The mean increase in Hb$_{mass}$ observed after 11 days (approximately 300 h) of altitude training was beyond the measurement error and consistent with the mean increase after 300 h of simulated live high:train low altitude. The results suggest that in elite cyclists, hemoglobin mass increases progressively with 3 weeks of natural altitude exposure, with greater increases expected as exposure persists [10108].

Several physiologic changes occur following acclimatization at high altitude. They can be divided into immediate (taking place over a few days) and long-term changes, which require weeks to a few months. Immediate changes include decrease in maximum cardiac output, decreased maximum heart rate, increased erythropoiesis, increased excretion of base via the kidneys to restore acid-base balance, and an increase in the number of mitochondria and oxidative enzymes in RBCs that allow a more efficient unloading of oxygen to peripheral tissues. The combination of altitude and time necessary to achieve significant athletic benefits, lasting 2 to 3 weeks after return from altitude, has been conventionally established at 2200 m for 4 weeks. This convention is supported by the practical evidence that a significant (77 to 92 %) and stable (over 24 h) increase in Epo production occurs rapidly (within 6 h), once a threshold altitude of 2100 to 2500 m is reached. Below these altitudes, Epo increases are modest (24 to 30 %) and unsteady, reaching a peak at 6 h after exposure. Accordingly, 4 weeks of training at an altitude of 1740 m produce no changes in hemoglobin and a negligible increase in maximum oxygen consumption in highly trained athletes. The mechanism of the greater hematologic response to high altitude includes improved oxyhemoglobin desaturation that usually occurs as the oxygen partial pressure (pO$_2$) falls to the steep portion of the oxyhemoglobin dissociation curve. Hypothetically, the higher the altitude, the greater the ergogenic benefits achieved. However, an upper limit of altitude should be set to limit the side effects of altitude exposure, such as chronic mountain sickness with depression, headache, loss of appetite, sickness, and sleeplessness. The concentration of Epo in blood starts to increase from the first day of living at an altitude of 2500 m; by 2 weeks it stabilizes and after 4 weeks returns to the baseline. Therefore, a period lasting not less than 3 to 4 weeks appears most appropriate. Like astronauts who enter microgravity, people acclimatized to high altitude who descend to sea level undergo a rapid adaptive mechanism that modifies the excessive blood volume and RBC mass for the new environment. This physiologic process, called neocytolysis, results in selective hemolysis of young circulating erythrocytes. While both RBC production and survival remain normal, the RBC mass decreases up to 15 percent over a few days. Serum Epo levels are also profoundly suppressed on descent. Therefore, Epo dynamically suppresses erythropoiesis by initiating neocytolysis when serum levels are reduced below a nadir threshold [10353].

A review of the current literature supports the benefits of altitude training, though there is a
large variation in outcomes, possibly due to the heterogeneity of settings and periods of living at altitude or to the effects of active stresses and confounding variables (athletic discipline, training regimen, diet). Since RBCs carry oxygen through the bloodstream, a substantial increase in the packed cells following altitude exposure allows more efficient oxygen delivery to the muscles at sea level, reducing fatigue and giving the athlete an edge. With acclimatization, there is convincing evidence of decreased production or increased clearance of lactate in muscles, moderate evidence of enhanced muscle buffering capacity and tenuous evidence of improved mechanical efficiency of cycling. Acclimatization to high altitude induces further central and peripheral adaptations that improve oxygen delivery and utilization. Moreover, hypoxic exercise may increase the training stimulus, thus magnifying the effects of endurance training. Several strategies have been proposed to optimize the acclimatization changes: live high and train high, live high and train low, and intermittent exposure. The current literature indicates that continuous living and training at moderate altitude does not improve sea-level performance of high-level athletes. Although oxygen delivery and utilization may be slightly improved, a lack of adequate training adaptation and/or decreased exercise intensity due to hypoxia can lead to a relative detraining effect that may overwhelm any advantage gained through altitude-induced acclimatization.

Therefore, acclimatization to a moderately high altitude, accompanied by training at low altitude (the so-called “living high-training low” theory), is likely the most effective variant of altitude training to improve sea-level endurance performance. Such improvement is due to a wide series of physiological and biochemical adaptations, including a consistent improvement in maximal oxygen uptake, a rise of circulating Epo, the levels of which can be nearly double those of the initial sea-level baseline, and an increase of up to 10 g/l of hemoglobin concentration in blood. Despite promising results, some later investigations described wide interindividual variability in adaptive response and athletic performance after a traditional altitude training camp. Although the concentration of Epo in plasma rises significantly, the aggregate variation is definitely broad, ranging from 41 to 400 percent, and responders had a significantly larger and more persistent increase in absolute terms and when expressed as a percentage of sea-level baseline. Taken together, current evidence suggests that short-term intermittent exposure to moderate hypoxia and hypobaric chambers may not be sufficient to improve aerobic capacity and to induce altitude acclimatization, whereas longer periods, up to 4 weeks, may be effective in eliciting hematologic modifications and improvement in endurance performance [10353].

In general, the efficacy of simulated altitude is achieved by an increased erythropoietic response and improved performance in athletes of endurance sport disciplines. Although earlier evidence in untrained subjects was promising, it is as yet questionable whether elite athletes, who may be closer to the maximal structural and functional adaptive capacity of the respiratory system, may achieve real gains from simulated altitude devices. Most clinical discrepancies are due to the marked heterogeneity in exercise, environmental conditions, nutritional therapies, duration, repetition rate, and intensity of the artificial hypoxic stimulus. If a distinct advantage is needed in endurance events, the use of high-altitude simulators would appear less risky than other forms of blood doping. However, there are some ethical and clinical concerns among coaches, athletes, and the scientific community that the use of these expedients may be unsafe and unethical for use in sports. Just as training preparation that gives an athlete a technological or athletic advantage that is too expensive or innovative for most other competitors to use is considered unfair, simulated altitude may currently be considered unethical. Although this position has been questioned, as the biological changes induced by altitude simulation are almost indistinguishable from those of real exposure and training at altitude, simulated altitude, called “holistic blood doping,” ultimately reproduces most of the physiological and pathological effects of rHuEpo and is now considered an illegal means of performance improvement. Finally, it is recognized that some athletes experience considerable reduction in the ability to train at the appropriate intensity...
A factor of importance to the outcome of an altitude-training programme is the training undertaken during the intervention period. The severity of altitude, time spent training at altitude, history of altitude training and timing of training leading into competition represent important factors to consider when designing a training programme at altitude. A considerable interindividual variability in the reduction of aerobic power at altitude exists, and this should be considered. Consequently, individual adjustments of training intensity and periodisation of training at altitude are required to avoid over-reaching and/or detraining. The proposed actions to individualise the ‘altitude dose’ and training content should include daily assessment of sleep quality, mood state and frequent monitoring of the changes in HR-derived measures. Training load during the altitude sojourn should also be carefully monitored. Ideally, this can be achieved by quantifying the duration and the intensity (CR-10 Borg scale) for each training session. Although monitoring perceived training load and wellness using psychometric and the Lake Louise acute mountain sickness questionnaires are also useful to help prevent the risk of negative adaptations. Careful daily monitoring of indirect measures of cardiac autonomic activity such as heart rate variability or heart rate responses, together with ratings of perceived exertion (RPE) responses to a submaximal run (e.g. 4-8 min at 10-12 km/h over 20-40 m shuttles) can help predict/prevent sickness and maintain the training process. In addition to the higher physiological stress, some critical aspects of sport-specific decision-making processes together with skill execution (short-passing ability) and perceived well-being are likely to be negatively affected by acute moderate altitude exposure as a result of exacerbated fatigue levels. When 28 international male football players belonging to the English national squad were tested in preparation of the 2010 FIFA World Cup, exposure to a simulated altitude of 1800 m compromised their ability to sustain work output during 10 min of constant-load cycling at 85 percent of maximum heart rate, and was also associated with higher RPE values. Cognitive function (as measured during the last 5 min of the 10 min constant-load test) was also impaired by acute altitude exposure with a 9 percent reduction in simple reaction time. As such, careful monitoring of decision-making responses (i.e., ideally assessed daily in the initial stages during a hypoxic intervention) undoubtedly has merit [13327].

Team sports are increasingly popular, with millions of participants worldwide. Athletes engaged in these sports are required to repeatedly produce skilful actions and maximal or near-maximal efforts (e.g., accelerations, changes in pace and direction, sprints, jumps and kicks), interspersed with brief recovery intervals (consisting of rest or low-intensity to moderate-intensity activity), over an extended period of time (1–2 h). While performance in most team sports is dominated by technical and tactical proficiencies, successful team-sport athletes must also have highly-developed, specific, physical capacities. Much effort goes into designing training programmes to improve these physical capacities, with expected benefits for team-sport performance. Recently, some team sports have introduced altitude training in the belief that it can further enhance team-sport physical performance. Until now, however, there is little published evidence showing improved team-sport performance following altitude training, despite the often considerable expense involved. In the absence of such studies, this review will identify important determinants of team-sport physical performance that may be improved by altitude training, with potential benefits for team-sport performance. These determinants can be broadly described as factors that enhance either sprint performance or the ability to recover from maximal or near-maximal efforts. There is some evidence that some of these physical capacities may be enhanced by altitude training, but further research is required to verify that these adaptations occur, that they are greater than what could be achieved by appropriate sea-level training and that they translate to improved team-sport performance [13331].
The internationalism of field-based team sports (TS) such as football and rugby requires teams to compete in tournaments held at low to moderate altitude (1200–2500 m). In TS, acceleration, speed and aerobic endurance are physical characteristics associated with ball possession and, ultimately, scoring. While these qualities are affected by the development of neuromuscular fatigue at sea level, arterial hypoxaemia induced by exposure to altitude may further hinder the capacity to perform consecutive accelerations (CAC) or sprint endurance and thereby change the outcome of a match. The higher the altitude, the more severe the hypoxaemia, and thus, the larger the expected decline in aerobic endurance, CAC and match running performance. Therefore, it is critical for athletes and coaches to understand how arterial hypoxaemia affects aerobic endurance and CAC and the magnitude of decline they may face at altitude for optimal preparation and increased chances of success. One mini review summarises the effects of acute altitude/hypoxia exposure on aerobic endurance, CAC and activity profiles of TS athletes performing in the laboratory and during matches at natural altitude, and analyses the latest findings about the consequences of arterial hypoxaemia on the relationship between peripheral perturbations, neural adjustments and performance during repeated sprints or CAC. Finally, it was briefly discussed how altitude training can potentially help athletes prepare for competition at altitude [13332].

Over the past two decades, intermittent hypoxic training (IHT), that is, a method where athletes live at or near sea level but train under hypoxic conditions, has gained unprecedented popularity. By adding the stress of hypoxia during “aerobic” or “anaerobic” interval training, it is believed that IHT would potentiate greater performance improvements compared to similar training at sea level. A thorough analysis of studies including IHT, however, leads to strikingly poor benefits for sea-level performance improvement, compared to the same training method performed in normoxia. Despite the positive molecular adaptations observed after various IHT modalities, the characteristics of optimal training stimulus in hypoxia are still unclear and their functional translation in terms of whole-body performance enhancement is minimal. To overcome some of the inherent limitations of IHT (lower training stimulus due to hypoxia), recent studies have successfully investigated a new training method based on the repetition of short (<30 s) “all-out” sprints with incomplete recoveries in hypoxia, the so-called repeated sprint training in hypoxia (RSH). The aims of one review are therefore threefold: first, to summarise the main mechanisms for interval training and repeated sprint training in normoxia. Second, to critically analyse the results of the studies involving high-intensity exercises performed in hypoxia for sea-level performance enhancement by differentiating IHT and RSH. Third, to discuss the potential mechanisms underpinning the effectiveness of those methods, and their inherent limitations, along with the new research avenues surrounding this topic [13333].

To examine with a parallel group study design the performance and physiological responses to a 14-day off-season ‘live high-train low in the heat’ training camp in elite football players 17 professional Australian Rules Football players participated in outdoor football-specific skills (32 ± 1°C, 11.5 h) and indoor strength (23 ± 1°C, 9.3 h) sessions and slept (12 nights) and cycled indoors (4.3 h) in either normal air (NORM, n=8) or normobaric hypoxia (14 ± 1 h/day, FiO\textsubscript{2} 15.2–14.3 %, corresponding to a simulated altitude of 2500–3000 m, hypoxic (HYP), n=9). They completed the Yo-Yo Intermittent Recovery level 2 (Yo-YoIR2) in temperate conditions (23 ± 1°C, normal air) precamp (Pre) and postcamp (Post). Plasma volume (PV) and haemoglobin mass (Hb\textsubscript{mass}) were measured at similar times and 4 weeks postcamp (4WPost). Sweat sodium concentration [\textsuperscript{+}Na\textsubscript{sweat}] was measured Pre and Post during a heat-response test (44°C). Both groups showed very large improvements in Yo-YoIR2 at Post (+44 %), with no between-group differences in the changes. Postcamp, large changes in PV (+5.6 %) and [\textsuperscript{+}Na\textsubscript{sweat}] (+29 %) were observed in both groups, while Hb\textsubscript{mass} only moderately increased in HYP (+2.6 %). At 4WPost, there was a likely slightly greater increase in Hb\textsubscript{mass} (+4.6 %) and PV (+6 %) in HYP than in NORM. It was concluded that the combination of
heat and hypoxic exposure during sleep/training might offer a promising 'conditioning cocktail' in team sports [13334].

Water polo requires high aerobic power to meet the demands of match play. Live high:train low (LHTL) may enhance aerobic capacity at sea level. Before the Olympics, the Australian women's water polo team utilised LHTL in an attempt to enhance aerobic fitness. Over 6 months, 11 players completed three normobaric LHTL exposures (block 1:11 days at 3000 m; block 2+3:9 days at 2500 m, 11 days normoxia, 10 days at 2800 m). Haemoglobin mass (Hbmass) was measured through carbon monoxide-rebreathing. Before each block, the relationship between Hbmass and water polo-specific aerobic fitness was investigated using the Multistage Shuttle Swim Test (MSST). Effect size statistics were adopted with likely, highly likely and almost certainly results being >75, >95, >99 percent, respectively. A Pearson product moment correlation was used to characterise the association between pooled data of Hbmass and MSST. Hbmass (pre 721 ± 66 g) likely increased after block 1 and almost certainly after block 2+3 (% change: block 1: 3.7 %; block 2+3: 4.5 %) and the net effect was almost certainly higher after block 2+3 than before block 1 (pre) by 8.5 percent. There was a very large correlation between Hbmass (g/kg) and MSST score. It was concluded that LHTL exposures of <2 weeks induced approximately 4% increase in Hbmass of water polo players. Extra Hbmass may increase aerobic power, but since match performance is nuanced by many factors it is impossible to ascertain whether the increased Hbmass contributed to Australia's Bronze medal [13335].

Repeated sprint ability (RSA) is a critical success factor for intermittent sport performance. Repeated sprint training has been shown to improve RSA, it was hypothesised that hypoxia would augment these training adaptations. Thirty male well-trained academy rugby union and rugby league players participated in this single-blind repeated sprint training study. Participants completed 12 sessions of repeated sprint training (10×6 s, 30 s recovery) over 4 weeks in either hypoxia (13 % F\textsubscript{O}\textsubscript{2}) or normoxia (21 % F\textsubscript{O}\textsubscript{2}). Pretraining and post-training, participants completed sports specific endurance and sprint field tests and a 10×6 s RSA test on a non-motorised treadmill while measuring speed, heart rate, capillary blood lactate, muscle and cerebral deoxygenation and respiratory measures. Yo-Yo Intermittent Recovery Level 1 test performance improved after RS training in both groups, but gains were significantly greater in the hypoxic (33 ± 12 %) than the normoxic group (14 ± 10 %). During the 10×6 s RS test there was a tendency for greater increases in oxygen consumption in the hypoxic group (hypoxic 7 ± 9 %, normoxic (~0.3 ± 8.8 %) and reductions in cerebral deoxygenation after hypoxic than normoxic training. Twelve RS training sessions in hypoxia resulted in twofold greater improvements in capacity to perform repeated aerobic high intensity workout than an equivalent normoxic training. Performance gains are evident in the short term (4 weeks), a period similar to a preseason training block [13336].

It was described the 3-year process underpinning a multinational collaboration to investigate soccer played at high altitude – La Paz, Bolivia (3600 m). There were two main aims: first, to quantify the extent to which running performance would be altered at 3600 m compared with near sea level; and second, to characterise the time course of acclimatisation of running performance and underlying physiology to training and playing at 3600 m. In addition, this project was able to measure the physiological changes and the effect on running performance of altitude-adapted soccer players from 3600 m playing at low altitude. A U20 Bolivian team (n=19) played a series of five games against a U17 team from sea level in Australia Town=n=20). 2 games were played near sea level (Santa Cruz 430 m) over 5 days and then three games were played in La Paz over the next 12 days. Measures were (1) game and training running performance – including global positioning system (GPS) data on distance travelled and velocity of movement; (2) blood – including haemoglobin mass, blood
volume, blood gases and acid–base status; (3) acclimatisation – including resting heart rate variability, perceived altitude sickness, as well as heart rate and perceived exertion responses to a submaximal running test; and (4) sleep patterns. It was concluded that pivotal to the success of the project were the strong professional networks of the collaborators, with most exceeding 10 years, the links of several of the researchers to soccer federations, as well as the interest and support of the two head coaches [13337].

Altitude training is used by elite athletes to improve sports performance, but it may also disrupt sleep. The aim of one study was to examine the effects of 2 weeks at high altitude on the sleep of young elite athletes. Participants (n=10) were members of the Australian under-17 soccer team on an 18-day (19-night) training camp in Bolivia, with six nights at near sea level in Santa Cruz (430 m) and 13 nights at high altitude in La Paz (3600 m). Sleep was monitored using polysomnography during a baseline night at 430 m and three nights at 3600 m (immediately after ascent, 1 week after ascent and 2 weeks after ascent). Data were analysed using effect size statistics. All results are reported as comparisons with baseline. Rapid eye movement (REM) sleep was likely lower immediately upon ascent to altitude, possibly lower after 1 week and similar after 2 weeks. On all three nights at altitude, hypopneas and desaturations were almost certainly higher; oxygen saturation was almost certainly lower; and central apnoeas, respiratory arousals and periodic breathing were very likely higher. The effects on REM sleep were common to all but one participant, but the effects on breathing were specific to only half the participants. It was concluded that the immediate effects of terrestrial altitude of 3600 m are to reduce the amount of REM sleep obtained by young elite athletes, and to cause 50 percent of them to have impaired breathing during sleep. REM sleep returns to normal after 2 weeks at altitude, but impaired breathing does not improve [13338].

Recovery

During an altitude sojourn the whole squad should be carefully monitored to try to avoid over-reaching, dehydration or upper respiratory tract infection, taking into account that hypoxia may impact on sleep quality/quantity and therefore player recovery. Avoiding illness is not always possible; however, by allowing adequate rest (first 1-2 days) and easing into training at altitude (following 2–3 days) before taking up regular training a player's immune system is not placed under excessive stress from both hypoxia and hard training. Higher heart rates and lower SpO2 values reflect the inadequate ability of a player to adjust to the hypoxic environment. Practically, we encourage monitoring a range of haematological and immune function parameters including iron status, vitamins, oxidative stress, as well as self-reported wellness and session RPE, before leaving for altitude, particularly during the early phase of chronic altitude training (within the first 2 days of ascent/exposure) and if possible every week thereafter. As training sessions in hypoxia increase the use of carbohydrates during exercise, appropriate nutrition is important. Reportedly, a high-CHO meal consumed prior to moderate exercise in hypoxic conditions reduced oxygen desaturation compared with a high-protein meal. Further, football players competing in the 2008 European Championships (Switzerland-Austria with venues elevation <600 m) experienced a decrease in extracellular and body mass (indicating fluid loss), which may be caused by a loss of appetite, dehydration or a change in energy balance (energy expenditure or food availability). In team-sport players, a diet high in carbohydrate could therefore improve tolerance to intense and stressful hypoxic training sessions, which is important when looking at increasing sport-specific fitness. At altitude, respiratory alkalosis during the first few days of exposure initiates a chronic loss of bicarbonate, which may be restored in order to help effectively buffer acidosis during high-intensity exercise and thereby maximise the potential for interval-training quality. Dehydration is common at altitude (diuresis) and may also be caused by sweating and fluid loss through the upper airways (low humidity) due to increased ventilation to defend
the immediate fall in SpO2 due to the reduction in the partial pressure of oxygen at altitude. Because the combination of hypoxia and a strenuous training programme could lead to the development of a chronic state of hypohydration, checking hydration status and electrolyte balance before, during and after training or the game is recommended. Practically, quantifying urine osmolality (>700 mOsm/kg), urine specific gravity (>1.020 g/mL) and/or body mass change (>2% body mass loss from water deficit) can be used as index of dehydration. Because sweat rates and sweat electrolyte content vary greatly among individuals, the development of individualised fluid and electrolyte replacement strategies is required for the preservation of performance and protection of player health. Global sleep quality (number of arousals and awakenings) can ideally be monitored using polysomnography, but alternatives including actigraphy, sleep questionnaires and other sleep monitoring devices are available in situations where this tool is not practicable [13339].

To examine the time course of wellness, fatigue and performance during an altitude training camp (La Paz, 3600 m) in two groups of either sea-level (Australian) or altitude (Bolivian) native young soccer players. Wellness and fatigue were assessed using questionnaires and resting heart rate (HR) and HR variability. Physical performance was assessed using HR responses to a submaximal run, a Yo-Yo Intermittent recovery test level 1 (Yo-YoIR1) and a 20 m sprint. Most measures were performed daily, with the exception of Yo-YoIR1 and 20 m sprints, which were performed near sea level and on days 3 and 10 at altitude. Compared with near sea level, Australians had moderate-to-large impairments in wellness and Yo-YoIR1 relative to the Bolivians on arrival at altitude. The acclimatisation of most measures to altitude was substantially slower in Australians than Bolivians, with only Bolivians reaching near sea-level baseline high-intensity running by the end of the camp. Both teams had moderately impaired 20 m sprinting at the end of the camp. Exercise HR had large associations with changes in Yo-YoIR1 in both groups. Thus, despite partial physiological and perceptual acclimatisation, 2 weeks is insufficient for restoration of physical performance in young sea-level native soccer players. Because of the possible decrement in 20 m sprint time, a greater emphasis on speed training may be required during and after altitude training. The specific time course of restoration for each variable suggests that they measure different aspects of acclimatisation to 3600 m; they should therefore be used in combination to assess adaptation to altitude [13339].

Performance in athletic activities that include a significant aerobic component at mild or moderate altitudes shows a large individual variation. Physiologically, a large portion of the negative effect of altitude on exercise performance can be traced to limitations of oxygen diffusion, either at the level of the alveoli or the muscle microvasculature. In the lung, the ability to maintain arterial oxyhaemoglobin saturation (SaO2) appears to be a primary factor, ultimately influencing oxygen delivery to the periphery. SaO2 in hypoxia can be defended by increasing ventilatory drive; however, during heavy exercise, many athletes demonstrate limitations to expiratory flow and are unable to increase ventilation in hypoxia. Additionally, increasing ventilatory work in hypoxia may actually be negative for performance, if dyspnoea increases or muscle blood flow is reduced secondary to an increased sympathetic outflow (e.g. the muscle metaboreflex response). Taken together, some athletes are clearly more negatively affected during exercise in hypoxia than other athletes. With careful screening, it may be possible to develop a protocol for determining which athletes may be the most negatively affected during competition and/or training at altitude [13340].

What recommendations to overcome some of the limitations of the current studies? It was commonly agreed that the current level of evidence for the efficacy of hypoxic methods to improve acclimatisation at moderate or high altitude is well established, but rather low when it comes to improved sea level exercise performance. Part of this inconsistency is linked to the fact that various hypoxic methods (hypobaric vs normobaric
hypoxia), training modalities or training states of the players have been employed within as well as between studies with discrepancies between measuring methods frequently seen (e.g. Hbmass). In addition, performance changes resulting from altitude training are not that reproducible even when the mean improvements in underlying physiology are more consistent. Furthermore, the ability to detect a relatively small signal is swamped by the noise of the range of factors that can impinge on individual performance, let alone that of a team. Important methodological limitations of some of the current literature also include uncontrolled trials, non-randomised study protocols and neither single-blinded nor double-blinded designs. Lack of blinding in interventions, leading to expectation (placebo and nocebo) effects, should be avoided wherever possible (ie, double-blinding natural LHTH studies is impossible), especially in a population of team-sport players where team connection has a widespread effect on performance. Future studies should avoid methodological shortcomings such as absence of lead-in period, undefined training cycle or players' recent training history. Such trial specifications are standard in many other exercise physiology fields and need to be adhered to in the area of altitude-training research in order to move this growing field forward. In addition, it would be worth recording through questionnaire the players' belief in the efficacy of altitude training before they go to the camp to further clarify if expectation is any way associated with benefit. When possible it is also encouraged to use double baseline measures, and carefully documenting training content/load before, during and after the altitude-training intervention will allow a more systematic comparison of various hypoxic methods. Performance changes should not only be monitored shortly (ie, few days) after the intervention but also for few weeks after the last day of exposure to distinguish the short from middle/long-term (or delayed) effects. Ideally, players would need to be accustomed to performing similar (if not identical) performance tests as part of their usual battery of team fitness testing in order to facilitate this process. It is also important to report eventual dropouts, which indirectly reflect how players coped with the altitude intervention. Further, the level of adherence of the players to the intervention must be measured: “How do the players think the intervention worked for them?” Finally, there is a need for consensus between practitioners and researchers to define what difference in magnitude, in terms of peak sprint or maximal aerobic velocities for instance, after any altitude-training intervention can realistically be considered a meaningful ‘improvement’ (ie, greater than the ‘smallest worthwhile change’ or the typical error of measurement) relevant for competitive team-sport performance. In this context, developing a long history of standardised performance tests is important to obtain an indication of each player’s sensitivity to a given altitude-training intervention. Only then meaningful recommendations for team-sport players could be derived. Some of the key research gaps in the field of altitude-training methods relevant for team sports can be addressed [13327]:

- determining whether performance and physiological changes induced by altitude-training protocols are actually transferred to competitive match outcomes: how to accurately measure these effects?
- verifying the usefulness of new hypoxic training methods (e.g. live high and train low and high interspersed, repeated sprints in hypoxia, live high – train low under heat stress or altitude training combined with blood occlusion) in a range of professional team sports, to determine whether the capacities meant to be improved actually are.
- evaluating the combination of altitude-training methods and the effect that they have on the magnitude and time course of several aspects of match-related performance and adaptive responses during isolated or periods of intensified competition
- validating the efficacy of LLTH methods when attempting to improve sea-level performance, preacclimation, prevention of detraining during off-season/injury periods or to prolong the beneficial effects of an extended altitude-training block
- determining whether the breathing abnormalities that occur during sleep at high altitude by many players, and the accompanying sleep fragmentation, affect the efficacy of altitude training
- clarifying how increased oxygen delivery/utilisation conferred by hypoxic interventions improves match-related performance, prevents premature and excessive neuromuscular fatigue and improves recovery processes in team-sport-related activities
- determining under which circumstances altitude exposure can be used either as a substitute to reduce the inevitable detraining effect seen in long-term injured players or to further stimulate the cardiovascular and metabolic systems, while keeping training load lower than at sea level
- clarifying some of the jet lag-related methodological (circadian rhythms) issues, and establish whether teams should train or be tested at the new destination or at the origin time, also taking into account the delay before competition and the details of the altitude stress
- understanding whether the cellular and molecular basis of hypoxic adaptations (downstream targets of HIF-1alpha) differ between the various altitude-training interventions, and the impact of titrated “hypoxic doses”
- hedging more light on putative adaptive mechanisms (eg, running economy, lactate metabolism and muscle/blood buffer capacity along with compensatory vasodilation associated to reduced oxygen content and potential on fibres behaviour and fatigability) and signalling pathways (e.g. mitochondrial efficiency and biogenesis, capillarisation and sodium/potassium handling) of non-haematological adaptations important for team-sport physical performance
- identifying physiological (with a particular emphasise on genetic and ventilatory responses) and psychosociological factors of primary influences affecting individual player responses to hypoxic training
- quantifying the extent of biomechanical/skill-based adaptations associated with hypobaric hypoxia, and the optimal dosing and timing of those aerodynamics and neuromuscular control adjustments in regards to physiological ones
- measuring the magnitude and rate of changes and the underpinning physiological (Hbmass, oxygen cost of breathing) and biomechanical (neural activation strategies, kinetic/kinematic adjustments) adaptations when altitude-resident players descend from altitude and when sea-level players live high and train low
- investigating if hypobaric and normobaric hypoxia hold the same potential for improvement in match-specific fitness and share similar underlying physiological mechanisms, and therefore determining whether they can be used interchangeably.

**Effects on sleep**

Sleeping at moderate altitude does not cause major disruption to players’ sleep in general, but it does cause minor to moderate disruption to rapid eye movement sleep, which is important for mental recovery. These symptoms, present in about 25 percent of the players in a team when sleeping at moderate altitude, should improve over 2–3 nights. Depending on the altitude and the individual, sleep disturbance can be caused by periodic breathing resulting from the interplay between hypercapnia and hypoxia leading to central sleep apnoea. Nearly 40-50 percent of the members of a team may experience moderate/severe disordered breathing at high altitude (3600 m). This disruption is unavoidable in terrestrial altitude, but it could be avoided in simulated altitude with the use of a ‘rest high, sleep low, train low’ paradigm for affected individuals. If this new paradigm is used, the potential benefit associated with avoiding disordered breathing during sleep should be considered against the potential cost of spending less time at altitude. Finally, when considering the effects of altitude training on sleep, it is also important to consider any potential effects of travel fatigue.
Altitude exposure causes acute sleep disruption in non-athletes, but little is known about its effects in elite athletes. The aim of one study was to examine the effects of altitude on two groups of elite athletes, that is, sea-level natives and high-altitude natives. Sea-level natives were members of the Australian under-17 soccer team (n=14). High-altitude natives were members of a Bolivian under-20 club team (n=12). Teams participated in an 18-day (19 nights) training camp in Bolivia, with 6 nights at near sea level in Santa Cruz (430 m) and 13 nights at high altitude in La Paz (3600 m). Sleep was assessed on every day/night using activity monitors. The Australians’ sleep was shorter, and of poorer quality, on the first night at altitude compared with sea level. Sleep quality returned to normal by the end of the first week at altitude, but sleep quantity had still not stabilised at its normal level after 2 weeks. The quantity and quality of sleep obtained by the Bolivians was similar, or greater, on all nights at altitude compared with sea level. The Australians tended to obtain more sleep than the Bolivians at sea level and altitude, but the quality of the Bolivians’ sleep tended to be better than that of the Australians at altitude. It was concluded that exposure to high altitude causes acute and chronic disruption to the sleep of elite athletes who are sea-level natives, but it does not affect the sleep of elite athletes who are high-altitude natives.

Hypoxic tents

Hypoxic tents (chambers) function in the same fashion as that of altitude in the live high – train low approach. The athlete sleeps in the hypoxic tent to simulate sleeping at altitude and then trains during the day at or near sea level in normoxia, which allows the maintenance of training intensity. A study using the live high – train low strategy, but incorporating an enriched nitrogen tent to simulate altitude, showed that hypoxic exposure increases muscle buffering capacity and reduces VO₂ for a given submaximal workload during sea-level exercise, suggesting improved exercise efficiency. These improvements are similar to the live high – train low altitude studies. However, two of the benefits of using a hypoxic tent (artificial altitude) are reduced daily travel time to and from altitude and reduced cost of travel to an altitude-training camp if not already in proximity. Much of the debate on the potential banning of hypoxic environments has focused primarily on the use of artificially-produced hypoxic environments and the passive mode of operation, such as hypoxic tents (nitrogen enriched) and not altitude training per se. However, there are many progressive arguments for the use of hypoxic tents. To summarize, the empirical evidence strongly suggests that hypoxic tents can enhance performance and are safe if used as directed.

There is actual debate on a position of the World Anti-Doping Agency (WADA), which has cautiously refrained from banning hypoxic tents (written in 2007) and intends to monitor their health risk. Regardless of teleological and deontological concepts, it was highlight that the health risks inherent to the widespread use of these artificial performance-enhancing devices would make them as unsafe as other forms of blood doping.

As altitude training has some logistical and biological disadvantages, scientists have investigated alternative techniques of simulating altitude exposure which may offer the same physiological benefits while minimizing the drawbacks. Since the introduction of the hypoxic training theory in 1930, several techniques have been developed, including regular high-altitude flights, training in altitude chambers, and training by inhalation of low-oxygen-gas mixtures. These techniques were used primarily for altitude pre-acclimatization and for the treatment of chronic disorders including lung disease, bronchial asthma, hypertension, diabetes mellitus, Parkinson’s disease, emotional disorders, radiation toxicity,
and prophylaxis of certain occupational diseases. The basic mechanisms of the beneficial effects of hypoxic training involve regulation of respiration, free-radical production, and mitochondrial respiration. In general, hypoxic exposure can be divided into hypoxia at rest, with the primary goal of stimulating altitude acclimatization, and hypoxia during exercise, to enhance the training stimulus. Moreover, the hypoxic stimulus may be continuous or intermittent. A sleep chamber that enables endurance athletes to “train while they sleep” has been developed. The chamber simulates the reduced air pressure of altitude environments equivalent to approximately 2000 to 3000 m. Athletes who use a hypoxic apartment typically “live and sleep high” in the hypoxic apartment but train at or near sea-level conditions, thus enabling them to achieve a similar advantage to those living at high altitude. The appropriate use of the chamber (6 to 8 h a day for 2 to 3 weeks) produces substantial increases in serum Epo, reticulocyte count, and RBC mass (up to 23 %), which, in turn, may lead to improvement in post-altitude endurance performance. Altitude simulation technology has continued to evolve. The hypoxic tent system is one of the first alternatives to the sleep chamber. The hypoxic tent system creates a hypoxic environment via a patented air separation unit that continually pumps low-oxygen content air. Inside the tent, the total pressure is unchanged, but the pO2 is reduced, allowing athletes to obtain the advantages of altitude training at any location. Unlike the constant hypoxia at higher altitudes, the intermittent hypoxia generated by the hypoxic tent system promotes gradual biological adaptation to yield better performance not only in a low-oxygen environment but also in normoxic conditions, such as at sea level. Earlier studies on simulated altitude by normobaric or hypobaric hypoxia convincingly demonstrated increases in Epo of a magnitude similar to those observed in response to a real altitude exposure of about 2.500 m. Hypoxic sleeping devices (hypobaric chamber) may simulate altitudes of up to approximately 4575 m and 4270 m. An alternative technique to simulate altitude is breathing hypoxic, normobaric gas mixtures. In these conditions, athletes breathe air with progressive decreases of the fraction of inspired oxygen (from 12.2 % to 10.0 %). This method is a modification of the “high-low” strategy, since athletes live in a natural terrestrial altitude environment while they train at sea level breathing supplemental oxygen. Limited data regarding the efficacy of hyperoxic training suggests that high-intensity workouts at moderate altitude and endurance performance at sea level may be enhanced when supplemental oxygen training is employed at altitude over several weeks [10353].

Living high, training low

Altitude training first became popular with athletes as part of their physical preparation for competition nearly fifty years ago, and over the intervening period many different altitude training protocols have evolved. In the past 15 years, numerous investigations have been conducted to examine the effects of Live high:train low (LHTL) altitude training, where athletes live at moderate altitude (2000-3000 m) but train near sea-level, on subsequent sports performance. Several researchers have concluded that, provided athletes are exposed to an adequate ‘dose’ of altitude (a combination of the duration and severity of hypoxic exposure), LHTL can lead to worthwhile performance improvements of 1-2 percent. An hypoxic dose of > 12 hours per day for at least 3 weeks at an elevation of 2100 m to 2500 m has been suggested as sufficient for athletes to benefit from the exposure. Despite other researchers finding no performance benefit from LHTL, including one recent placebo-controlled double-blind study this form of altitude training remains popular amongst elite athletes. The specific facilities required for LHTL altitude protocols can be logistically and financially inaccessible to many athletes. Either a location with rapid travel options between a low altitude training venue and a moderate altitude residential facility, or a special purpose ‘altitude house’ is required where the hypoxic environment can be simulated by reducing the oxygen content of the ambient air. One alternative, acute (60-90 min daily) intermittent
hypoxic exposure (IHE), was highlighted by a recent meta-analysis as one of the most beneficial forms of altitude training in sub-elite athletes. However, the authors of two recent reviews of the literature held an opposing view: that more studies suggest unchanged or impaired performance resulting from IHE than improved performance. Although the effectiveness of IHE is highly debated, this method offers major practical advantages over LHTL in terms of cost-effectiveness, time-efficiency, accessibility and portability. The combination of these practical advantages and the 1-5 percent performance improvements that have been reported by some researchers ensure that IHE remains a method of interest to elite athletes and coaches. However, there have been no previous studies that have directly compared these two methods in the same population and very few studies have used elite athletes. In light of the debate surrounding the purported effects of LHTL and IHE on sports performance, insight might be gained by examining the underlying physiological effects of these protocols. The erythropoietic effect of prolonged hypoxic exposure has been studied in detail due to the positive relationship between haemoglobin mass (Hbmass) and maximal oxygen consumption (VO2max). Haemoglobin mass increases of 1-4 percent following an adequate dose of LHTL have been demonstrated by several researchers, although it has been suggested recently that athletes with already high Hbmass may benefit less. The effect of IHE on Hbmass has not been examined, although, evidence of increased haemoglobin concentration ([Hb]) and increased serum erythropoietin concentration following IHE are suggestive of a positive haematological adaptation. However, due to the confounding influence of plasma volume fluctuations on [Hb], measurement of Hbmass is a more relevant method for the true effect of IHE on athletes' blood to be determined. Considerations of non-haematological adaptations to hypoxia reveal a potentially common mechanism of physiological adaptation following IHE and LHTL; there is evidence of similar improvements in sub-maximal exercise efficiency in runners from two separate studies, following IHE. The aim of one study was to compare directly the effects of LHTL and IHE on the running and blood characteristics of elite triathletes. Changes in total haemoglobin mass (Hbmass), maximal oxygen consumption (VO2max), velocity at VO2max (vVO2max), time to exhaustion (TTE), running economy, maximal blood lactate concentration ([La]) and 3 mM [La] running speed were compared following 17 days of LHTL (240 h of hypoxia), IHE (10.2 h of hypoxia) or placebo treatment in 24 Australian National Team triathletes (7 female, 17 male). There was a clear 3.2 ± 4.8 percent (mean ± 90% confidence limits) increase in Hbmass following LHTL compared with placebo, whereas the corresponding change of -1.4 ± 4.5 percent in IHE was unclear. Following LHTL, running economy was 2.8 ± 4.4% improved compared to IHE and 3mM [La] running speed was 4.4 ± 4.5% improved compared to Placebo. After IHE, there were no beneficial changes in running economy or 3mM [La] running speed compared to Placebo. There were no clear changes in VO2max, vVO2max and TTE following either method of hypoxia. The clear difference in Hbmass response between LHTL and IHE indicated that the dose of hypoxia in IHE was insufficient to induce accelerated erythropoiesis. Improved running economy and 3mM [La] running speed following LHTL suggested that this method of hypoxic exposure may enhance performance at submaximal running speeds. Overall, there was no evidence to support the use of IHE in elite triathletes. Despite a clear 3.2 percent increase in haemoglobin mass following 17 days of Live high: train low altitude training, no change in maximal aerobic capacity was observed. There were positive changes in running economy and the lactate-speed relationship at submaximal running speeds following Live high: train low altitude training. There was no evidence to support the use of daily 60-90 minute intermittent hypoxic exposure in elite triathletes [13326].

At the Olympic level, differences in performance are typically less than 0.5 percent. This helps explain why many contemporary elite endurance athletes in summer and winter sport incorporate some form of altitude/hypoxic training within their year-round training plan, believing that it will provide the "competitive edge" to succeed at the Olympic level. The
The purpose of one paper was to describe the practical application of altitude/hypoxic training as used by elite athletes. Within the general framework of the paper, both anecdotal and scientific evidence will be presented relative to the efficacy of several contemporary altitude/hypoxic training models and devices currently used by Olympic-level athletes for the purpose of legally enhancing performance. These include the three primary altitude/hypoxic training models:

- live high+train high (LH+TH)
- live high+train low (LH+TL)
- live low+train high (LL+TH)

The LH+TL model has been examined in detail including its various modifications: natural/terrestrial altitude, simulated altitude via nitrogen dilution or oxygen filtration, and hypobaric normoxia via supplemental oxygen. A somewhat opposite approach to LH+TL is the altitude/hypoxic training strategy of LL+TH, and data regarding its efficacy will be presented. Recently, several of these altitude/hypoxic training strategies and devices underwent critical review by the World Anti-Doping Agency (WADA) for the purpose of potentially banning them as illegal performance-enhancing substances/methods. The paper concluded with an update on the most recent statement from WADA regarding the use of simulated altitude devices [07135].

Live high-train low (LH+TL) altitude training was developed in the early 1990s in response to potential training limitations imposed on endurance athletes by traditional live high-train high (LH+TH) altitude training. The essence of LH+TL is that it allows athletes to "live high" for the purpose of facilitating altitude acclimatization, as manifest by a profound and sustained increase in endogenous erythropoietin (EPO) and ultimately an augmented erythrocyte volume, while simultaneously allowing athletes to "train low" for the purpose of replicating sea-level training intensity and oxygen flux, thereby inducing beneficial metabolic and neuromuscular adaptations. In addition to "natural/terrestrial" LH+TL, several simulated LH+TL devices have been developed to conveniently bring the mountain to the athlete, including nitrogen apartments, hypoxic tents, and hypoxicator devices. One of the key questions regarding the practical application of LH+TL is, what is the optimal hypoxic dose needed to facilitate altitude acclimatization and produce the expected beneficial physiological responses and sea-level performance effects? The purpose of one paper was to objectively answer that question, on the basis of an extensive body of research by a group in LH+TL altitude training. On the basis of consistent findings from the research group, it was recommended that for athletes to derive the physiological benefits of LH+TL, they need to live at a natural elevation of 2000-2500 m for >4 weeks for >22 h/day [07136].

To determine and compare the erythropoietic response and exercise performance of adolescent cross-country skiers, as a result of "living high-training high" (HH) and "living high-training low" (HL). Nine female and six male adolescent cross-country skiers volunteered to participate in separate trials. In the first trial (HH), the skiers lived and trained for 21 days at 1550-2050 m, while in the second trial (HL) they trained near sea level (450-500 m) but resided at 1550 m. All participants underwent maximal cycle ergometer tests for the determination of VO₂max and cardiorespiratory parameters via an open circuit system at sea level before ascent to altitude, and 1-2 days after descent from altitude. Blood samples were drawn prior to and immediately after maximal cycle exercise testing, at sea level prior to ascent, on days 1 (D1) and 21 (D21) at altitude (1740 m), and 1-2 days post-altitude, for the determination of serum erythropoietin (EPO) concentration, haemoglobin (Hb), hematocrit (Ht), and red blood cell (RBC) volume. The results showed that both boys and girls cross-country skiers, significantly improved their sea level VO₂max after 21 days of living at moderate altitude and training near sea level. The study demonstrates that living at moderate
altitude, 1550-2050 m and training low, near sea level (450-500 m) significantly increases VO$_{2\text{max}}$ and RBC mass for both boys and girls. Results indicate that applying the training concept "living high – training low" in adolescent athletes may improve their endurance performance [11144].

Methods such as the intake of erythropoietin (EPO, a natural hormone) and blood doping are banned by sport governing bodies owing to the invasive nature of the injections, potentially harmful outcomes, and the fact that they are a biomedical short cut allowing the body to exceed its genetic potential for adaptation. For example, EPO, which is injected subcutaneously, is now mass-produced and readily available as recombinant EPO. Its mechanism of action is the same as the endogenous EPO secreted by the kidneys, stimulating the production of RBCs to ultimately increase the RBCM. Research indicates that injection of recombinant EPO can significantly increase the level of hemoglobin (152 ± 4.2 g/L to 169 ± 9.3 g/L) after 6 weeks of injections, with analogous changes demonstrated in hematocrit (44.5 % ± 1.5 % to 49.7 % ± 1.9 %). Similarly, blood doping, the reinfusion of whole blood or packed RBCs, serves to increase total hemoglobin level, bypassing the need to produce EPO to stimulate the production of RBCs. Thus, blood doping's result is effectively the same as injecting recombinant EPO. These techniques have known dangers; for example, the injection of recombinant EPO can have side effects such as hypertension, seizures, and the development of thrombi. In contrast, the use of altitude training and (or) hypoxic devises (i.e. hypoxic tents) is not banned, but much debate has focused on the use of these methods to improve aerobic performance. Scientific research supported by universities and sport governing bodies has focused on the use of altitude training and hypoxic tents now more than ever before. Depending on the training system used, athletes can either sleep and train at altitude, or sleep at altitude and train at sea level. The latter strategy of "live high–train low" is arguably the preferred method. The benefits of increased RBCM occur as a result of sleeping in the hypoxic environment (e.g. the tent) and the benefits of physical training are realized by training at or near sea level (maintaining training intensity). If the live high–train low strategy is used, it makes the use of hypoxic sleeping tents a valuable part of the training regimen. No longer is it necessary to travel to altitude to sleep and then travel back down to train at or near sea level, and anecdotal guidelines for hypoxic training regimens are outlined by a body of accumulating research literature [07133].

Research suggests that a minimum of 8-10 h/day exposure to hypoxic environments is needed to elicit an adaptive response in the RBCM. Based on this timeline for daily exposure, the physiological benefits of the hypoxic environment may be evident in approximately 4 weeks. However, the rapidity and amplitude of the physiological response to the hypoxic environment is dependent on each individual’s specific response. The guidelines for altitude or simulated altitude show some variability, but it is suggested that a range of approximately 2200-4000 m above sea level is sufficient to stimulate an increase in RBC production and thus aerobic athletic performance. Hypoxic tents can achieve an altitude equivalence of 4285 m, which is capable of inducing performance-enhancing changes in RBCM. The development of protocols with the inclusion of hypoxic environments has evolved through scientific research, and two of the pre-eminent scientists are B. Levine and J. Stray-Gundersen. These investigators came to be recognized with their initial study, entitled "Live High – Train Low", using moderately to highly trained club- and collegiate-level runners. The concept of sleeping in hypoxia (moderate altitude), but training at or near sea level was in contrast to the more traditional live high – train high approach. The benefit of living high and training low was to spend 8-12 h/day in hypoxia, which stimulated an increase in RBCM, but allowed for the maintenance of training intensity and fitness level by training at or near sea level in normoxia. The study's performance-based outcome measure was a 5000 m time trial, while the laboratory-based outcome measures were maximal O$_2$ uptake (i.e. VO$_{2\text{max}}$), anaerobic capacity (i.e. accumulated O$_2$ deficit), maximal steady state at
ventilatory threshold, running economy, velocity at VO\textsubscript{2,max}, and blood compartment volumes. Four weeks of hypoxic acclimatization with the maintenance of sea-level training intensity (living high, training low) improved sea-level running performance and increased RBCM and VO\textsubscript{2,max}. A follow-up study was then completed using elite distance runners. The purpose was to determine if elite athletes who may be near their maximal structural and functional adaptive capacity of the respiratory system might achieve similar performance gains as the moderately to highly trained distance runners. The findings indicated that 3000 m time trial performance at sea level could be improved by 1.1 percent and VO\textsubscript{2,max} could be increased by 3 percent, with one third of the runners achieving personal best times for their competitive running distance after the altitude training camp. Twenty hours after ascent, the circulating erythropoietin levels were nearly double the initial sea-level values (8.5 ± 0.5 to 16.2 ± 1.0 IU/mL). The hemoglobin concentration measured at sea level increased by 1 g/dL (13.3 ± 0.2 to 14.3 ± 0.2 g/dL) over the duration of the training camp. The results indicated that the natural stimulation of the kidneys to produce endogenous EPO could improve sea-level endurance performance even in elite distance runners. In general, hematocrit studies suggest that training at or near sea level in coordination with sleeping in a hypoxic environment can increase RBC production, improving both laboratory and performance outcome measures [07133].

The concept of living at “high” altitude and training at “low” altitude (“live high-train low,” LHTL) has been increasingly used in recent years by endurance athletes with the expectation that sea-level performance may as a consequence be improved. The LHTL strategy combines living at moderate altitude, to increase hemoglobin mass (Hb\textsubscript{mass}) and red cell volume (RCV), with training at low altitude to maintain a high absolute training intensity. This concept has been shown to be superior to normal sea-level training or classical live high-train high (LHTH) altitude training for improving sea-level performance in elite endurance athletes. However, studies of whether exposure to moderate altitude increases Hb\textsubscript{mass} and RCV in elite endurance athletes have given controversial results. Results from the only published LHTL study that reported increase in RCV have been discussed, because RCV was measured indirectly with the Evans blue dye method, for which the adequacy for estimating RCV after hypoxic exposure has been questioned. LHTL studies that directly measured Hb\textsubscript{mass} with the carbon monoxide (CO)-rebreathing method did not report increased Hb\textsubscript{mass} and RCV. However, two LHTH studies, in which subjects generally spend more time at altitude, have recently reported increased Hb\textsubscript{mass} after exposure to moderate altitude. Thus it has been hypothesized that the hypoxic dose (living altitude combined with the duration of the altitude exposure) is the key factor. No controlled LHTL study has been published that has used a presumably adequate hypoxic dose at real altitude similar to the study by Levine and Stray-Gundersen and measured Hb\textsubscript{mass} directly with the CO-rebreathing technique [06165].

The effect of live high-train low on hemoglobin mass (Hb\textsubscript{mass}) and red cell volume (RCV) in elite endurance athletes is still controversial. It was expected that Hb\textsubscript{mass} and RCV would increase, when using a presumably adequate hypoxic dose. An altitude group (AG) of 10 Swiss national team orienteers (5 men and 5 women) lived at 2,500 m (18 h per day) and trained at 1,800 and 1,000 m above sea level for 24 days. Before and after altitude, Hb\textsubscript{mass}, RCV (carbon monoxide rebreathing method), blood, iron, and performance parameters were determined. Seven Swiss national team cross-country skiers (3 men and 4 women) served as "sea level" (500-1,600 m) control group (CG) for the changes in Hb\textsubscript{mass} and RCV. The AG increased Hb\textsubscript{mass} and RCV whereas there was no change for the CG. Serum erythropoietin, reticulocytes, transferring, soluble transferrin receptor, and hematocrit increased, whereas ferritin decreased in the AG. These changes were associated with an increased maximal oxygen uptake and improved 5,000-m running times from pre- to postaltitude. Living at 2,500 m and training at lower altitudes for 24 days increases Hb\textsubscript{mass} and RCV. These changes
may contribute to enhance performance of elite endurance athletes [06165].

The "living high-training low" model (Hi-Lo) may improve aerobic performance in athletes, and the main mechanism of this improvement is thought to be augmented erythropoiesis. A positive effect of Hi-Lo has been demonstrated previously by using altitudes of 2,000-3,000 m. Since the rate of erythropoiesis is altitude-dependent, it was tested whether a higher altitude (3,500 m) during Hi-Lo increases erythropoiesis and maximal aerobic performance. Nordic skiers trained for 18 days at 1,200 m, while sleeping at 1,200 m in ambient air (control group, n=5) or in hypoxic rooms (Hi-Lo, n=6; 3 x 6 days at simulated altitudes of 2,500, 3,000 and finally 3,500 m, 11 h/day). Measurements were done before, during (blood samples only) and 2 weeks after the intervention (POST). Maximal aerobic performance was examined from \( \text{VO}_{2\text{max}} \) and time to exhaustion (T\text{exh}) at \( v\text{VO}_{2\text{max}} \) (minimum speed associated with \( \text{VO}_{2\text{max}} \)), respectively. Erythropoietin and soluble transferrin receptor responses were higher during Hi-Lo, whereas reticulocytes did not change. In POST (vs before): hematological parameters were similar to basal levels, as well as red blood cell volume, being 2.68 + 0.83 L (v. 2.64 + 0.54 L) in Hi-Lo and 2.62 + 0.57 L (vs 2.87 + 0.59 L) in controls. At that time, neither \( \text{VO}_{2\text{max}} \) nor T\text{exh} were improved by Hi-Lo, \( \text{VO}_{2\text{max}} \) being non-significantly decreased by 2.0 percent (controls) and 3.7 percent (Hi-Lo). The present results suggest that increasing the altitude up to 3,500 m during Hi-Lo stimulates erythropoiesis but does not confer any advantage for maximal \( \text{O}_2 \) transport [06166].

**Short-term normobaric hypoxia**

One study aimed to determine the impact of short-term normobaric hypoxia on physiology and performance in highly trained athletes. Twelve (7 male and 5 female) athletes were randomly assigned into two groups and spent 8 h per night for two consecutive nights a week over 3 weeks under either short-term normobaric hypoxia (simulating 3636 m altitude, inspired \( \text{O}_2=13 \% \)) or in normobaric normoxia in a single-blind study. Following a 3 week washout period, athletes were then exposed to the other condition. Athletes were tested for maximal oxygen consumption and time to exhaustion on an electromagnetically braked cycle ergometer before and after each treatment in addition to being tested for anaerobic performance (Wingate test) on a modified Monark cycle ergometer. Blood samples were taken throughout the experiment and vastus lateralis muscle biopsies were taken before and after each treatment. Increases in red blood cell count, haematocrit, haemoglobin, platelet number and erythropoietin concentration were observed following short-term normobaric hypoxia. Except for a modest decrease in phosphofructokinase activity following short-term normobaric hypoxia, no changes were observed in muscle enzyme activities, buffer capacity, capillary density or morphology. No performance measures were changed following short-term normobaric hypoxia or normobaric normoxia. Although short-term normobaric hypoxia exposure increased levels of a number of haematological parameters, this was not associated with improved aerobic or anaerobic performance in highly trained athletes [06167].

**Effect of intermittent hypoxic training on cycling performance**

One study aimed to investigate the effects of a short-term period of intermittent hypoxic training (IHT) on cycling performance in athletes. Nineteen participants were randomly assigned to two groups: normoxic (NT, n=9) and intermittent hypoxic training group (IHT, n=10). A 3-week training program (5 x 1 h-1 h 30 min per week) was completed. Training sessions were performed in normoxia (approximately 30 m) or hypoxia (simulated altitude of 3,000 m) for NT and IHT group, respectively. Each subject performed before (W0) and after
(W4) the training program, three cycling tests including an incremental test to exhaustion in normoxia and hypoxia for determination of maximal aerobic power \((\text{VO}_{2\text{max}})\) and peak power output (PPO) as well as a 10-min cycle time trial in normoxia (TT) to measure the average power output \((P(\text{aver}))\). No significant difference in \(\text{VO}_{2\text{max}}\) was observed between the two training groups before or after the training period. When measured in normoxia, the PPO significantly increased by 7 and 7 percent in NT and IHT groups, respectively. However, only the IHT group significantly improved (11 %) PPO when measured in hypoxia. The NT group improved in TT by 8 percent, whereas IHT group did not show any significant difference. Intermittent training performed in hypoxia was less efficient for improving endurance performance at sea level than similar training performed in normoxia. However, IHT has the potential to assist athletes in preparation for competition at altitude [07139].

**Effects on laboratory parameters of intermittent hypoxic training**

The aim of one study was to assess the effect of intermittent hypoxia exposure on direct and indirect methods used to evaluate recombinant human erythropoietin (rhEPO) misuse. Sixteen male triathletes were randomly assigned to either the intermittent hypoxia exposure group (experimental group) or the control normoxic group (control group). The members of the experimental group were exposed to simulated altitude (from 4000 to 5500 m) in a hypobaric chamber for 3 h per day, 5 days a week, for 4 weeks. Blood and urine samples were collected before and after the first and the final exposures, and again 2 weeks after the final exposure. While serum EPO significantly increased after the first and final exposures, hemoglobin, percentage of reticulocytes, and soluble transferrin receptor were not elevated. Second-generation ON/OFF models (indirect rhEPO misuse detection) were insensitive to intermittent hypoxia exposure. The distribution of the urinary EPO isoelectric profiles (direct rhEPO misuse detection) was altered after intermittent hypoxia exposure with a slight shift towards more basic isoforms. However, those shifts never resulted in misinterpretation of results. The intermittent hypoxia exposure protocol studied did not produce any false-positive result for indirect or direct detection of rhEPO misuse in spite of the changes in EPO serum concentrations and urinary EPO isoelectric profiles, respectively [07140].

Aim of one study was to determine the influence of classic altitude training on hemoglobin mass (Hb-mass) in elite swimmers under the following aspects: normal oscillation of Hb-mass at sea level; time course of adaptation and de-adaptation; gender influences; influences of illness and injury; and interaction of Hb-mass and competition performance. Hb-mass of 45 top swimmers (male 24; female 21) was repeatedly measured (about 6 times) over the course of 2 years using the optimized CO-rebreathing method. Twenty-five athletes trained between one and three times for 3-4 weeks at altitude training camps at 2,320 m and 1,360 m. Performance was determined by analyzing 726 competitions according to the German point system. The variation of Hb-mass without hypoxic influence was 3.0 percent (males) and 2.7 percent (females). At altitude, Hb-mass increased by 7.2 ± 3.3 percent (2,320 m) and by 3.8 ± 3.4 percent (1,360 m). The response at 2,320 m was not gender-related, and no increase was found in ill and injured athletes \((n=8)\). Hb-mass was found increased on day 13 and was still elevated 24 days after return \((4.0 \pm 2.7 \%)\). Hb-mass had only a small positive effect on swimming performance; an increase in performance was only observed 25-35 days after return from altitude. In conclusion, the altitude \((2,320 \text{ m})\) effect on Hb-mass is still present 3 weeks after return, it decisively depends on the health status, but is not influenced by sex. In healthy subjects it exceeds by far the oscillation occurring at sea level. After return from altitude performance increases after a delay of 3 weeks [13291].
Position statement on hypoxic training

Team sports are activities that enjoy worldwide participation with large numbers of players training and competing at all levels. As skill proficiency increases, it is clear that overall technical and tactical effectiveness – rather than (competitive) physical performance per se – have a greater impact on winning. Over the last two decades, however, it is indisputable that team sports have experienced a tremendous increase in the tempo of play and energy demands imposed on players during matches. In this context, coaches and their staff are continuously looking for innovative ways to improve match outcomes, and moderate altitude training (2000–3000 m) has emerged as a popular ergogenic aid. Precompetition acclimatisation while residing at altitude (e.g. training for 1-2 weeks at the competition venue elevation) versus using altitude training to improve players’ “trainability” and competition performance in the days and weeks following return to sea level (e.g. 2-3 weeks of living high and training low during the preseason) are two distinct forms of altitude interventions that were debated by the expert panel. Despite altitude training being an area of interest for many sporting organisations — for example, Fédération Internationale de Football Association (FIFA), symposium on playing football at altitude and the International Olympic Committee (IOC), consensus statement on thermoregulatory and altitude challenges for all high-level athletes — research on the impact of altitude training for team sports is still in its infancy. An Altitude Training and Team Sports conference was held in Doha, Qatar on 24–25 March 2013. The original aims of the conference were to present cutting-edge research on the basic and applied aspects of altitude training and its impact on the physical performance of team-sport players. To this end, a panel of international experts was invited to address specific issues (detailed in the different review papers of this supplement) related to this topic. This position statement provides an overview of research and practical issues that may be of importance to consider when intending to use altitude training with team-sport players [13327].

To quantify the year-to-year variability of altitude-induced changes in haemoglobin mass (Hb_mass) in elite team-sport athletes 12 Australian-Footballers completed a 19-day (ALT1) and 18-day (ALT2) moderate altitude (2100 m), training camp separated by 12 months. An additional 20 participants completed only one of the two training camps (ALT1 additional n=9, ALT2 additional n=11). Total Hb_mass was assessed using carbon monoxide rebreathing before (PRE), after (POST1) and 4 weeks after each camp. The typical error of Hb_mass for the pooled data of all 32 participants was 2.6 percent. A contemporary statistics analysis was used with the smallest worthwhile change set to 2 percent for Hb_mass. POST1 Hb_mass was very likely increased in ALT1 (3.6 ± 1.6 %, n=19) as well as ALT2 (4.4 ± 1.3 %, n=23) with an individual responsiveness of 1.3 and 2.2 percent, respectively. There was a small correlation between ALT1 and ALT2 for a change in Hb_mass, but a moderately inverse relationship between the change in Hb_mass and initial relative Hb_mass (g/kg). It was concluded that two preseason moderate altitude camps 1 year apart yielded a similar (4 %) mean increase in Hb_mass of elite footballers, with an individual responsiveness of approximately half the group mean effect, indicating that most players gained benefit. Nevertheless, the same individuals generally did not change their Hb_mass consistently from year to year. Thus, a “responder” or “non-responder” to altitude for Hb_mass does not appear to be a fixed trait [13328].

Aviability of Hb_mass in altitude training

To quantify the year-to-year variability of altitude-induced changes in haemoglobin mass (Hb_mass) in elite team-sport athletes 12 Australian-Footballers completed a 19-day (ALT1) and 18-day (ALT2) moderate altitude (2100 m), training camp separated by 12 months. An additional 20 participants completed only one of the two training camps (ALT1 additional n=9, ALT2 additional n=11). Total Hb_mass was assessed using carbon monoxide rebreathing
before (PRE), after (POST₁) and 4 weeks after each camp. The typical error of Hb\text{mass} for the pooled data of all 32 participants was 2.6 percent. A contemporary statistics analysis was used with the smallest worthwhile change set to 2 percent for Hb\text{mass}. POST₁ Hb\text{mass} was very likely increased in ALT₁ (3.6 ± 1.6 %, n=19) as well as ALT₂ (4.4 ± 1.3 %, n=23) with an individual responsiveness of 1.3 and 2.2 percent, respectively. There was a small correlation between ALT₁ and ALT₂ for a change in Hb\text{mass}, but a moderately inverse relationship between the change in Hb\text{mass} and initial relative Hb\text{mass} (g/kg). It was concluded that two preseason moderate altitude camps 1 year apart yielded a similar (4 %) mean increase in Hb\text{mass} of elite footballers, with an individual responsiveness of approximately half the group mean effect, indicating that most players gained benefit. Nevertheless, the same individuals generally did not change their Hb\text{mass} consistently from year to year. Thus, a “responder” or “non-responder” to altitude for Hb\text{mass} does not appear to be a fixed trait [13327].

**For players of which team sports might altitude training be relevant?**

The physical (total distance covered, high-speed running or sprinting) and physiological (cardiovascular load, blood lactate concentration) demands of major team (football, rugby or Australian football) and racket sports (tennis and squash) during training and competition have been described by using miniaturised smart sensor devices (e.g. Global Positioning System technology, video tracking, portable gas analysers). In many team sports, the running distance during matches has considerably increased in recent years due to new tactical approaches having been adopted by many teams, thereby increasing the importance of endurance capacity. Team sports share the common feature of high-intensity, intermittent exercise patterns with continuously changing pace and also experience marked variability of game characteristics between sports, between playing positions and playing styles within the same sport and even from one match to the next. This creates a diversity of physiological challenges and performance needs across team-sport players. While elite team-sport players do not exhibit the specific physical/physiological capacities of elite endurance and sprint athletes, they generally possess an efficient combination of ‘aerobic’ and ‘anaerobic’ potential, though the relative contribution of oxidative versus glycolytic component varies widely across players and sports. Although aerobic metabolism dominates the energy delivery during most team sports, decisive actions (eg, sprints, jumps and tackles) are covered by means of anaerobic metabolism. As a result, the demands of team sports lend themselves towards a potential gain from adaptations to hypoxia from aerobically (maximal oxygen uptake (VO\text{2max}), economy and PCr resynthesis) and anaerobically (muscle buffer capacity) derived mechanisms. However, because the extent to which a player may benefit from different altitude-training methods may differ according to both their general and specific training focus (more aerobic vs anaerobic type of adaptations), no uniform recommendations can be made across all team sports. Nonetheless, it is anticipated that those activities displaying shorter exercise-to-rest ratios and/or requiring prolonged time spent at a high relative exercise intensity are more likely to benefit from altitude training. It was agreed that the effectiveness of any altitude-training programme might be worthwhile for some, but certainly not all, team members. For instance, it is intuitive that altitude training may have greater benefits for players covering large distances (>100 m/min) with high-intensity repeated efforts (ie, ‘invasion’ sports), as Australian football players do, compared with volleyball players, who run relatively less (distance covered generally <50 m/min) during a match. It is worth noting that since the impact of fitness on match running performance is likely player-dependent, playing style-dependent and position-dependent, the possible performance benefits of altitude training for team sports might not be as straightforward as for individual sports, where physical capacities strongly determine final performance. Considering soccer for instance, while it is appealing that altitude training may positively affect midfielders’ or attackers’ activity patterns, less evident is its possible impact for central defenders and goalkeepers. However, these assumptions await scientific evidence. Although
it is difficult to derive sound conclusions based on the limited literature available, it is proposed that incorporating information in accordance with time-dependent metabolic and match profiling (exercise-to-rest ratios), along with supplemented data from position-specific and player profile-related requirements could enable more informed judgements of the relevance of altitude training for a given player. It has been acknowledged that in elite endurance as well as team-sport athletes the effect of altitude training on red cell mass may depend on the initial haemoglobin mass (Hbmass). The proposed rationale is that an initial high Hbmass will not allow Hbmass to increase substantially following altitude training, whereas an initial low value will likely lead to meaningful enhancements in Hbmass. Noteworthy, however, is the observation that meaningful increases in Hbmass also occur in highly trained endurance athletes – that is, with some of the highest reported preintervention Hbmass values – from different sports and after various forms of altitude training. In team sports, where a high Hbmass is not necessarily a pre-requisite in all positions, players are generally characterised by a low to moderate Hbmass (or VO2max values usually ranging from 55 to 65 mL/min/kg) in comparison with endurance athletes, whose performance is largely related to aerobic capacity. There remains considerable controversy about the extent to which Hbmass increases in response to altitude training and two recent meta-analyses also offer somewhat conflicting viewpoints [13327].

In elite junior soccer players, the potential for altitude training to increase Hbmass was 3 percent after 12 days at 3600 m, and it is likely also present at the lower altitudes usually used for altitude-training camps by team-sport players. The rationale for attempting to increase Hbmass in team-sport players would be to increase their VO2max and enhance blood buffer capacity, and thereby decrease relative exercise intensity during games and increase tolerance for repeated-sprint exercises, respectively. While it is noted that, in some players, those values might already be near the upper limit of aerobic power, the expert panel agreed that any improvement in blood oxygen carrying capacity needs to be balanced so as not to limit explosive-type performance gains. What type of altitude-training interventions should be recommended for team-sport players? Contemporary altitude-training practices among athletes include: living high and training high (LHTH), living high and training low (LHTL) as well as living low and training high (LLTH). These paradigms can be achieved with natural altitude, simulated altitude or a combination, but it is important to note that the physiological responses to natural and simulated altitude may be quite different and controversy still exists as to what is the most efficient hypoxic exposure. In a 2009 meta-analysis of sea-level performance after hypoxic exposure, it was found that in elite endurance athletes, an enhancement of maximal aerobic power output was only possible with natural LHTL (4.0 %) and unclear with LHTH (1.6 %) and LLTH (0.6 %). While it is arguably easier to accumulate hours of hypoxia with LHTH, a recent meta-analysis concluded that Hbmass increases at approximately 1.1 percent per 100 h of altitude exposure regardless of the type of exposure (i.e. LHTH (>2100 m) or LHTL (3000 m)). Owing to possible inter-individual variability (e.g. individual responsiveness is approximately half the group mean effect in professional football players completing an LHTH training camp), when it comes to improving player’s fitness, one may question whether having all the team members residing and training at the same natural altitude is a sound approach if no individual adjustments in training content are made. Another recognised concern of hypoxic exposure is the large and individual decrease in maximal aerobic power (VO2max, 7 % per 1000 m altitude ascent), which may slow down the process of phosphocreatine resynthesis when recovering from high-intensity efforts. Compared to sea-level, VO2max was reduced by 20 percent in a cohort of non-acclimatised soccer players at a natural altitude of 3600 m, while at the same simulated altitude a single 5 s treadmill sprint performance was preserved. However, after repetitive efforts of short duration a larger fatigability is commonly observed in hypoxia, this effect may also be dependent on other factors such as training background, work-to-rest ratio and hypoxia severity [13327].
In order to maintain high-intensity training effectiveness (i.e. prevent premature fatigue), which represents a significant portion of competitive teams' training content, regular training practices of LHTH altitude camps may need to be modified. These modifications could be avoided by descending the whole squad to lower training venues but the logistical constraints and extended travel times may actually result in additional fatigue. Alternatively, work : rest ratios could be altered during sessions also taking into account the altitude of the training venue and players’ background. Practically, this requires adjusting distance or time of efforts and/or recovery times in order to modify the intensity or duration of practice bouts at altitude. Only with these adjustments can dramatic reductions in training quality along with accompanying negative alterations in mechanical and neuromuscular stimuli be avoided. Another solution could be to live in a natural, hypobaric hypoxic environment, but train at or near ‘simulated’ sea level with the aid of supplemental oxygen. While scientific evidence is still lacking, training with oxygen cylinders requires a stationary training situation, which is clearly unpractical for training sport-specific, technically complex activities commonly associated with team sports. Conversely, artificial altitude models (LHTL) may be more convenient for the team-sport players with the possibility of remaining in one training venue, while individualising the ‘altitude dose’ and training contents in line with their characteristics and field positioning. Exercise capacity during high-intensity intermittent exercise not only depends on the blood oxygen-carrying capacity, but also on molecular adaptations in the skeletal muscle and the efficiency of the neuromuscular system. Although not a consensus, LLTH altitude training regimes including near or maximal-intensity efforts (repeated sprint training in hypoxia) have proved superior to training at sea level in enhancing peripheral adaptations (i.e. oxidative capacity, capillary density and muscle glycolytic potential as well as increased expression of hypoxia inducible factor 1alpha (HIF-1alpha) and downstream genes to oxygen and transport) and, thereby, high-intensity intermittent performance. Likewise, resistance training combined with systemic hypoxia has been reported to further increase muscle strength, although other studies have shown no additional effect of hypoxia on strength gains. With only two known studies recruiting team-sport players, it was recognised that there needs to be more research to determine which form of LLTH altitude-training intervention may be more effective for maximising strength gains and multiple-sprint performance, while taking into account the specific characteristics of the different team sports and of the player for a given activity [13327].

What are the most relevant performance tests to provide ecologically valid data of the benefits of altitude training in a population of team-sport players? Assessment of the physiological determinants of physical performance is an integral part of sport science support for elite teams. At present, however, virtually all performance tests commonly used to judge the efficacy of any altitude-training intervention have been based on indicators of endurance-like performance (e.g. time trials). As such, the extent to which altitude training affects anaerobic performance is largely unaccounted for in the available literature. While altitude training is thought to improve some aspects of performance by only a small amount, high reliability (i.e. typical errors of 1-2 % with the tested-dependent variable having an error of measurement smaller than the smallest important effect) is a fundamental criterium guiding the selection of a particular field-based or laboratory-based test in the plethora of tests available today. It would be worthwhile to centre the test battery on the key elements of aerobic-type and anaerobic-type performance meant to be improved by the altitude-training intervention in question. As a single performance test cannot address the full complexity of team-sport performance, a broad suite of tests is expected. It was agreed that, in a population of team-sport players, relevant tests would at least include an evaluation of acceleration/peak sprinting and maximal aerobic velocities, while jump, repeated-sprints (with or without agility sequences) and running economy (e.g. 10-12 km/h for 5 min) tests can elegantly complete the test battery. In the absence of a gold-standard for repeated-sprint
testing for instance, coaching teams should adjust sprint distances, frequency, recovery time/type according to their players and sport requirements, ensuring that the tests are valid and reliable. Sport scientists have been tempted to directly measure the acute effects of an altitude exposure or the efficacy of a period of altitude acclimatisation on the occurrence of repeated high-intensity actions (frequency of maximal accelerations) and match running performance, as recently garnered from total distance covered or distances completed across different preselected time intervals. However, given the numerous confounding factors such as temporal changes in a team, opposition’s tactics and playing system and/or the contributions of substitutions, it must be questioned whether time motion analysis data can realistically be used in isolation to identify the benefits of any altitude-training intervention on athletic performance. In other words, one should proceed cautiously when inferring physical performance of team-sport players from their activity profiles since distances covered only reflect the “external physical output” of players. Important, high-intensity activity in professional soccer is not always related to team success, while those players performing more high-intensity work are also often covering lower total distances. Practically, it was therefore recognised that it is important to simultaneously measure the external (e.g., distance covered within different velocity zones) and internal loads (e.g., heart rate, perceptual responses) — irrespective of whether total distance covered has increased or not in response to altitude training — in order to objectively determine if a player is working easier physiologically to produce the same external physical output. Controlled experimental simulations of match-play activity performed on the field such as the Yo-Yo Intermittent Recovery test (level 2) and the 30-15 Intermittent Fitness Test or simulating team-sport running performance on a non-motorised treadmill in the laboratory environment are recommended to evaluate altitude training usefulness. Standardised drills in the form of small-sided games that replicate to a certain extent the physical intensity, movement (running performance) patterns and the technical requirements (skill component) of competitive match play for instance, with a simultaneous evaluation of total distance covered and distance ran at high speed, are also likely suitable [13327].

What is the optimal altitude dose to be used?

In individual athletes, the success of altitude training requires living high enough (>2000 m), for enough hours/day (>14–16 h/day), for a sufficient period of time (>19–20 days) in order to sustain an erythropoietic effect of hypoxia; that is, the so-called altitude dose (300–400 h). The time course of the erythropoietic response to altitude training is highly individual ranging from no response until 15 percent after 3–4 weeks. Training camps as short as 2 weeks have also been shown to increase Hbmass substantially in elite youth soccer (i.e. LHTH), elite water polo (i.e. LHTL) and Australian football players (i.e. LHTL and LHTH). Limited data currently exists regarding the time course of non-haematological adaptations, which may also be potentially beneficial for team-sport performance, during and after an altitude-training camp, thereby limiting the possibility to offer scientifically based recommendations about these adaptations. Most altitude-training venues around the world, which are equipped with the necessary facilities to suit team-sports (i.e. comfortable rooms and playing fields), are in the 1800–2500 m range. In the majority of research studies moderate altitudes (2000–3000 m) have been used arguably because at those heights robust and reliable erythropoietin (EPO)-induced expansion of red cell mass is usually observed, with athletes suffering from only minor side effects. Limited data are available on how these entities should be balanced and how far the boundaries of hypoxic exposure can be extended. At present, the optimal altitude for a team to reside at is unknown, but there is a widespread belief that elevations higher than 3000 m should be used with caution because of the excessive loss of training intensity and the characteristics of ball flight will change substantially due to the thinness of the air. On the one hand, the degree of hypoxia determines the magnitude of the induced physiological changes in a dose–response relationship, with higher altitudes triggering
larger/faster increases in red cell mass. On the other hand, exposure to chronic (several days to several weeks) hypoxia using elevations >3000–3500 m can be unproductive for some individual players as the stress on their body and the resultant side effects – for example, loss of appetite, inhibition of protein synthesis, muscle wasting, prevalence and severity of acute mountain sickness, excessive ventilatory work and/or metabolic compensation – from such high altitude could outweigh any erythropoietic benefits and thereby impair performance gains. Reportedly, however, sleep quality is rapidly increased with acclimatisation and may not even be adversely affected by acute (1–2 days) or chronic (1–2 weeks) exposures to high altitudes (>3500 m). This reinforces the potential value of individualising altitude-training “prescription” with artificial exposures as a prerequisite in order to maximise the performance of each player, and thereby reduce some of the individual responses seen today [13327].

Players who have had previous hypoxic exposure may adapt sooner to hypoxic conditions due to an increase in the magnitude of hyperventilation in the first few days of re-exposures. Although absolute mean changes in physiological capacities (i.e., Hbmass) appear to be repeatable after both LHTL and LHTH, individual athletes do not exhibit consistency in altitude-induced Hbmass changes from year-to-year, that is, the magnitude of the correlations between Hbmass changes are only small to moderate with differences in the individual responses to each intervention as large as 8 to 10 percent. More importantly, subsequent physical performance benefits may be even more variable from an intervention to another one. However, even though altitude training (at least in elite endurance athletes) results in an increase in VO_{2max} of more than half the magnitude of the increase in Hbmass, the weak (but significant) correlation found between these factors suggests that other non-haematological factors are also likely to be important. Although irrefutable scientific support is lacking, the effects of altitude training on some of the determinants of physical performance in team players may depend on the training phase of the competitive season. Importantly, altitude training needs to fit within the busy competition schedule of a team, without compromising the quality of the technical and tactical training. With the advent of hypoxic facilities (hypoxic chambers and/or altitude dormitories) in a growing number of high-level professional clubs and sport institutes, the prospect of implementing altitude interventions in a congested calendar is no longer as daunting. Larger physiological changes are generally expected for altitude training conducted preseason compared to in-season, likely due to lower initial fitness levels. Preseason generally provides a window of about a month or two to embark on a 2-week to 4-week sojourn at a natural or simulated altitude aiming to primarily enhance convective oxygen transport. The increased oxygen transport capacity of blood in response to altitude training may allow training at higher intensities during subsequent training in normoxia (improved lactate metabolism), thereby optimising the training stimuli by enhancing some neuromuscular and cardiovascular determinants of team-sport performance [13327].

Today, the busy competition schedules of major team sports often makes prolonged (>2 weeks) stays at altitude (at least for natural altitude exposure) unrealistic for anything other than preseason camps and the most important international tournaments. During the competition period, a 2-week camp implemented during the mid-season break for instance – be it LHTL or LLTH – may boost physical performance; nevertheless, longer exposures are certainly required to maximise the magnitude of these responses. Coaching teams involved in sports (e.g. water-polo and rugby) with a competition calendar targeting major international tournaments (i.e., Olympics and World Cup) in addition to regular league matches could accommodate an LHTL intervention during the competition preparation phase to maximise physiological adaptations of their squads. With minimal travel, modest expense and relatively minor disruption of training and daily life, a few blocks of LLTH altitude intervention (simulated altitude of 2500-3500 m; 2-3 sessions/week for 2-4 weeks; supra-maximal...
intensity workouts) could also be included in their yearly programme in order to add variety to training and help maintain in-season sprint speed and maximise explosive power/maximal strength capacity. Upon removal of the hypoxic stimulus, a reversal of some altitude-specific adaptations can occur relatively quickly (within few weeks; ie, neocytolysis, red blood cell destruction). Nevertheless, with a typical exposure of 300-400 h, the increase above prealtitude Hbmass values persists for 2-3 weeks, which does not support the proposal of short-term neocytolysis after altitude descent. Accordingly, the ability of the players to train at a high level for several weeks on return to sea level, due to the positive acclimatisation responses to altitude, may allow them to achieve a higher level of fitness (i.e. one that may last longer than the acclimatisation effects themselves) and more importantly performance. While the entire physiological acclimatisation is mostly undetectable 4 weeks of postdescent, performance gains seem to be more resilient and may last up to 4 weeks after the altitude camp. However, coaches should not expect any altitude-induced physiological changes to be maintained throughout the entire duration of a team-sport season if no additional hypoxic stimulus is added thereafter to the training programme. Although this awaits stronger scientific evidence, it was recognised that some of the side effects (decrease in Na\(^+/\)K\(^+\) ATPase activity and decreased plasma volumes) of each of the individual altitude-training interventions could potentially be attenuated when using combined (or mixed) methods. For players and coaches looking to elicit ‘aerobic’ and ‘anaerobic’ benefits to improve sea-level performance, living high and training low and high is an attractive altitude intervention for team sports. Proposed LHTL modifications which involve interspersing ‘blocks’ of nightly exposure to hypoxia, with several nights of normoxia (“intermittent” LHTL), to lessen any adverse psychological and physiological (eg, minimising the detrimental effects of chronic hypoxic exposure on muscle Na\(^+/\)K\(^+\) ATPase activity, especially in athletes undertaking heavy training) impacts of prolonged (>20 h/day) room confinement. Reportedly, a combined approach of LHTL plus additional hypoxic training sessions resulted in greater enhancement in the physiological capacities (VO\(_{2}\text{max}\) and Hbmass) that underpin endurance performance (3 km time trial) compared with LHTL or LLTH. Currently, however, the optimal characteristics of exercise in hypoxia or the combination of the various methods are unclear. Although altitude-training interventions combined with other challenging environmental conditions (eg, heat exposure to increase plasma volume) could potentially be useful to improve selected aspects of team-sport performance, at this stage, there is insufficient evidence to recommend such innovative mixed methods [13327].

Does the reduced air resistance with terrestrial altitude (hypobaric hypoxia) significantly modify match-related performance and the aerodynamics of the ball compared to exposure to simulated altitude (normobaric hypoxia)? What is the impact on training or competition? LHTH altitude has been, and will remain, widely used by teams to acclimatise before matches at altitude. This approach is supported by the lack of direct transfer of the benefits induced by a normobaric acclimation to the hypobaric situation and, in comparison, larger ventilatory acclimatisation, minimised acute mountain sickness prevalence and improved performance using terrestrial altitude or hypobaric chambers. As a general recommendation, suitable strategies to maximise physiological acclimatisation (oxygen transport and acid–base balance) should last 3-7 days for low altitude (500–2000 m), 1-2 weeks for moderate altitude (2000–3000 m) and at least 2 weeks if possible for high altitude (>3000 m). When designing acclimatisation strategies it is of utmost importance to consider the altitude of residence of the team and the ultimate competition altitude. The expert panel agreed that for squads who must compete at a moderate altitude within 2 weeks of ascent, living at the competition altitude (whenever possible at the competition venue) and not higher is advisable. Practically, shorter recovery periods before players can repeat high-intensity efforts and improved willingness to possess the ball can be viewed as signs of positive physiological acclimatisation. Because it does not simulate the reductions in air density, which affect motor ball trajectory and consequently motor skill proficiency, using normobaric
hypoxia is not optimal for preparation for competition at a natural altitude. The major
determinants of air density are barometric pressure, temperature and, to a lesser extent,
humidity. Upon ascent to natural altitude, changes in these variables will have a
proportionate effect on air density (air density reduces by about 10 percent for every 1000 m
increase in altitude) and, consequently, physical performance and player behaviour. This is a
serious concern in team sports where performance relies directly on repeated high-intensity
activities such as sprinting and involves a large technical skill component essential for
training and competition. At natural altitude, any potential advantage associated with reduced
air resistance (increased single sprint performance) is offset by the increased metabolic
challenge in hypoxic conditions (impaired repeated sprint ability). Regarding the effects of
moderate altitude exposure on activity profiles during actual soccer match play, not only is
total distance covered reduced above 1200 m, but also, a larger reduction in match running
performance is seen at 1600 m for higher intensity tasks such as high velocity running or
maximal acceleration. Thirteen days of acclimatisation nor life-long residence at high-altitude
(3600 m) protected against detrimental effects of altitude on match activity profile.
Additionally, for sea-level players a significant number of repetitions are arguably necessary
to make the appropriate motor skill adjustments required for competitive success in a
reduced air density environment [13327].

The decreasing air density associated with increasing altitude also results in changes in the
drag and lift forces acting on the flying object (ball, missile), thereby altering its flight
characteristics. This is typically manifested as a reduction in the lateral deflection or curve of
the projectile and an increased flight, as the projectile will travel more easily through the
thinner air. As a result, a soccer player's technical skills may be impacted when shooting,
controlling long passes and clearing the ball using punted and long kicks out of defence.
Undoubtedly, the goalkeeper could also be deceived by shots at goal, owing to the faster
flight of the ball and its altered trajectory. However, those effects have yet to be quantified,
and whether a technical acclimatisation to altitude takes place, beyond physiological
acclimatisation, needs to be researched, with a careful monitoring of the extent and the time
course of these adaptations for a range of heights. Despite the absence of scientific
evidence, it is reasonable to suggest that extra time and practice is probably required to
allow adequate adjustments in motor skills and movement timing as the terrestrial altitude
where teams reside, train and compete increases. Because physiological and aerodynamic
(also likely to be highly individual) adaptations may not necessarily share the same time
course, it is advised that teams experience these responses in a training camp setting well
ahead of the competitive event. Arguably, when a team prepares for competition at one
altitude but has to contest games at various altitudes during the tournament (eg, 1986 and
2010 FIFA World Cups), without a suitable time period to readjust to the biomechanical
constraints, the coach may need to make tactical changes. Likewise, teams will also have to
readjust upon return to sea level and whether LHTH should not be recommended when
competing at sea level in a short window (<7-10 days) requires research [13327].

One meta-analysis uses raw data from 17 studies that used carbon monoxide rebreathing to
determine Hbmass prealtitude, during altitude and postaltitude. Seven studies were classic
altitude training, eight were live high train low (LHTL) and two mixed classic and LHTL.
Separate linear-mixed models were fitted to the data from the 17 studies and the resultant
estimates of the effects of altitude used in a random effects meta-analysis to obtain an
overall estimate of the effect of altitude, with separate analyses during altitude and
postaltitude. In addition, within-subject differences from the prealtitude phase for altitude
participant and all the data on control participants were used to estimate the analytical SD.
The 'true' between-subject response to altitude was estimated from the within-subject
differences on altitude participants, between the prealtitude and during-altitude phases,
together with the estimated analytical SD. During-altitude Hbmass was estimated to increase
by 1.1 percent/100 h for LHTL and classic altitude. Postaltitude Hbmass was estimated to be
3.3 percent higher than prealtitude values for up to 20 days. The within-subject SD was
constant at 2 percent for up to 7 days between observations, indicative of analytical error. A
95 percent prediction interval for the “true” response of an athlete exposed to 300 h of
altitude was estimated to be 1.1–6.0 percent. It was concluded that camps as short as 2
weeks of classic and LHTL altitude will quite likely increase Hbmass and most athletes can
expect benefit [13329].

Correlation between Hbmass and altitude training result

Endurance athletes have been using altitude training for decades to improve near sea-level
performance. The predominant mechanism is thought to be accelerated erythropoiesis
increasing hemoglobin mass [Hbmass] resulting in a greater maximal oxygen uptake (O2max).
Not all studies have shown a proportionate increase in O2max as a result of increased Hbmass.
The aim of one study was to determine the relationship between the two parameters in a
large group of endurance athletes after altitude training. 145 elite endurance athletes (94
male and 51 female) who participated in various altitude studies as altitude or control
participants were used for the analysis. Participants performed Hbmass and O2max testing
before and after intervention. For the pooled data, the correlation between per cent change
in Hbmass and per cent change in O2max was significant with a slope of 0.48 intercept free to
vary and 0.62 when constrained through the origin. When separated, the correlations were
significant for the altitude and control groups, with the correlation being stronger for the
altitude group (slope of 0.57 to 0.72). It was concluded that, with high statistical power,
altitude training of endurance athletes will result in an increase in O2max of more than half the
magnitude of the increase in Hbmass, which supports the use of altitude training by athletes.
But race performance is not perfectly related to relative O2max, and other non-haematological
factors altered from altitude training, such as running economy and lactate threshold, may
also be beneficial to performance [13330].

Physiological markers worthwhile monitoring to identify altitude responders?

At present, there is no gold-standard test battery to facilitate the detection of team members
who are unlikely to cope well with the stress of altitude or who will respond positively. As a
general rule, however, preascent evaluations should ensure that players are free of illness,
injury and fatigue. A comprehensive initial assessment would also include other measures
such as “normal” iron and nutritional-hydration status, body mass and psychological
attributes. Only players who fulfil these criteria should add the stress of hypoxia to their
training. A conservative approach might be prudent for at-risk players – those who are
currently unfit and not coping well with altitude stress – to ensure that they are not worked
too hard at hypoxia until fully recovered. Arterial oxyhaemoglobin saturation (SpO2) in
hypoxia is largely controlled by the hypoxic ventilatory response. An enhanced resting
ventilatory response to hypoxia, which is mediated primarily by the peripheral
chemoreceptors in the carotid bodies, is arguably beneficial as the body responds to the
hypoxic stimulus more quickly. Reportedly, the ability to maintain SpO2 during heavy exercise
at sea level has a strong influence on the ability to maintain VO2max and exercise
performance with acute altitude exposure. As such, although their mode of evaluation (rest
vs exercise; hypoxic dosage) is still debated, determining chemosensitivity parameters (ie,
desaturation and ventilatory response to hypoxia) may help detect at-risk players before a
sojourn to altitude. Although irrefutable scientific support is lacking, coaches and support
staff can also be proactive by implementing short-term, intermittent normobaric hypoxia
exposures (30-60 min at altitude ranging 3000-4500 m) before travel for those with a blunted
hypoxic ventilatory response in order to reduce the prevalence and severity of acute
mountain sickness [13327].
Another explanation proposed to account for the lack of adaptation to altitude training is depleted iron stores prior to and as a result of altitude exposure. In iron deficient athletes (serum ferritin <35 ng/mL for females and <50 ng/mL for males) the likelihood of an altitude-induced increase in Hbmass is minimal, suggesting that normalisation with oral (ferrous sulfate) supplements and monitoring iron status of each team member is an absolute necessity before exposure to hypoxia. Iron-deficiency per se could result in decreased training potential or physical performance in team-sport players, not only because of blunted erythropoiesis but also due to its negative impact on other iron-dependent physiological processes at the mitochondrial level and in myoglobin content. We recommend that iron deficient players receive iron supplementation, in order to normalise serum ferritin stores before departing for altitude, and maintenance of iron supplementation for all players while at altitude in order to prevent bias arising from iron deficiency. As with any other training stimulus, there is considerable variation in the response to altitude training. This is evidenced by decreased sympathetic activity and strong erythropoietic responses to altitude in some participants, while others see little or no changes in such variables with chronic exposure. Likewise, most players experience significant impairment of training velocities and oxygen uptake at a moderate altitude, while few would be able to maintain training and oxygen flux near what they would be able to at sea level. The concept of “responders” and “non-responders” was created without offering plausible mechanisms. While factors influencing the magnitude of individual response to hypoxia may be genetically inherited traits (i.e. HIF-1alpha functions as a master regulator of many genes, notably of erythropoiesis, pH regulation and glycolysis), it remains possible that certain psychosociological concerns, not physiological ones, may also determine how specific team members will respond. For some players leaving their family (spouse and parents) and regular training environment for the duration of a camp can be problematic. This may partly explain the within and between years variability observed in the response of an individual athlete. As such, it may not be appropriate to divide team members into “responders” and “non-responders” but rather to question whether the intervention had a measurable impact on player performance. A proposition would be to identify those who will respond with a fast/high, moderate/medium and slow/low response compared to the group mean response. Importantly, in the same individuals, changes in physiological and performance measures (i.e. the former to a higher extent than the latter) after two virtually identical altitude-training camps are not necessarily consistent. This reinforces that altitude training-related gains may not only be dependent on positive physiological adaptations but also on a complex interaction of other factors including fitness, training status and fatigue. Substantially increased feelings of fatigue (players’ perception of how hard they are training along with their fatigue, stress and muscle soreness levels), submaximal heart rates, poorer training quality and disrupted sleep structure, as measured from validated tools also give the coach invaluable insight to help delineate those players who are coping well with the stress of hypoxia from those who are not. The majority of training benefits at sea level are accrued with adequate attention given to consistent training, suitable recovery/nutrition and skill development. How are these factors taken into account when training at altitude? It was recognised that disrupted training and recovery are expected at altitude, especially for novice players, and therefore require careful management [13327].

The ethics of hypoxic training

Unlike many forms of doping, the use of hypoxic chambers within sports does not involve synthetic substances that can easily be characterized as artificial or unnatural. Moreover, it cannot easily be aligned with the antisocial connotations of drug abuse, which are so
effective at garnering political sport. At most, the arguments surrounding its use involve its
effect as a form of cheating or as a health risk. Yet, for some time now it has not been
possible to describe the use of such chambers as a form of cheating since they have been
legal. Moreover, a number of high profile athletes have used them extensively without any
moral outrage reported. The science of hypoxia involves changes in the partial pressure of
oxygen within an environment, which increases the body’s hematocrit level. These changes
reduce the partial pressure of oxygen in the pulmonary capillaries, which leads to an
increased need to breathe. In turn, the body senses the changes and increases the
production of red blood cells, which are rich in oxygen carrying protein (hemoglobin). This
enhanced production leads to a greater aerobic potential for the individual. The pop singer
Michael Jackson was photographed within such a chamber. Such a context easily frames
this technology as something alien to “normal” human practices. Indeed, the characteristics
of the technology tend to have required obstructive practices for athletes who will need to
spend extensive time in these isolated booths. Such spaces conjure up images of athletes as
rats in laboratories simply growing stronger almost by magic. Such images forces one to
question whether the WADA Code seeks to protect an athletically moral practice. Hypoxic training has also been particularly interesting because it seems to have divided the scientific community and its support for WADA’s work [06168].

Yet, the more intriguing characteristics of this issue relate to the ethical debate that has
ensued. During 2006, the ethical status of hypoxic chambers was put to the recently formed Ethical Issues Review Panel in WADA. The Panel’s report raises a number of specific arguments as critical to the ethical status of hypoxic training, beginning its discussion paper by asking what it is about sport that people find honorable, admirable, and beautiful. Their position concludes that hypoxic training is a violation of the “spirit of sport” (WADACode) insofar as it does not require the “virtuous perfection of natural talents” matters to sport. In short, their view was that the use of such chambers was “passive” requiring no skill, knowledge, or effort on the part of the athlete. They state: “my responsibility for my performance is diminished by technologies that operate upon me, independent of any effort on my part.” As was mentioned earlier, the “spirit of sport” concerns constitute only one element of the process by which a technology might be deemed a doping technology. The final outcome of this inquiry made in September 2006 was that the hypoxic chambers should remain legal [06168].

It seems remarkable that, for so many years, athletes have used hypoxic training without it
giving rise to moral outrage. The idea of spending time locked in a room doing nothing
cannot easily be associated with the practice ethos of sports. Yet, this view of what hypoxic
training entails is also ambiguous or, at least, contingent. For instance, there already exist
rooms, which resemble regular rooms within a home. Moreover, one could envision its
construction as a space of reflection on an athletic life or for learning essential information
about the practice of sports. The point is that a hypoxic chamber is a work in progress and
that the moral judgment of this technology on how it seems to occupy a quite different social
space compared with the idea of athletes running in mountains is neither accurate nor
relevant. Moreover, the development of this technology is only likely to become more
"seamless" in the way that I mentioned earlier [06168].

In short, it is possible for a performance-enhancing technology to be of no detriment to the
spirit of sport, but simply involve a reskilling of the activities an athlete undertakes in order to
remain competitive. The intrinsic value of sports – the skills required to bring about sporting
performance – are unaffected by hypoxic chambers. At the very most, their use will raise the
standard of sporting achievements, which is precisely what gives elite sports their unique
social value. While it is inevitable that circumstances arise where an athlete is simply
introduced to a new performance-enhancing technology, it is crucial to remember that every
part of that technology's development has involved members of the athletic community. Indeed, as is true of other technologies, it is likely that open access to this innovation will lead to a more nuanced culture of use [06168].
PLATELET-RICH PLASMA

Biomedical sciences have made major advances in understanding how tissues repair, and the signalling mechanisms required to achieve this goal are progressively being dissected. Advances in the understanding of tissue repair mechanisms and the pivotal role of growth factors have stimulated the use of platelet-rich therapies by orthopaedic surgeons and sports physicians, mainly with the aim of stimulating and enhancing tissue healing. Autologous activated platelets retained in fibrin matrices are used as a source of molecular signals that control cell fate, including cell growth, cell differentiation and the synthesis of diverse functional proteins. Thus far, platelet-rich technologies have spawned additional ambitious endeavours, including surgical and non-surgical treatments in sports orthopaedics. Reconstruction of anterior cruciate ligament and tendon surgery and treatment of joint injuries, tendinopathy or muscle tears are but a few examples of the potential applications of this technology in the field of orthopaedic sports medicine. In the present article, some of the most important therapeutic applications using these approaches – especially preparation rich in growth factor technology – are presented, as are some of the limitations, anti-doping concerns and future challenges in this field. In view of a general state of confusion, the concept of platelet-rich plasma needs rigorous definition associated with well characterized products and re-administration procedures. There is evidence that reconstruction of anterior cruciate ligament and tendon surgery combined with preparation rich in growth factor technology enhances healing and functional recovery; clinical evidence is also appearing in the literature regarding treatment of tendinopathies and osteoarthritis. Currently, the challenge lies in conducting randomized, controlled clinical trials to determine the essential qualities of these technologies. If anti-doping agencies clarify their regulatory guidelines, robust studies in athletes are expected to emerge. Although much research work lies ahead, the current knowledge points to a future in which platelet-rich therapies will continue improving existing conventional approaches to treatment of sports injuries.

Autologous platelet-rich plasma (PRP) is perceived to accelerate healing in musculoskeletal injuries. PRP is increasingly used in situations that require rapid return-to-play, which, in the professional sports arena, translates to fame and money. Human blood platelet counts are approximately 200,000/ml. PRP is an autologous concentration of human platelets above this in a small volume of plasma. Reports vary regarding the platelet concentration and different growth factors present in the PRP concentrate. Also, there are many preparation protocols, kits, centrifuges and methods to trigger platelet activation before use. The same is true for application methods, including using injectable activated PRP liquid concentrate versus implanting a fibrin scaffold, optimal timing of injection and the specific volume to use. Almost every major manufacturer in the orthopaedic and sports medicine world markets a different commercial kit. Some claim to produce a better quantity and quality of PRP than their competitors from the same amount of blood from the same patient. Costs vary tremendously: a commercial kit yields a PRP concentrate at the cost of several hundred dollars, but inhouse non-automatised techniques produce a PRP concentrate for approximately US$10. Each method to concentrate platelets leads to a different product with different biology and potential uses, with a high variation (3 to 27-fold) in growth factor concentration and in the kinetics of release. Most techniques yield a PRP concentrate of approximately 10 percent of the blood volume taken (e.g. 20 ml of whole blood would result in approximately 2 ml of PRP). These differences might be of relevance to clinical management, although they have not been systematically studied. PRP preparations containing only moderately elevated platelet concentrations may be the ones to induce optimal biological benefit, with lower platelet concentrations leading to suboptimal effects, and higher platelet concentrations to inhibitory effects. Other authors have stated that the
“therapeutic dose” of PRP would be at least four to six times higher than the normal platelet count. To complicate things, the actual growth factor content is not well correlated with the platelet count in whole blood or in PRP, and there is no evidence that gender or age affects platelet count or growth factor concentrations [10360].

A few studies have categorised the different platelet concentrates according to the presence or absence of white blood cells (WBC): either pure platelet rich plasma (P-PRP), in which WBC have been intentionally eliminated from the PRP, or leucocyte and platelet-rich plasma (L-PRP), possibly from the inability of the kit to differentiate between the WBC and the PRP layers. A positive or negative effect of WBC cannot be generalised to all tissues and clinical conditions. This issue requires further investigation, as the WBC content in the preparation injected has never been systematically studied. It is known that neutrophils promote additional muscle damage soon after the initial injury, but there is no direct evidence that neutrophils play a beneficial role in muscle repair or regeneration. This exacerbation of injury and/or delay in muscle regeneration may be of major importance for injuries managed with PRP. This, together with the improved homogeneity of P-PRP and its reduced donor-to-donor variability, supports some PRP production techniques that claim to be clinically superior. L-PRP injected for soft tissue injuries might induce more local pain than P-PRP. Platelets by themselves do reduce pain. L-PRP reduces postoperative pain, although here the contribution of the WBC to the overall effect observed remains unclear [10360].

Platelets contain many biologically active factors, including many of the proteins responsible for haemostasis, synthesis of new connective tissue and revascularisation. They can stimulate a supraphysiological release of growth factors to jumpstart healing in chronic injuries, or speed up an acute injury repair process. The idea behind PRP treatment is that all stages of the repair process are controlled by a wide variety of cytokines and growth factors acting locally as regulators of the most basic cell functions, using endocrine, paracrine, autocrine and intracrine mechanisms. More than 95 percent of presynthesised growth factors are secreted within 1 h of activation from the granules. After the initial burst of PRP-related growth factors, platelets synthesise and secrete additional growth factors for the remaining 7–10 days of their lifespan. Typically, blood, such as the haematoma formed in a muscle tear, contains approximately 94 percent red blood cells (RBC), a small amount of platelets (6 %) and less than 1 percent leucocytes. The rationale for PRP therapy lies in reversing the blood ratio by decreasing RBC, which are less useful in the healing process, to approximately 5 percent, and increasing the platelet amount to 94 percent to stimulate recovery. The main growth factors in the PRP concentrate are the transforming growth factor-beta1, platelet-derived growth factor, vascular endothelial growth factor, epithelial growth factor, hepatocyte growth factor and insulin-like growth factor 1. Most of these growth factors play key roles in tendon, muscle, ligament, cartilage and bone healing by stimulating angiogenesis, epithelialisation, cell differentiation–replication–proliferation and the formation of extracellular matrix [10360].

Most early studies concentrated on purified isolated growth factors that were known to play a specific role in tissue healing. Only in the past decade has it been recognised and put into practice that the need to target various signalling pathways requires the administration of a balanced combination of mediators, as isolated growth factors would not be able to satisfy the multiple requirements of the injured tissue. PRP has been used to enhance the healing of meniscus defects and muscle injuries, and stimulate chondrocytes to engineer cartilaginous tissue, reduce pain and produce better and more balanced synovial fluid in arthritic knees, improve outcomes after total knee arthroplasty and subacromial decompression, accelerate bone formation, stimulate the healing of an anterior cruciate ligament injury central defect, its primary repair or its reconstruction, improve the outcome of operated ruptured Achilles tendons, reduce pain in chronic tendinopathies and prevent and reverse intervertebral disc
degeneration. It is remarkable that very few randomised controlled trials have been performed, and even fewer trials are adequately powered, use appropriate outcome measures and have decent follow-up [10360].

The 2008 “Aspetar Consensus”, organised by the World Anti-doping Association (WADA) and the International Olympic Committee to debate possible conflicts with the WADA code, discussed the use of PRP in muscle injuries, concluding that further research is necessary. “The application of the WADA Therapeutic Use Exemption (TUE) process is the preferred approach when wishing to utilise growth factors technology in elite athletes, however the ability of the TUE committee to appropriately evaluate such applications is inhibited by the current level of scientific evidence.” The position statement concludes that “WADA will not be in a position to evaluate its clinical utility for either assessment of TUE applications or the prohibited list.” In section S2 of the Aspetar Consensus, concerning any autologous product that contains growth factors, the only actual factor mentioned in connection with PRP is insulin-like growth factor 1, which, while present in PRP, is systemically subtherapeutic by a factor of 500; only 1 percent of it is unbound, available and active, with a half life of 10 minutes [10360].

Another concern is that PRP might produce genetic instability, potentially leading to neoplasms. Growth factors act on cell membranes rather than on the cell nucleus, and activate gene expression by internal cytoplasmic signal proteins, which promote normal, not abnormal, gene expression. Growth factors are not directly mutagenic, and act through gene regulation and normal wound healing feedback control mechanisms. Furthermore, the systemic effects on circulating growth factors from a local PRP injection showed a very brief reduction of blood growth factors. The modalities of use of PRP vary. The use of non-steroidal anti-inflammatory drugs in the early post-injection period may exert an inhibitory effect on healing, and the use of local anaesthesia at the injection site is controversial. Extrarticular injections are performed under ultrasound guidance, and it is suggested that the haematoma, if present, should be evacuated and replaced with PRP [10360].

Regarding platelet-rich plasma, the sceptics point out that, given the well concerted healing cascade that has evolved over millions of years, it is not easy to understand how a single or even a few injections of a cocktail of growth factors at variable, and at present not well codified, times from the injury will produce a lasting beneficial effect on a wide variety of conditions. Despite the hype of the technique and its biological plausibility, the anecdotal nearly miraculous recovery reported in the lay press in some famous athletes, and the myriad of extremely favourable retrospective and prospective studies published, level I investigations are lacking [10360].

Platelet-rich plasma is defined as autologous blood with a concentration of platelets above baseline values. Platelet-rich plasma has been used in maxillofacial and plastic surgery since the 1990s; its use in sports medicine is growing given its potential to enhance muscle and tendon healing. In vitro studies suggest that growth factors released by platelets recruit reparative cells and may augment soft-tissue repair. Although minimal clinical evidence is currently available, the use of platelet-rich plasma has increased, given its safety as well as the availability of new devices for outpatient preparation and delivery. Its use in surgery to augment rotator cuff and Achilles tendon repair has also been reported. As the marketing of platelet-rich plasma increases, orthopaedic surgeons must be informed regarding the available preparation devices and their differences. Many controlled clinical trials are under way, but clinical use should be approached cautiously until high-level clinical evidence supporting platelet-rich plasma efficacy is available [09180].

Biomedical sciences have made major advances in understanding how tissues repair, and
the signalling mechanisms required to achieve this goal are progressively being dissected. Advances in the understanding of tissue repair mechanisms and the pivotal role of growth factors have stimulated the use of platelet-rich therapies by orthopaedic surgeons and sports physicians, mainly with the aim of stimulating and enhancing tissue healing. Autologous activated platelets retained in fibrin matrices are used as a source of molecular signals that control cell fate, including cell growth, cell differentiation and the synthesis of diverse functional proteins. Thus far, platelet-rich technologies have spawned additional ambitious endeavours, including surgical and non-surgical treatments in sports orthopaedics. Reconstruction of anterior cruciate ligament and tendon surgery and treatment of joint injuries, tendinopathy or muscle tears are but a few examples of the potential applications of this technology in the field of orthopaedic sports medicine. In the present article, some of the most important therapeutic applications using these approaches - especially preparation rich in growth factor (PRGF) technology – are presented, as are some of the limitations, anti-doping concerns and future challenges in the field. In view of a general state of confusion, the concept of platelet-rich plasma needs rigorous definition associated with well characterized products and re-administration procedures. There is evidence that reconstruction of anterior cruciate ligament and tendon surgery combined with PRGF enhances healing and functional recovery; clinical evidence is also appearing in the literature regarding treatment of tendinopathies and osteoarthritis. Currently, the challenge lies in conducting randomized, controlled clinical trials to determine the essential qualities of these technologies. If anti-doping agencies clarify their regulatory guidelines, robust studies in athletes are expected to emerge. Although much research work lies ahead, the current knowledge points to a future in which platelet-rich therapies will continue improving existing conventional approaches to treatment of sports injuries [09181].

In Europe and the United States, there is an increasing prevalence of the use of autologous blood products to facilitate healing in a variety of applications. Recently, it has been learned more about specific growth factors, which play a crucial role in the healing process. With that knowledge there is abundant enthusiasm in the application of concentrated platelets, which release a supra-maximal quantity of these growth factors to stimulate recovery in non-healing injuries. For 20 years, the application of autologous PRP has been safely used and documented in many fields including; orthopedics, sports medicine, dentistry, ENT, neurosurgery, ophthalmology, urology, wound healing, cosmetic, cardiothoracic, and maxillofacial surgery. One article introduces the reader to PRP therapy and reviews the current literature on this emerging treatment modality. In summary, PRP provides a promising alternative to surgery by promoting safe and natural healing. However, there are few controlled trials, and mostly anecdotal or case reports. Additionally the sample sizes are frequently small, limiting the generalization of the findings. Recently, there is emerging literature on the beneficial effects of PRP for chronic non-healing tendon injuries including lateral epicondylitis and plantar fasciitis and cartilage degeneration [09182].

Circulation-derived cells play a crucial role in the healing processes of tissue. In early phases of tendon healing processes, circulation-derived cells temporarily exist in the wounded area to initiate the healing process and decrease in number with time. It was assumed that a delay of time-dependent decrease in circulation-derived cells could improve the healing of tendons. In one study, it was injected platelet-rich plasma (PRP) containing various kinds of growth factors into the wounded area of the patellar tendon, and compared the effects on activation of circulation-derived cells and enhancement of tendon healing with a control group (no PRP injection). To follow the circulation-derived cells, we used a green fluorescent protein (GFP) chimeric rat expressing GFP in the circulating cells and bone marrow cells. In the PRP group, the numbers of GFP-positive cells and heat-shock protein (HSP47; collagen-specific molecular chaperone)-positive cells were significantly higher than in the control group at 3 and 7 days after injury. At the same time, the immunoreactivity for types I and III collagen
was higher in the PRP group than in the control group at early phase of tendon healing. These findings suggest that locally injected PRP is useful as an activator of circulation-derived cells for enhancement of the initial tendon healing process [09183].

Mechanical stimulation improves the repair of ruptured tendons. Injection of a platelet concentrate (platelet-rich plasma, PRP) can also improve repair in several animal models. In a rat Achilles tendon transection model, 1 postoperative injection resulted in increased strength after 4 weeks. Considering the short half-lives of factors released by platelets, this very late effect calls for an explanation. It was studied the effects of platelets on Achilles tendon regenerates in rats 3, 5 and 14 days after transection. The tendons were either unloaded by Botulinum toxin A (Botox) injections into the calf muscles, or mechanically stimulated in activity cages. No Botox injections and ordinary cages, respectively, served as controls. Repair was evaluated by tensile testing. At 14 days, unloading (with Botox) abolished any effect of the platelets and reduced the mechanical properties of the repair tissue to less than half of normal. Thus, some mechanical stimulation is a prerequisite for the effect of platelets at 14 days. Without Botox, both activity and platelets increased repair independently of each other. However, at 3 and 5 days, platelets improved the mechanical properties in Botox-treated rats. Platelets influence only the early phases of regeneration, but this allows mechanical stimulation to start driving neo-tendon development at an earlier time point, which kept it constantly ahead of the controls [09184].

It was described the therapeutic utilization of separated and isolated autologous growth factors in semiconservative treatment of type III injury to the ankle ligamentous complex. Eleven patients, two women and nine men, with acute injury to the lateral ligamentous complex of the ankle were treated by plasma rich in growth factors (PRGF) infiltration. The injured patients were clinically examined and standard forced inversion radiographs were made using topical anesthesia. Autologous PRGF activated with calcium chloride was used to infiltrate the injured tissues. The treatment was followed by immobilization of the joint and its subsequent rehabilitation. Clinical examination of injured tissues was carried out at 4 and 6 weeks of follow-up, using stability assessment tests and functional radiography of the ankle. Physical therapy included standard procedures, but faster regeneration of the soft tissues allowed for more exercises. The average time of healing was 5 weeks. Five patients showed no signs of instability at 4 weeks after therapy and could return to their previous sports activities. One patient had lateral ankle instability at 5 weeks and therefore the therapy continued with prolonged immobilization and then rehabilitation at a slower pace. According to the authors presence of growth factors facilitates the healing and remodeling of soft tissues and regeneration may begin before leukocytes infiltrate the affected site. At a relatively low level of interleukins, the inflammatory phase of healing is suppressed, pain is reduced and the process of reparation and regeneration is accelerated [08291].

Tendon and muscle injuries are common in elite and weekend athletes. Treatment of these injuries in both groups is rapidly evolving. In this context, platelet-rich plasma (PRP) has emerged as a potential solution. PRP is a fraction of whole blood containing concentrated growth factors and proteins. These cytokines direct tissue healing through autocrine and paracrine effects. The number of basic science, animal, and human investigations of PRP for tendon and muscle injuries worldwide has risen sharply in recent years. These studies are helping clinicians better understand the mechanisms of PRP and are guiding novel treatment protocols. In one paper, the value of PRP as a treatment for acute or chronic tendon and muscle disorders is Explorer [08292].

In recent years there have been rapid developments in the use of growth factors for accelerated healing of injury. Growth factors have been used in maxillo-facial and plastic surgery with success and the technology is now being developed for orthopaedics and sports
medicine applications. Growth factors mediate the biological processes necessary for repair of soft tissues such as muscle, tendon and ligament following acute traumatic or overuse injury, and animal studies have demonstrated clear benefits in terms of accelerated healing. There are various ways of delivering higher doses of growth factors to injured tissue, but each has in common a reliance on release of growth factors from blood platelets. Platelets contain growth factors in their alpha-granules (insulin-like growth factor-1, basic fibroblast growth factor, platelet-derived growth factor, epidermal growth factor, vascular endothelial growth factor, transforming growth factor-beta) and these are released upon injection at the site of an injury. Three commonly utilised techniques are known as platelet-rich plasma, autologous blood injections and autologous conditioned serum. Each of these techniques has been studied clinically in humans to a very limited degree so far, but results are promising in terms of earlier return to play following muscle and particularly tendon injury.

The use of growth factors in sports medicine is restricted under the terms of the World Anti-Doping Agency (WADA) anti-doping code, particularly because of concerns regarding the insulin-like growth factor-1 content of such preparations, and the potential for abuse as performance-enhancing agents. The basic science and clinical trials related to the technology are reviewed, and the use of such agents in relation to the WADA code is discussed [09185].

PRGF (plasma rich in growth factors) had been introduced with much basic and some clinical research for maxillofacial surgery by Dr Eduardo Anitua in Vitoria, Spain. After first having published impressive results from his own research field, his group followed up with the first studies in sports medicine as well. In 2007, Peter AM Everts from Eindhoven in The Netherlands defended his impressive thesis on “Autologous platelet-leucocyte enriched gel. Basics and efficacy,” where he used the system to support soft tissue and bone healing. Finally, a method that will accelerate the healing of muscle, tendon, bone and cartilage injuries, shorten the rehabilitation time after musculoskeletal injuries and rejuvenate sleeping cells? is it not perfect? Well, it seems that even though more is better, much more may not be so good, at least not in every term of the word. The issues of preparation procedure, with or without leucocytes, volume of autologous blood, the concentration of growth factors, method of delivery, dose and frequency, the effect of local anaesthesia and anti-inflammatory medication are all unresolved issues. Even more importantly, the risks of side effects have not been fully elucidated, even though it all looks good at this stage. To discuss the use of PRP in a clinical setting, and the need for further research, the International Olympic Committee (IOC) assembled an expert group in May 2010 to critically review the current state of PRP treatment among athletes, aiming to provide recommendations for clinicians, athletes and individual sports governing bodies. The purpose of this consensus paper was furthermore to review the evidence for the clinical effectiveness of PRP, its ergogenic potential and safety, and attempt to reconcile any possible disparity between its increasing popularity and the underlying science supporting its use. Firm recommendations on the effectiveness of PRP in the clinical setting to support the healing processes of muscle, tendon, ligament and cartilage injuries cannot be given. Results of clinical studies on PRP are difficult to interpret, as the methodological quality of published investigations varied substantially. More attention should be paid to the use of solid methods when designing, performing and reporting clinical trials. The final recommendation of the IOC consensus group is to proceed with caution in the use of PRP in clinical practice of sports medicine. The group believes that more work on the basic science needs to be undertaken and that greater rigour should be implemented in developing robust clinical trials to demonstrate the efficacy of PRP [10474].

In 2008, the International Olympic Committee (IOC) published a consensus document on the importance of molecular mechanisms in connective tissue and skeletal muscle injury and healing. This document predicted an increase in the use of autologous growth factors, as it
has indeed happened following that publication. Platelet-rich plasma (PRP) (also referred to as platelet-rich in growth factors, platelet-rich fibrin matrix, platelet-rich fibrin, fibrin sealant, platelet concentrate) is now being widely used to treat musculoskeletal injuries in sports and draws widespread media attention despite the absence of robust clinical studies to support its use. Of the few studies on the effectiveness of PRP in clinical settings published, very few are of sufficient methodological quality that would enable evidence-based decision-making. PRP and its variant forms were originally used in clinical practice as an adjunct to surgery to assist in the healing of various tissues. PRP in an injectable form has been used for the management of common muscle, tendon and cartilage injuries. Currently, PRP is not considered as a drug or a therapeutic substance, and so it does not have the usual regulatory requirements that would generally be needed for a substance used in regular clinical practice [10432].

In broad terms, PRP may be defined as a volume of the plasma fraction of autologous blood having a platelet concentration above baseline, and is therefore a concentrated source of autologous platelets. Platelets contain a number of growth factors that play an important role in the healing of injured tissue. PRP is prepared from a volume of autologous blood using extracorporeal blood processing techniques such as blood cell savers/separators, table-top devices (centrifuges) and filtration methods. This volume may contain variable concentrations of red and white cells depending on the specific preparation technique that is used. Not only can PRP be prepared in a variety of methods, but it can be administered in various forms; this diversity is reflected by the number of terms used to describe the product. These variations will inevitably influence the composition and potential effectiveness of the biologically active material. Allogenic fibrin glue was originally described in 1970, and is formed by polymerising fibrinogen with thrombin and calcium. The first reference in the scientific literature to the use of PRP in clinical practice dates back to 1987, when PRP was used as an autologous transfusion component after open heart surgery to prevent the need for a homologous blood product transfusion. In 1990, an autologous fibrin gel (fibrin sealant or fibrin glue) was introduced; a biomaterial with haemostatic and adhesive properties. In 1999, the first autologous PRP prepared from a small quantity of blood was described. Despite limited scientific support, musculoskeletal practitioners began using PRP for the management of cartilage problems as early as 2003. The use of PRP in many fields of medical practice has recently expanded rapidly, with many articles being published. This results in part from its relative ease of use, relatively low cost and a strong commercial industry investment, with the yet unsubstantiated promise that it may prove to be highly effective. In particular, in athletes with sporting injuries, especially in elite athletes where there is a relative urgency to facilitate a rapid return to competition, the use of PRP has expanded rapidly [10432].

Platelets are cytoplasmic fragments of megakaryocytes that are formed in the bone marrow. They are the smallest of the blood components, with an irregular shape and a diameter of 2-3 µm. They lack nuclei, but contain organelles and structures such as mitochondria, microtubules and three forms of granules (alpha, beta and gamma). The alpha-granules, bound by a membrane, are formed during megakaryocytes maturation and are about 200 to 500 nm in diameter. There are approximately 50 to 80 granules per formed platelet. They contain more than 30 bioactive proteins, many of which play a role in haemostasis or tissue healing. However, the entire and exact function of these proteins remains to be elucidated. These proteins are accumulated in alpha granules, and platelets contain distinct subpopulations of α granules that undergo differential release during activation, a potentially important point in understanding how PRP is activated and acts. Platelets contain, synthesise and release large amounts of biologically active proteins that promote tissue regeneration. Researchers have identified more than 1100 types of proteins inside platelets or on their surface. The most commonly studied platelet proteins include platelet-derived
growth factor (PDGF), transforming growth factor (TGF-β), platelet-derived epidermal growth factor (PDEGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), fibroblastic growth factor (FGF), epidermal growth factor (EGF) and cytokines including proteins such as platelet factor 4 (PF4) and CD40L [10432].

The basic premise of PRP use in clinical practice is to facilitate the application of autologous plasma and platelet-derived proteins, in addition to developing at the desired location a fibrin scaffold that can act as a temporary matrix for cell growth and differentiation to assist repair in the injured tissue. PRP can be prepared in a laboratory, an operating theatre or an appropriate room in the outpatient clinic from blood collected in the immediate pretherapeutic period. A sterile technique is followed when blood withdrawing, preparing and applying PRP. PRP can be applied percutaneously or during an open surgical procedure as fluid injections, gel or releasate serum, or mixed with other biological active materials such as bone and ligament grafts. During open procedures, PRP is activated to form a gelatinous mass to facilitate ease of application. During closed procedures, more applicable to sporting injuries such as soft tissue muscle and tendon injuries, PRP is injected by a syringe in a fluid form. It is recommended that the injections are administered under ultrasound guidance, thus ensuring the exact location of the product placements. Platelets begin to actively secrete these proteins within 10 min of clotting, and more than 95 percent of the presynthesised growth factors are secreted within 1 h. After the initial burst of growth factors, the platelets synthesise and secrete additional growth factors for the remaining several days of their life span. When using anticoagulated PRP, activation is critical, as clotting results in the release of growth factors from the α-granules (degranulation) of the platelets. PRP may be activated immediately before application. Alternatively, activation can occur in vivo, that is, with or after the injection in the tissue of interest. There is no consensus on the timing of PRP activation, or even whether activation is necessary at all. Furthermore, there is currently no consensus on whether the PRP is better activated in vitro and placed in vivo, or whether we allow the local environment (in vivo) to activate. In vitro, the application of PRP enhances gene expression of the extracellular matrix proteins, collagen production and tenocyte proliferation. Studies demonstrated the mitogenic activity of PRP, and also that stimulated tenocytes synthesise important growth factors such as VEGF and HGF, suggesting a beneficial effect for the management of tendon injuries by inducing cell proliferation and promoting the synthesis of angiogenic factors during the healing process [10432].

In addition to healing ability, PRP may also contain antibacterial effects that could demonstrate clinical benefits. PRP or platelet-leucocyte rich plasma prepared by two-step centrifugation of whole blood contains high concentrations of platelets and leucocytes. Both platelets and leucocytes play an important role in antimicrobial defence by performing opsonophagocytosis, chemotaxis and oxidative microbialic activity. Furthermore, platelets and leucocytes can release a variety of small cationic peptides (antibacterial peptides) that, upon contact with pathogens, exert bactericidal activity via a non-oxidative mechanism. Another potential advantage is that in vitro and in vivo data have shown that these peptides possess potent microbicidal activities with minor cytotoxicity for relevant mammalian cells. Thus, PRP may act in cooperation with the host immune defence system to defend invasion of pathogens. There are no published applications of the antibacterial effect of PRP in sports medicine. The antibacterial effects of PRP are transient, lasting for only 2-6 h. In summary, the antimicrobial effect of PRP and its use in clinical practice is, as its role in healing and repairing cells and tissue, yet to be fully elucidated. However, there could be a future use for PRP in both the prophylaxis of infection, in particular for surgical wounds, and as adjuvant to normal treatment regimes [10432].

Muscle strain and contusion injuries are common in sports, and result in time loss from training and competition. In many sports, particularly the football codes, muscle injuries are
the single largest cause of time loss from injury. Historically, the management of muscle injuries has involved the use of various stretching and strengthening regimes underpinned by a graduated return to activity and subsequent return to sporting competition. These management strategies lack sound scientific support. The rapid return to functional activity and minimisation of recurrence is the goal of any management intervention. In the past, there has been little direct intervention. However, to facilitate an earlier return to sporting competition and with less risk of injury recurrence, invasive techniques using various substances are currently being considered for use. These include traumeel (a homeopathic anti-inflammatory), actovegin (protein-free extract obtained from filtered calf blood), growth factors such as IGF-1 and PRP. None of these proposed interventions, however, have any evidence base for their use in the treatment of muscle injuries. While the use of recombinant growth factors for muscle injuries has a strong theoretical and scientific basis, cost, side effects and prohibition by World Anti-Doping Agency (WADA) contraindicate their use in athletes. While acknowledging that the mode of delivery of growth factors (bolus vs sustained release) may impact significantly upon the clinical outcome in injured muscle tissue, the recognised physiological benefits of recombinant growth factors include the enhancement of muscle regeneration and minimisation of scarring. By contrast, while anecdotally being widely used in elite sport, the use of PRP for acute muscle injuries has little scientific support with very few studies in either animals or athletes. While not strictly PRP, one study investigated the potential of autologous growth factors to enhance recovery from muscle strain injury using autologous conditioned serum (ACS). By injecting 5 ml of autologous ACS, they compared the return to play time of 18 professional athletes with muscle strain injuries treated with ACS, with 11 athletes treated with traumeel and actovegin. While the authors report a significant reduction in return to play time for the treated group (16 vs 22 days), the large number of methodological concerns, including choice of control, lack of randomisation, lack of blinding and potential bias of the MRI, limits its interpretation. In summary, at present there is little scientific support for the use of PRP for the management of muscle strain injuries. This provides challenges for clinicians hoping to utilise this technology to treat this common sporting injury. Optimal timing, dose, volume, frequency, content and postinjection rehabilitation techniques require future clarification in order to provide any coherent guidelines, and future research should address these areas. However, as basic science supports the use of specific growth factors in muscle regeneration with minimisation of muscle scarring, further investigation of the utility of PRP injection is warranted.

Despite the morbidity associated with tendon problems in athletes and an abundance of therapeutic options, management is far from scientifically based, and many of the therapeutic options in common use lack scientific support. Although tendon biopsies show an absence of inflammatory cell infiltration, anti-inflammatory agents (non-steroidal anti-inflammatory drugs and corticosteroids) are commonly used, but their efficacy and effectiveness are dubious. In most instances, the rate of success using anti-inflammatory agents, defined as an improvement of symptoms and return to sport, is in the region of 65 percent, and the time to return to sport ranges from several weeks to several months. PRP is one treatment that is considered option for management of chronic tendon injuries in athletes, with a positive effect of PRP on tendon healing having been established in several animal studies. In one of these studies, PRP was percutaneously injected into the transected rat achilles tendon. This increased tendon callus strength and stiffness by about 30 percent after 1 week, and mechanical testing indicated an improvement in maturation of the tendon callus when compared with controls. Another study showed that locally injected PRP in the rat patella tendon increased the activation of circulation-derived cells and the immunoreactivity for types I and III collagen at the early stages of tendon healing. Finally, the osteoinductive effect of PRP on tendon-to-bone healing was evaluated on a sheep infraspinatus repair model using MRI scan and histology. This study demonstrated an increased formation of new bone and fibrocartilage at the healing site. In summary, there is a lack of well-designed studies to
support the use of PRP in clinical settings in the management of tendon injuries. More research on basic science and the clinical application of PRP needs to be undertaken before there is any comprehensive recommendation for PRP administration in injured human tendons. For each individual athlete and circumstance, a risk/benefit analysis should be performed before embarking on this as yet scientifically unproven therapeutic modality [10432].

Based on long-term clinical experience and thousands of patients being treated, the use of PRP is safe. In musculoskeletal tissues, although no long-term clinical studies with PRP exist, a large number of patients have been treated worldwide. Recently, Wang-Saugusa et al reported that no adverse effects were observed when plasma rich in growth factors was infiltrated in more than 800 patients, many of whom suffered from knee osteoarthritis. As, theoretically, PRP is an autologous preparation, immunogenic reactions or disease transmission should be prevented. However, development of antibodies against clotting factors V and IX leading to life-threatening coagulopathies has been reported. To date, there is no compelling evidence of any systemic effect of local PRP injection. Furthermore, there are no scientific reports suggesting potential cause–effect relationships between growth factors present in PRP and carcinogenesis. Some potential arguments for these considerations include the limited need of PRP injections in clinics (as PRP is not chronically administered) and the short in vivo half-lives and local bioavailability of growth factors produced by PRP [10432].

In 2010, PRP was specifically mentioned in the prohibited list for the first time. Intramuscular PRP injections were prohibited. All other routes of administration, such as intra-articular, intra- or peritendinous were permitted and required only a declaration of use. Note that specific purified or recombinant growth factors (eg, IGF-1, VEGF, PDGF) are explicitly prohibited elsewhere in the list. Growth factors are permitted only when part of platelet-derived preparations from the centrifugation of autologous whole blood. There was concern by the WADA List Expert Group that growth factors contained in PRP may stimulate muscle satellite cells and increase muscular size and strength (beyond normal healing). However, the different PRP formulations and treatment methodologies, as they exist now, have not been found to increase muscle growth beyond return to a normal physiological state. There are some animal studies that show faster muscle regeneration and recovery to full function following experimentally induced injury, but no enhancement of performance beyond normal. There is a suggestion, but no compelling evidence, of systemic effects. The risk of adverse reactions (fibrosis, infection, carcinogenesis) are theoretical and have not been documented clinically. The use of PRP injections for therapeutic purposes only does not violate the spirit of sport. The prohibition for intramuscular injections of PRP has been deleted in the 2011 Prohibited List. PRP is now permitted by all routes of administration [10432].

There is a limited amount of basic science research on the influence of PRP on the inflammation and repair of connective tissue and skeletal muscle. There is an even greater paucity of well-conducted clinical studies on the use of PRP to manage sport injuries. For clinicians, the generalisability of basic science must be tempered by clinical studies that inherently contain factors controlled for in basic science experiments. For these reasons, the design of robust clinical studies is essential for conclusions to be assigned sufficient validity to be used in clinical practice. Although PRP has been in clinical use for decades, some basic science issues still require further investigation. Several techniques are available to prepare PRP; however, there is no evidence of standardisation of preparation (in terms, for example, of length and speed of centrifugation) and use of PRP. In addition, different methods of preparation may produce different platelet concentrations such as storing the PRP for differing lengths of time before use, using different anticoagulants and variable degrees of other cells such as red and white cells in the PRP preparation. It is therefore
possible that each preparation method may lead to a different product with different biology and potential uses. As stated, all these variables may produce PRPs in which the amount and type of growth factors are different. Therefore, a classification system for different PRPs should be developed and should be used to define the PRPs used by different research and treatment groups. For clinical applications, based on different clinical conditions, the best time to inject PRP must be determined according to the different tissues and body districts. The kinetics of cytokine release from various PRPs with/without other biomaterials needs further investigation, as this may ultimately determine the best time for injection for a given PRP formulation. Furthermore, the tissue-specific effects of PRP should be compared, as the underlying cellular and molecular processes for a particular tissue healing may be markedly quite different. For instance, muscle and bone healing need vascularisation. However, a high degree of vascularisation may not be required for tendon and articular cartilage injuries. In fact, it is plausible that the effect of PRP on a given tissue is influenced by the microenvironment within that tissue, and therefore PRP activation may not be required prior its use. Lastly, the optimal use of PRP for regenerative medicine is still under investigation. Although application of the PRP may enhance mesenchymal stem cell proliferation and migration, exposure of cells to PRP may also limit differentiation of those cells into the appropriate cell lineages. The question arises in this consensus statement as to whether clinicians should use a treatment with very little scientific evidence supporting its clinical efficacy and with limited evidence supporting its safety. Medical ethics is anchored by the concepts of beneficence (doing good) and non-maleficence (do no harm). Medical ethics includes the concept of patient autonomy (self-determination). Western medicine tends to hold to the principle that patients can determine their treatment themselves, even if beneficence or non-maleficence is not proved. For the doctor, non-maleficence is the principal determinant of medical practice. While limited, current evidence suggests the use of PRP to be safe, and therefore the non-maleficence principal is probably upheld; however, there are few if any studies that document adverse or serious adverse events, and there are no studies at all looking at long-term effects. As there is little scientific evidence that PRP injections are of clinical benefit, beneficence is at this time not proven. Current medical ethics generally allows clinicians to make an individual choice to prescribe treatments that have not shown beneficence as long as the treatment is non-maleficient. With respect to PRP, its increasing popularity appears to have outreached in some respects the principle of medical ethics and the usual conservatism that new treatments are taken up by the clinicians. Part of the answer to this would be that PRP is presently marketed and widely perceived as a natural healing method with the implications of minimal maleficence [10432].

Platelet-rich plasma (PRP) is a new technology focused on enhancing the healing response after injury of different tissue types. PRP is prepared by withdrawal of patients’ peripheral blood and centrifugation to obtain a highly concentrated sample of platelets, which undergo degranulation to release growth factors with healing properties. It also contains plasma, cytokines, thrombin, and other growth factors that are implicated in wound healing and have inherent biological and adhesive properties. The prepared concentrate is then injected back into the patient at the site of morbidity. This may be intrasional, intra-articular, or surrounding the involved tissue bed. PRP preparations have been used therapeutically in various medical fields from implantology to vascular ulcers, with a more recent evolution and promising results in the field of sports medicine and arthroscopy. Sports medicine patients desire a rapid return to their preinjury level of function, and PRP may have certain applications that will speed recovery in cases of tendon, ligament, muscle, and cartilage disorders. In particular, anterior cruciate ligament reconstruction has shown better autograft maturation, improved donor site morbidity, and pain control, in addition to improved allograft incorporation. By acceleration of the biological integration of the graft by use of PRP, patients may undergo faster, more intensive rehabilitation programs and return to sports more rapidly. Because of its autogenous origin, easy preparation, and excellent safety profile, the advent
of PRP has opened another therapeutic door for sports medicine physicians and orthopaedic surgeons. Future directions of PRP include improving the results of arthroscopic and related surgery, in addition to delineating correct dosage, timing, and quantification, as well as ideal techniques of PRP application [10131].

Autologous platelet-rich plasma (PRP) is perceived to accelerate healing in musculoskeletal injuries. PRP is increasingly used in situations that require rapid return-to-play, which, in the professional sports arena, translates to fame and money. Human blood platelet counts are approximately 200 000/ml. PRP is an autologous concentration of human platelets above this in a small volume of plasma. Reports vary regarding the platelet concentration and different growth factors present in the PRP concentrate. Also, there are many preparation protocols, kits, centrifuges and methods to trigger platelet activation before use. The same is true for application methods, including using injectable activated PRP liquid concentrate versus implanting a fibrin scaffold, optimal timing of injection and the specific volume to use. Almost every major manufacturer in the orthopaedic and sports medicine world markets a different commercial kit. Some claim to produce a better quantity and quality of PRP than their competitors from the same amount of blood from the same patient. Each method to concentrate platelets leads to a different product with different biology and potential uses, with a high variation (3 to 27-fold) in growth factor concentration and in the kinetics of release. Most techniques yield a PRP concentrate of approximately 10 percent of the blood volume taken (e.g. 20 ml of whole blood would result in approximately 2 ml of PRP). These differences might be of relevance to clinical management, although they have not been systematically studied. PRP preparations containing only moderately elevated platelet concentrations may be the ones to induce optimal biological benefit, with lower platelet concentrations leading to suboptimal effects, and higher platelet concentrations to inhibitory effects. Other authors have stated that the “therapeutic dose” of PRP would be at least four to six times higher than the normal platelet count. To complicate things, the actual growth factor content is not well correlated with the platelet count in whole blood or in PRP, and there is no evidence that gender or age affects platelet count or growth factor concentrations. A few studies have categorised the different platelet concentrates according to the presence or absence of white blood cells (WBC): either pure platelet-rich plasma (P-PRP), in which WBC have been intentionally eliminated from the PRP, or leucocyte and platelet-rich plasma (L-PRP), possibly from the inability of the kit to differentiate between the WBC and the PRP layers. A positive or negative effect of WBC cannot be generalised to all tissues and clinical conditions. This issue requires further investigation, as the WBC content in the preparation injected has never been systematically studied. There is no direct evidence that neutrophils play a beneficial role in muscle repair or regeneration. This exacerbation of injury and/or delay in muscle regeneration may be of major importance for injuries managed with PRP. This, together with the improved homogeneity of P-PRP and its reduced donor-to-donor variability, supports some PRP production techniques that claim to be clinically superior. L-PRP injected for soft tissue injuries might induce more local pain than P-PRP. Platelets by themselves do reduce pain. L-PRP reduces postoperative pain, although here the contribution of the WBC to the overall effect observed remains unclear. Over this background, the sceptics point out that, given the well concerted healing cascade that has evolved over millions of years, it is not easy to understand how a single or even a few injections of a cocktail of growth factors at variable, and at present not well codified, times from the injury will produce a lasting beneficial effect on a wide variety of conditions [10132].

One article aimed to provide a comprehensive review of the current literature that pertains to the therapeutic use of autologous platelet-rich plasma (PRP). The basic science literature regarding the role of growth factors in mediating the healing process and the laboratory data from in vitro and in vivo studies that evaluated PRP are reviewed. Subsequently, the current evidence regarding PRP efficacy from animal models, human surgical studies, and human
Clinical studies are presented. A critical analysis of the literature follows, and the article concludes with the authors' perspectives on the state of PRP as a potentially efficacious bioregenerative treatment option for musculoskeletal and sports medicine applications. The relevant articles in this review were obtained via PubMed literature searches for PRP publications that pertain to musculoskeletal and sports medicine conditions. This article is not intended to be a formal meta-analysis [11151].

Growth factor technologies are increasingly used to enhance healing in musculoskeletal injuries, particularly in sports medicine. Two such products; platelet-rich plasma (PRP) and autologous blood, have a growing body of supporting evidence. No previous trial has directly compared the efficacy of these two methods. Growth factor administration improves tissue regeneration in patients who have failed to respond to conservative therapy. Study design A prospective, double-blind, randomised trial. Elbow tendinopathy patients who had failed conservative physical therapy were divided into two patient groups: PRP injection (N=80) and autologous blood injection (ABI) (n=70). Each patient received two injections at 0 and 1 month. Patient-related tennis elbow evaluation (PRTEE) was recorded by a blinded investigator at 0, 1, 3 and 6 months. The main outcome measure was PRTEE, a validated composite outcome for pain, activities of daily living and physical function, utilising a 0-100 scale. At 6 months the authors observed a 66 percent success rate in the PRP group versus 72 percent in the ABI group. There was a higher rate of conversion to surgery in the ABI group (20 %) versus the PRP group (10 %). In patients who are resistant to first-line physical therapy such as eccentric loading, ABI or PRP injections are useful second-line therapies to improve clinical outcomes. In one study, up to seven out of 10 additional patients in this difficult to treat cohort benefit from a surgery-sparing intervention [11152].

To review the evidence for the clinical utilization of autologous plasma products in the management of muscle strain injuries a systematic review using EMBASE and MEDLINE (up to March 2010) was done. There is no level 1, 2, and 3 evidence for the use of autologous plasma products in muscle strain injuries. Furthermore, significant methodological limitations impact on the interpretation of the few published studies in this field. Therefore it can be concluded that although basic science and the use of recombinant growth factors in animal models support the concept of applying growth factors to acute muscle injuries, it is unclear if this evidence can be directly translated to reflect outcomes from platelet-enriched plasma. There remain a large number of unanswered questions, including the principle questions regarding safety and efficacy, which require appropriate scientific investigation. It is incumbent on sports physicians wishing to enhance athlete care, together with researchers, to search for these answers [11153].

Platelet-rich plasma (PRP) is an autologous blood-derived product with an increased concentration of platelets in plasma, which are used to deliver supraphysiological levels of growth factors. Platelet-rich plasma has been used in many fields, including oral, maxillofacial, and plastic surgery. Its use in sports medicine has been increasing after recent evidence and media publicity suggest that it may augment the treatment of muscle strains, as well as tendon and ligament healing. Basic science and animal studies show promising results, but high-level clinical trials have yet to prove its efficacy. With increasing media coverage on the use of PRP in athletes, it is paramount that orthopedic surgeons and sports medicine physicians understand the various methods of preparation and administration, potential clinical applications, and available clinical results to best counsel patients on its advantages and disadvantages [11154].

At present, no drugs are available to hasten restoration of muscle function after injury. Platelet-rich plasma (PRP) therapies may help athletes by promoting muscle regeneration. A systematic review assessing the evidence base for PRP therapies in the management of
muscle injuries was based on a computerized literature search, citation tracking and hand searching for original studies assessing the effect of PRP therapies on skeletal muscle cell biology, skeletal muscle repair, or regeneration in animals or humans was performed. No randomized trials have studied the merits of PRP injections for muscle healing. Clinical studies indicated that PRP therapies may enhance muscle repair after strain or contusion, and laboratory data indicated that they can enhance diverse aspects of myogenesis. However muscle injuries present a complicated picture that includes many components other than muscle cells, such as blood vessels, connective tissue and neural components. The field is relevant but under-researched. No PRP formulation has yet displayed proven solid evidence for the stimulation of healing and recovery after sports muscle injuries. Therefore, major issues, including standardization of formulations and application procedures, need to be addressed to inform clinical studies before recommending best practice guidelines [11155]. Platelet-rich plasma (PRP) has been increasingly used in sports medicine applications. Platelets are thought to release growth factors important in wound healing, including transforming growth factor (TGF-beta1), platelet-derived growth factor (PDGF-AB), and vascular endothelial growth factor (VEGF). However, little is known about the effect of platelet activator choice on growth factor release kinetics. Platelet-rich plasma aliquots were activated with either thrombin or collagen. A control group of whole blood aliquots was clotted with thrombin. Supernatant containing the released growth factors was collected daily for 1 week. Levels of TGF-beta1, PDGF-AB, and VEGF were measured using enzyme-linked immunosorbent assay (ELISA). The use of thrombin as an activator resulted in immediate release of TGF-beta1 and PDGF-AB, while the collagen-activated PRP clots released similar amounts each day for 5 days. The use of collagen as an activator resulted in an 80 percent greater cumulative release of TGF-beta1 from the PRP aliquots over 7 days. Concentrating platelets to 3 times the systemic blood level resulted in a 3-fold higher release of TGF-beta1, 2.5-fold greater release of PDGF, and 5-fold greater release of VEGF when compared with whole blood control clots, but no significant differences in the timing of release were noted. These experiments demonstrated that the choice of platelet activator can significantly influence the release kinetics of cytokines from PRP, with thrombin resulting in an immediate release and collagen having a more sustained release pattern. The level and rate of growth factor release depends on the selected platelet activator, a factor that should be considered when selecting a PRP system for a given application [11156]. Tissue repair in musculoskeletal lesions is often a slow and sometimes incomplete process. In sports patients or professional athletes, the impact of musculoskeletal lesions on life and work is great, and the fast recovery of full efficiency and return to competition is of primary importance. The clinical improvement offered by available treatments is not always sufficient for highly demanding patients to return to their previous level of activity. The search for a minimally invasive solution to improve the status of the chondral surface of the injured joint is therefore highly desirable, especially in these patients. Platelet-rich plasma (PRP) is a procedure that allows to obtain a natural concentration of autologous growth factors. The attractive possibility to use the patients’ own growth factors to enhance reparative process in tissues with low healing potential, the promising preliminary clinical findings and the safety of these methods, explain the wide application of this biological approach. The aim of one review is to analyse the existing published studies to look for scientific evidence in preclinical studies or in the results obtained through PRP application in humans that supports the efficacy of PRP and its use for the treatment of tendinous, ligamentous, cartilaginous and muscular injuries. The analysis of the literature shows promising preclinical results but contradictory clinical findings for the treatment of sport injuries. High-quality studies are required to confirm these preliminary results and provide scientific evidence to support its use [11157].
The International Olympic Committee consensus panel have produced a clear summary of
the current understanding of the basic and clinical science relating to platelet-rich plasma
(PRP) as the body of published literature allows. While there was initially great hope in sports
medicine circles that PRP would become the magic bullet for injuries, recent trials have failed
to provide the conclusive evidence so desired, which is not surprising. The more we learn
about tissue regeneration, the more apparent it becomes how complex a process it is. Tissue
regeneration is not a passive phenomenon, instead it is a highly coordinated interplay of
multiple cell lines at different stages of maturation. Different cellular and humoral
components play their different roles. The process can be likened to the repair of a
collapsed building. Consider muscle injury. The initial cells on the scene, due to bleeding, are
platelets, but they appear to be relatively passively involved – alarm bells that sequester and
awaken the major players. Platelets release chemotactic factors that attract neutrophils to
clear the debris. However, within 24 h macrophages arrive, akin to the foreman, and it is
these cells that appear to regulate the process from this point onwards. If there is any
“brains” or “thinking” to tissue repair, it would seem to be the macrophages doing it. Next
come the actual builders. Fibroblasts are activated to produce a collagen infrastructure,
and satellite cells to form myocytes and finally myotubes, merging to become a single strand of
muscle fibre. So where do “growth factors” come into it? These proteins are simply the
communications being sent between the foreman and his workers. The messages are very
simple – move or stay put, divide or do not divide, live or die, make collagen, etc. In biology
we use the terms chemotaxis, mitosis, quiescence, apoptosis and protein biosynthesis. The
point is this: growth factors are just the messenger molecules used by one cell to send an
instruction to another, they are not the person giving the orders. Unfortunately the language
of growth factors is very different to English. We are used, pretty much, to one word having
roughly one meaning. However, growth factor “words” are more like a tonal language,
Mandarin. In these languages the same word can have multiple different meanings
depending on how you pronounce it. In the same manner, growth factors can produce
varying effects depending on their concentration, time of release, point in cell cycle and
recipient cell. Therefore, trying to pin any one growth factor down to one particular action can
be pointless. For example transforming growth factor beta1 is commonly associated with
fibrosis, but it can also stimulate regeneration or fibrosis, chemoattraction or stasis,
depending on its concentration, target cell, and sequence within the tissue regeneration
process. This is where PRP has the potential to fall down. PRP has variable and inconsistent
content and concentration. There is no consensus on the timing of the injection. What does a
random bolus injection into an injury achieve? Are we sending these effector cells a clear
and coordinated set of instructions with PRP? Or are they being sent a completely confusing
message? It would seem hopelessly optimistic and naive to presume that we are accurately
reproducing biological complexity. Whether or not PRP is eventually proved or disproved in
sports injuries, it is nevertheless a good start. The sports medicine world has woken to the
possibilities of regenerative medicine, and is trying to be scientific in the development of
novel therapies. In the future we will have improved understanding of how the complex and
overlapping processes of tissue regeneration are controlled by coordinating cells, stem cells,
effector cells and the messenger molecules that they employ, and more importantly, how to
manipulate these processes for a beneficial effect [11158].

Full-thickness chondral defects and early osteoarthritis continue to present major challenges
for the patient and the orthopaedic surgeon as a result of the limited healing potential of
articular cartilage. The use of bioactive growth factors is under consideration as a potential
therapy to enhance healing of chondral injuries and modify the arthritic disease process. It
was reviewed the role of growth factors in articular cartilage repair and identified specific
growth factors and combinations of growth factors that have the capacity to improve cartilage
regeneration. Additionally, it was discussed the potential use of platelet-rich plasma,
autologous-conditioned serum, and bone marrow concentrate preparations as methods of combined growth factor delivery. A PubMed search was performed using key words cartilage or chondrocyte alone and in combination with growth factor. The search was open for original manuscripts and review papers and open for all dates. From these searches it was selected manuscripts investigating the effects of growth factors on extracellular matrix synthesis and excluded those investigating molecular mechanisms of action. By modulating the local microenvironment, the anabolic and anticatabolic effects of a variety of growth factors have demonstrated potential in both in vitro and animal studies of cartilage injury and repair. Members of the transforming growth factor-beta superfamily, fibroblast growth factor family, insulin-like growth factor-I, and platelet-derived growth factor have all been investigated as possible treatment augments in the management of chondral injuries and early arthritis. It was concluded that the application of growth factors in the treatment of local cartilage defects as well as osteoarthritis appears promising; however, further research is needed at both the basic science and clinical levels before routine application [11467].

Previous studies of bioactive molecules in platelet-rich plasma (PRP) have documented growth factor concentrations that promote tissue healing. However, the effects of leukocytes and inflammatory molecules in PRP have not been defined. The hypothesis for one study was that the concentration of growth factors and catabolic cytokines would be dependent on the cellular composition of PRP. Platelet-rich plasma was made from 11 human volunteers using 2 commercial systems: Arthrex ACP (Autologous Conditioned Plasma) Double Syringe System (PRP-1), which concentrates platelets and minimizes leukocytes, and Biomet GPS III Mini Platelet Concentrate System (PRP-2), which concentrates both platelets and leukocytes. Transforming growth factor-beta1 (TGF-beta1), platelet-derived growth factor-AB (PDGF-AB), matrix metalloproteinase-9 (MMP-9), and interleukin-1beta (IL-1beta) were measured with enzyme-linked immunosorbent assay (ELISA). The PRP-1 system consisted of concentrated platelets (1.99×) and diminished leukocytes (0.13×) compared with blood, while PRP-2 contained concentrated platelets (4.69×) and leukocytes (4.26×) compared with blood. Growth factors were significantly increased in PRP-2 compared with PRP-1. The PRP-1 system did not have a higher concentration of PDGF-AB compared with whole blood. Catabolic cytokines were significantly increased in PRP-2 compared with PRP-1. Significant, positive correlations were found between TGF-beta1 and platelets, PDGF-AB and platelets, MMP-9 and neutrophils, IL-1beta and neutrophils, and IL-1beta and monocytes. It was thus concluded that growth factor and catabolic cytokine concentrations were influenced by the cellular composition of PRP. Platelets increased anabolic signaling and, in contrast, leukocytes increased catabolic signaling molecules. Platelet-rich plasma products should be analyzed for content of platelets and leukocytes as both can influence the biologic effects of PRP. Depending on the clinical application, preparations of PRP should be considered based on their ability to concentrate platelets and leukocytes with sensitivity to pathologic conditions that will benefit most from increased platelet or reduced leukocyte concentration [11468].

The therapeutic use of autologous platelet-rich plasma constitutes a relatively new biotechnology that has been a breakthrough in the stimulation and acceleration of soft-tissue and bone healing. The efficiency of this process lies in the local and continuous delivery of a wide range of growth factors and proteins, mimicking the needs of the physiological wound healing and reparative tissue processes. Consequently, the application of platelet-rich plasma has been extended to many different fields, including orthopedics, sports medicine, dentistry, cosmetic and periodontal medicine and cosmetic, plastic and maxillofacial surgery. This article highlights the use of this technology and discusses some of the obstacles and challenges that need to be addressed to maintain progress in this field [06169].
Mechanical stimulation improves the repair of ruptured tendons. Injection of a platelet concentrate (platelet-rich plasma, PRP) can also improve repair in several animal models. In a rat Achilles tendon transection model, 1 postoperative injection resulted in increased strength after 4 weeks. Considering the short half-lives of factors released by platelets, this very late effect calls for an explanation. It was studied the effects of platelets on Achilles tendon regenerates in rats 3, 5 and 14 days after transection. The tendons were either unloaded by Botulinum toxin A (Botox) injections into the calf muscles, or mechanically stimulated in activity cages. No Botox injections and ordinary cages, respectively, served as controls. Repair was evaluated by tensile testing. At 14 days, unloading (with Botox) abolished any effect of the platelets and reduced the mechanical properties of the repair tissue to less than half of normal. Thus, some mechanical stimulation is a prerequisite for the effect of platelets at 14 days. Without Botox, both activity and platelets increased repair independently of each other. However, at 3 and 5 days, platelets improved the mechanical properties in Botox-treated rats. It was interpreted that platelets influence only the early phases of regeneration, but this allows mechanical stimulation to start driving neo-tendon development at an earlier time point, which kept it constantly ahead of the controls [06170].

Platelet-rich plasma (PRP) is an autologous blood product used to treat acute and chronic tendon, ligament, and muscle injuries in over 86,000 athletes in the United States annually. The World Anti-Doping Agency (WADA) banned intramuscular PRP injections in competitive athletes in 2010 because of concerns that it may increase performance-enhancing growth factors. The ban on PRP was removed in 2011 because of limited evidence for a systemic ergogenic effect of PRP, but the growth factors within PRP remain prohibited. One study aimed to quantify the effect of PRP injection on systemic growth factors with performance-enhancing effects and to identify molecular markers to detect treated athletes. Six ergogenic growth factors monitored by WADA-human growth hormone (hGH), insulin-like growth factor-1 (IGF-1), insulin-like growth factor binding protein-3 (IGFBP-3), basic fibroblast growth factor (bFGF or FGF-2), vascular endothelial growth factor (VEGF), and platelet-derived growth factor-BB (PDGF-BB) were measured in 25 patients before (baseline) and at 0.25, 3, 24, 48, 72, and 96 hours after intratendinous leukocyte-rich PRP injection. Eating and exercise were prohibited for 3 hours before testing. Growth factors were quantified by enzyme-linked immunosorbent assay, and the change relative to each patient's baseline was calculated. Relative to serum, PRP contained significantly more bFGF (226 vs 5 pg/mL), VEGF (1426 vs 236 pg/mL), and PDGF-BB (26,285 vs 392 pg/mL), but IGF-1 and hGH were not elevated. Serum levels increased significantly for IGF-1 at 24 and 48 hours, for bFGF at 72 and 96 hours, and for VEGF at 3, 24, 48, 72, and 96 hours after PRP injection. Additionally, VEGF was increased in all 25 patients after PRP treatment. It was concluded that serum IGF-1, VEGF, and bFGF levels are significantly elevated after PRP injection, supporting a possible ergogenic effect of PRP. An indirect marker for hGH doping, the product of IGFBP-3 × IGF-1, also significantly increased after PRP. Platelet-rich plasma appears to trigger an increase in circulating growth factors through activating biological pathways rather than by serving as a vehicle for the direct delivery of presynthesized growth factors. Elevated VEGF was observed in all patients after PRP, and ≥88 percent of patients had elevated VEGF at each time point from 3 to 96 hours after PRP, suggesting that VEGF may be a sensitive molecular marker to detect athletes recently treated with PRP. This is the first and only adequately powered study of the systemic effects of PRP. It was provided evidence that VEGF could serve as a useful molecular marker to detect athletes treated with PRP [12267].

Platelet rich plasma (PRP) is a powerful new biologic tool in sports medicine. PRP is a fraction of autologous whole blood containing and increased number of platelets and a wide variety of cytokines such as platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factor beta-1 (TGF-B1), fibroblast growth factor (FGF), Insulin-like growth factor-1 (IGF-1) among many others. Worldwide interest in
this biologic technology has recently risen sharply. Basic science and preclinical data support the use of PRP for a variety of sports related injuries and disorders. The published, peer reviewed, human data on PRP is limited. Although the scientific evaluation of clinical efficacy is in the early stages, elite and recreational athletes already use PRP in the treatment of sports related injuries. Many questions remain to be answered regarding the use of PRP including optimal formulation, including of leukocytes, dosage and rehabilitation protocols. In one review, a classification for platelet rich plasma is proposed and the in-vitro, preclinical and human investigations of PRP applications in sports medicine will be reviewed as well as a discussion of rehabilitation after a PRP procedure. The regulation of PRP by the World Anti-Doping Agency will also be discussed. PRP is a promising technology in sports medicine; however, it will require more vigorous study in order to better understand how to apply it most effectively [12268].

There is strong evidence that exercise affects platelet haemostasis factors, but this potential effect on growth factor concentrations in platelet-rich plasma (PRP) has never been studied. In addition, there is a paucity of studies focusing on the effects of activating agents used in conjunction with PRP. The first aim of this study was to evaluate the effect of exercise on platelet and platelet-derived growth factors (PDGF)-AB, hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) concentrations in PRP. The second aim was to study the effect of the activating agent calcium chloride (CaCl₂) on growth factor concentration in relation to different exercise states. Ten healthy participants performed 1 h of submaximal exercise with blood being withdrawn immediately pre, post and 18 h following. PRP was prepared in each condition in both an activated CaCl₂ and non-activated form. Concentrations of PDGF-AB, HGF, IGF-1 and VEGF were evaluated using standard ELISA systems. Exercise had no significant effect on platelet concentration, but significantly suppressed both VEGF and PDGF-AB concentrations. Exercise state had no significant effect on IGF-1 or HGF concentration. Activation with CaCl₂ resulted in a significant increase in PDGF-AB and IGF-1 concentrations, unchanged VEGF and significantly reduced HGF concentrations. It was concluded that exercise significantly impacts on PDGFs in PRP with significantly reduced concentrations of VEGF and PDFG-AB. Furthermore, the activation of PRP with CaCl₂ results in a differentiated GF release from platelets. These relevant factors can potentially influence outcome in daily clinical practice and are recommended to be accounted for in future study design [13342].

Muscle strains are one of the most common injuries induced by sporting activities. According to the severity and location of the lesion different treatments can be delivered, including growth factor therapy through administration of autologous platelet-rich plasma (PRP) during the early period following the injury. PRP is easily obtained from centrifugation of the patient's own blood and contains high concentration of platelets, which release growth factors and cytokines by adding thrombin. This process improves tissue repair in cartilage, tendons, ligaments, muscles and bones by down-regulation of inflammatory mediators and synthesis of regenerative proteins. Furthermore, PRP has antimicrobial properties that may contribute to reduce pain⁸ and to prevent infections. PRP is administered by local injection or applied directly in the form of gel into the site of injury. Initially many PRP injections were done without imaging guidance by either palpating the site of tenderness or using a peppering technique to distribute the gel uniformly. The use of ultrasound (US)-guided injection has led to a more precise and direct visualisation of the exact site of the pathology and of the injected blood products in the region of lesion. Furthermore, sonography allows the position of the needle position to be adjusted in real time¹⁰-¹³. Considering these properties, local US-guided injection of PRP may improve the repair of tendons, muscles, ligaments, cartilage and bone injuries. The aim of one study was to demonstrate the efficacy of ultrasound-guided injection of platelet-rich plasma in muscle strains and the absence of
side effects. Fifty-three recreational athletes were enrolled in the study. The patients were recruited from the Emergency Room in the University Hospital at Parma according to a predefined protocol. Every patient was assessed by ultrasound imaging to evaluate the extent and degree of muscle injuries. Only grade II lesions were treated with three ultrasound-guided injections of autologous platelet-rich plasma every 7 days. Platelet concentrate was produced according to standard methods, with a 10 percent variability in platelet count. The platelet gel for clinical use was obtained by adding thrombin to the concentrates under standardised conditions. Outcomes assessed were: pain reduction, muscle function recovery and return to sports activity, ultrasound-imaging tissue healing, relapses, local infections, and any side effect during the treatment. In all cases muscle lesions healed fully on ultrasound-imaging, the pain disappeared, and muscle function recovery was documented with a return to sports activity. A single patient had a relapse 1 year after treatment. Platelet-rich plasma injected into the injury site is one of the most important factors rendering the treatment effective. To maximise its efficacy the preliminary ultrasound must be done accurately to localise the lesion and guide the needle into the corresponding lesion. According to the current results, which document full muscle recovery and no relapse except for one case, platelet-rich plasma ultrasound-guided injection represents a valid mini-invasive treatment for muscle injuries [13343].

Platelet-rich plasma (PRP) is an autologous blood product used to treat acute and chronic tendon, ligament, and muscle injuries in over 86,000 athletes in the United States annually. The World Anti-Doping Agency (WADA) banned intramuscular PRP injections in competitive athletes in 2010 because of concerns that it may increase performance-enhancing growth factors. The ban on PRP was removed in 2011 because of limited evidence for a systemic ergogenic effect of PRP, but the growth factors within PRP remain prohibited. To quantify the effect of PRP injection on systemic growth factors with performance-enhancing effects and to identify molecular markers to detect treated athletes six ergogenic growth factors monitored by WADA-human growth hormone (hGH), insulin-like growth factor-1 (IGF-1), insulin-like growth factor binding protein-3 (IGFBP-3), basic fibroblast growth factor (bFGF or FGF-2), vascular endothelial growth factor (VEGF), and platelet-derived growth factor-BB (PDGF-BB)-were measured in 25 patients before (baseline) and at 0.25, 3, 24, 48, 72, and 96 hours after intratendinous leukocyte-rich PRP injection. Eating and exercise were prohibited for 3 hours before testing. Growth factors were quantified by enzyme-linked immunosorbent assay, and the change relative to each patient's baseline was calculated. Relative to serum, PRP contained significantly more bFGF (226 vs 5 pg/mL), VEGF (1426 vs 236 pg/mL), and PDGF-BB (26,285 vs 392 pg/mL), but IGF-1 and hGH were not elevated. Serum levels increased significantly for IGF-1 at 24 and 48 hours, for bFGF at 72 and 96 hours, and for VEGF at 3, 24, 48, 72, and 96 hours after PRP injection. Additionally, VEGF was increased in all 25 patients after PRP treatment. It was concluded that serum IGF-1, VEGF, and bFGF levels are significantly elevated after PRP injection, supporting a possible ergogenic effect of PRP. An indirect marker for hGH doping, the product of IGFBP-3 × IGF-1, also significantly increased after PRP. Platelet-rich plasma appears to trigger an increase in circulating growth factors through activating biological pathways rather than by serving as a vehicle for the direct delivery of presynthesized growth factors. Elevated VEGF was observed in all patients after PRP, and ≥88 percent of patients had elevated VEGF at each time point from 3 to 96 hours after PRP, suggesting that VEGF may be a sensitive molecular marker to detect athletes recently treated with PRP. Thus, there are present evidence that PRP contains and may trigger systemic increases in substances currently banned in competitive athletes. Finally, it was provided evidence that VEGF could serve as a useful molecular marker to detect athletes treated with PRP [13344].

Platelet-rich plasma (PRP) is an autologous concentration of blood-derived human platelets in a small volume of plasma. The types of PRP vary according to the commercial preparation
system used, the platelet concentration, or the anticoagulant or activator used. Autologous conditioned plasma is an autologous concentration of human platelets in plasma 2 to 4 times greater than that which is found in blood at baseline. Platelets are important to the normal healing response of tissue by the local secretion of growth factors and recruitment of reparative cells in an area of injury. PRP is theorized to create an optimal healing environment in a region of tissue injury. This is a literature review of currently published studies using PRP in orthopedic injuries. It was performed a literature search in PubMed and Medline in April 2013 and concluded that given the number of variations of PRP available and the lack of high-level published studies, there is insufficient evidence to conclusively support its clinical use [13345].

Platelet-rich plasma (PRP) as a clinical treatment for bone, muscle, tendon, and cartilage injury has gained popularity in the field of orthopedic sports medicine. The use of a patient's own blood is an appealing aspect of PRP treatment, as the resulting plasma preparation is considered relatively benign in comparison with more common, potentially caustic treatments such as corticosteroids and anesthetics. Although appealing, the autologous nature of PRP introduces variability to plasma preparations, creating challenges for both the researcher and the clinician. Differences in patients at the time of blood draw result in plasma preparations that vary within as well as between patients. This variability is compounded by the multitude of protocols and devices available for procuring PRP. The variability of components and its effects on dosage should be considered in single or consecutive treatments of PRP [13346].

Platelet-rich plasma (PRP) has been advocated for the biological augmentation of tissue healing and regeneration through the local introduction of increased levels (above baseline) of platelets and their associated bioactive molecules. In theory, the increased levels of autologous growth factors and secretory proteins provided by the concentrated platelets may enhance the wound healing process, especially in degenerative tissues or biologically compromised individuals. Although PRP has been increasingly utilized in the treatment of a variety of sports-related injuries, improvements in healing and clinical outcomes have not been universally reported. One reason for this may be the fact that all PRP preparations are not the same. Variations in the volume of whole blood taken, the platelet recovery efficacy, the final volume of plasma in which the platelets are suspended, and the presence or absence of white blood cells, and the addition of exogenous thrombin to activate the platelets or calcium chloride to induce fibrin formation, can all affect the character and potential efficacy of the final PRP product. One article reviewed the basic principles involved in creating PRP and examine the potential basic scientific significance of the individual blood components contained in the various forms of PRP currently used in sports medicine [13347].

Platelets in PRP

Platelet rich plasma (PRP) therapies require blood to be processed prior to application, however, the full assessment of the output of platelet sequestration devices is lacking. In this study the products of the Autologous Fluid Concentrator (Circle BiologicsTM, Minneapolis, MN) and the Gravitational Platelet Separation System (GPS, Biomet, Warsaw, IN, USA) were evaluated in terms of platelet viability and PRP constituents. The AFC and GPS produced 6.4 mL and 6.3 mL of PRP, with platelet recovery of 46 and 60 percent producing fold increases of platelets of 4.19 and 5.19, respectively. Fibrinogen concentration was increased above baseline PPP produced with the AFC. pH was lower for both of the processed samples than for whole blood. White Blood Cell count was increased around 5 fold. Functional tests showed preserved viability with both devices. This represents essential
knowledge that every treating physician should have before they can confidently administer PRP therapy produced by any method. These are the first published results of platelet function for the GPS system and the first performance results of the AFC system. The PRP produced is classified according to broad classifications as Leukocyte-PRP (L-PRP) for both devices [13348].

A randomized study

Animal studies have shown that local application of platelet-rich plasma (PRP) stimulates tendon repair. Preliminary results from a retrospective case series have shown faster return to sports. Thirty patients were recruited consecutively to a randomized study. During surgery, tantalum beads were implanted in the Achilles tendon proximal and distal to the rupture. Before skin suture, randomization was performed, and 16 patients were injected with 10 mL PRP (10 times higher platelet concentration than peripheral blood) whereas 14 were not. With 3-dimensional radiographs (roentgen stereophotogrammetric analysis; RSA), the distance between the beads was measured at 7, 19, and 52 weeks while the patient resisted different dorsal flexion moments over the ankle joint, thereby estimating tendon strain per load. An estimate of elasticity modulus was calculated using callus dimensions from computed tomography. At 1 year, functional outcome was evaluated, including the heel raise index and Achilles Tendon Total Rupture Score. The primary effect variables were elasticity modulus at 7 weeks and heel raise index at 1 year. The mechanical variables showed a large degree of variation between patients that could not be explained by measuring error. No significant group differences in elasticity modulus could be shown. There was no significant difference in heel raise index. The Achilles Tendon Total Rupture Score was lower in the PRP group, suggesting a detrimental effect. There was a correlation between the elasticity modulus at 7 and 19 weeks and the heel raise index at 52 weeks. The results suggest that PRP is not useful for treatment of Achilles tendon ruptures. The variation in elasticity modulus provides biologically relevant information, although it is unclear how early biomechanics is connected to late clinical results [11159].

Rotator cuff

Long-standing rotator cuff tendon tearing is associated with retraction, loss of work capacity, irreversible fatty infiltration, and atrophy of the rotator cuff muscles. Although continuous musculotendinous relengthening can experimentally restore muscular architecture, restoration of atrophy and fatty infiltration is hitherto impossible. Continuous relengthening with pharmacological stimulation of muscle growth using an anabolic steroid or insulin-like growth factor (IGF) can reverse atrophy and fatty infiltration as well as improve the work capacity of chronically retracted rotator cuff muscles in sheep. Sixteen weeks after tenotomy of the infraspinatus (ISP) tendon, atrophy and fatty infiltration had developed in the retracted ISP muscle. The musculotendinous unit was continuously relengthened in 14 sheep during 6 weeks: Four sheep were treated without pharmacological stimulation, 4 with intramuscular administration of an anabolic steroid, and 6 with IGF before final repair and rehabilitation (12 weeks). Changes were documented by intraoperative measurements of muscle work capacity, histology, and computed tomography/magnetic resonance imaging. Musculotendinous relengthening by continuous traction resulted in gains of length ranging from 0.7 cm in the IGF group to 1.3 cm in the control group. Fatty infiltration progressed in all groups, and the muscle's cross-sectional area ranged from 71 to 74 percent of the contralateral side at sacrifice and did not show any differences between groups in weight, volume, histological composition, or work capability of the muscle. The contralateral muscles
in the anabolic steroid group, however, showed significantly higher (mean ± standard deviation) muscle work capacity of 10 ± 0.9 Nm than the contralateral muscles of the control group (6.8 ± 2.4 Nm). This was accompanied by an increased mean muscle fiber area as well as by an unusual gain in the animals' weight after injection of the anabolic steroid. Subcutaneous continuous relengthening of a chronically retracted musculotendinous unit is feasible and advances the retracted musculotendinous junction toward its original position. This does not change the muscle work capacity. Whereas anabolic steroids have been shown to be effective in preventing classic degenerative muscle changes after tendon tears, neither an anabolic steroid nor IGF contributes to regeneration of the muscle once degenerative changes are established. The findings demonstrate that muscle cells lose reactivity to an anabolic steroid and IGF once retraction has led to fatty infiltration and atrophy of the muscle. Retraction of the muscle after tendon tears must be avoided by early repair, particularly in an athlete, as no regeneration can be achieved by mechanical or pharmacological means at this time [12269].

Despite the theoretic basis and interest in using platelet-rich plasma (PRP) to improve the potential for rotator cuff healing, there remains ongoing controversy regarding its clinical efficacy. The objective of one systematic review was to identify and summarize the available evidence to compare the efficacy of arthroscopic rotator cuff repair in patients with full-thickness rotator cuff tears who were concomitantly treated with PRP. It was searched the Cochrane Central Register of Controlled Trials, Medline, Embase, and PubMed for eligible studies. Two reviewers selected studies for inclusion, assessed methodologic quality, and extracted data. Pooled analyses were performed using a random effects model to arrive at summary estimates of treatment effect with associated 95 percent confidence intervals. Five studies (2 randomized and 3 nonrandomized with comparative control groups) met the inclusion criteria, with a total of 261 patients. Methodologic quality was uniformly sound as assessed by the Detsky scale and Newcastle-Ottawa Scale. Quantitative synthesis of all 5 studies showed that there was no statistically significant difference in the overall rate of rotator cuff retear between patients treated with PRP and those treated without PRP (risk ratio, 0.77; 95% confidence interval 0.48 to 1.23). There were also no differences in the pooled Constant score; Simple Shoulder Test score; American Shoulder and Elbow Surgeons score; University of California, Los Angeles shoulder score; or Single Assessment Numeric Evaluation score. It was concluded that PRP does not have an effect on overall retear rates or shoulder-specific outcomes after arthroscopic rotator cuff repair. Additional well-designed randomized trials are needed to corroborate these findings [12270].

Tendopathies

Chronic degenerative tendinopathies are frequent and difficult to treat. Tendon healing and regeneration may be improved by injecting autologous growth factors obtained from the patient’s blood. Autologous growth factors can be injected with autologous whole blood or platelet-rich plasma (PRP). Electronic databases were searched for prospective clinical trials on treatment with autologous growth factors of patients with chronic tendinopathy. Chronic tendinopathy in this study included wrist extensors, flexors, plantar fasciopathy and patellar tendinopathy. Studies examining the treatment of other tendinopathies were not identified. The Physiotherapy Evidence Database score was used to examine the methodological quality of the assessment, and a qualitative analysis was performed with the levels of evidence. There are many proposed treatment options for chronic tendinopathy. Treatments in the form of injections with autologous whole blood or PRP are increasingly used in clinical practice. There are high expectations of these regenerative injections, and there is a clear need for effective conservative therapies. All studies showed that injections of autologous
growth factors (whole blood and PRP) in patients with chronic tendinopathy had a significant impact on improving pain and/or function over time. However, only three studies using autologous whole blood had a high methodological quality assessment, and none of them showed any benefit of an autologous growth factor injection when compared with a control group. At present, there is strong evidence that the use of injections with autologous whole blood should not be recommended. There were no high-quality studies found on PRP treatment. There is limited evidence to support the use of injections with PRP in the management of chronic tendinopathy. There is growing interest in the working mechanisms of autologous growth factors. The amount and mixture of growth factors produced using different cell separating systems are largely unknown and it is also uncertain whether platelet activation prior to injection is necessary. These variables should be taken into account when starting clinical studies. A good experimental model for studying tendinopathy would be helpful for basic research. Future clinical studies using a proper control group, randomization, blinded and validated disease-specific outcome measures for pain and function are needed [10133].

Tendinopathy is a failed healing response of the tendon. Despite an abundance of therapeutic options, very few randomized prospective, placebo-controlled trials have been carried out to assist physicians in choosing the best evidence-based management. Eccentric exercises have been proposed to promote collagen fiber cross-link formation within the tendon, thereby facilitating tendon remodeling. Overall results suggest a trend for a positive effect of eccentric exercises, with no reported adverse effects. Combining eccentric training and shock wave therapy produces higher success rates compared with eccentric loading alone or shock wave therapy alone. The use of injectable substances such as platelet-rich plasma, autologous blood, polidocanol, corticosteroids, and aprotinin in and around tendons is popular, but there is minimal clinical evidence to support their use. The aim of operative treatment is to excise fibrotic adhesions, remove areas of failed healing, and make multiple longitudinal incisions in the tendon to detect intratendinous lesions and to restore vascularity and possibly stimulate the remaining viable cells to initiate cell matrix response and healing. New operative procedures include endoscopy, electrocoagulation, and minimally invasive stripping. The aim of these techniques is to disrupt the abnormal neoinnervation to interfere with the pain sensation caused by tendinopathy. Randomized controlled trials are necessary to better clarify the best therapeutic options for the management of tendinopathy [10472].

The therapeutic use of platelet-rich plasma (PRP) is an autologous biotechnology that relies on the local delivery of a wide range of growth factors and cytokines with the aim of enhancing tissue healing. Understanding both tendon healing and PRP therapies is an area of research that is critically important in developing optimal formulations and protocols to achieve the intended therapeutic effects. It was summarise recent information on the mechanisms inherent to the earliest response to tendon injury. We then describe the positive effect of PRP therapies on tendon healing. Research on tendinopathy has produced several biological hypotheses based on histopathological, biochemical and clinical findings showing that cell apoptosis, angiofibroblastic features or abnormal biochemical adaptations underlie the condition. The article provided insights into early healing mechanisms and the influence of PRP therapies on inflammation, cell migration, angiogenesis and the proliferation and synthesis of extracellular matrix. The knowledge gained helps to better understand and optimize tendon therapies. The use of endogenous therapies has a positive effect on experimental tendon healing. However, several obstacles need to be addressed to optimise medical practice in this field [10473].

**Hamstrings**
Injury to the hamstring muscle complex (HMC) is extremely common in the athletic community. Anatomical and functional aspects of the HMC predispose it to injury, including the fact that the muscles cross two joints and undergo eccentric contraction during the gait cycle. Injury most commonly occurs at the muscle tendon junction but may occur anywhere between the origin and insertion. There is increasing interest in the use of growth factors to accelerate healing after muscle and tendon injury. Animal studies have demonstrated clear benefits in terms of accelerated healing. There are various methods of delivery of the growth factors, all involving the release of growth factors from platelets. These include plasma rich in platelets and autologous blood. Clinical studies in humans are very limited at this stage but are promising. At present the World Anti-Doping Authority bans the intramuscular administration of these agents [10134].

**The knee**

Platelet-rich plasma (PRP) is a natural concentrate of autologous blood growth factors experimented in different fields of medicine in order to test its potential to enhance tissue regeneration. The aim of one study was to explore this novel approach to treat degenerative lesions of articular cartilage of the knee. One hundred consecutive patients, affected by chronic degenerative condition of the knee, were treated with PRP intra-articular injections (115 knees treated). The procedure consisted of 150-ml of venous blood collected and twice centrifugated: 3 PRP units of 5 ml each were used for the injections. Patients were clinically prospectively evaluated before and at the end of the treatment, and at 6 and 12 months follow-up. IKDC, objective and subjective, and EQ VAS were used for clinical evaluation. Statistical analysis was performed to evaluate the significance of sex, age, grade of OA and BMI. A statistically significant improvement of all clinical scores was obtained from the basal evaluation to the end of the therapy and at 6-12 months follow-up. The results remained stable from the end of the therapy to 6 months follow up, whereas they became significantly worse at 12 months follow up, even if still significantly higher respect to the basal level. The preliminary results indicate that the treatment with PRP injections is safe and has the potential to reduce pain and improve knee function and quality of life in younger patients with low degree of articular degeneration [10135].

**Achilles tendon**

Non-insertional Achilles tendinopathy commonly impedes the functioning of active persons. Treatment methods vary, as do their results. The aim of one study was to evaluate the effectiveness of non-insertional Achilles tendinopathy treatment with autologous platelet-rich plasma (PRP). Autologous PRP was injected into the affected Achilles tendon of 14 prospectively selected patients (15 Achilles tendons). Before PRP administration, all patients were evaluated using the American Orthopedic Foot and Ankle Society (AOFAS) scale for the hind foot, and the Victorian Institute of Sport Assessment – Achilles (VISA-A) scale. Ultrasonography (US) and Power-Doppler ultrasonography (PDUS) of the area was also performed. Identical physical and imaging evaluations were performed at 6 weeks, and at 3, 6, and 18 months after injection. During follow up, a significant improvement was observed in the clinical and imaging results. The AOFAS scale improved significantly from a baseline median of 55 points to 96 points at 18 months, while the VISA-A scale improved from a baseline of 24 to 96 in the final evaluations. During the final evaluation, one subject experienced minor pain following prolonged daily activity, while another subject complained of pain following overloading activity. Local, accurate PRP administration improved
symptoms of non-insertional Achilles tendinopathy [10136].

**Muscle injuries**

Muscle strains and contusions are extremely common in sport and account for significant time loss. The healing process can be slow, and reinjury is common. Recently there has been significant interest in the use of platelet-rich plasma (PRP) to enhance healing. There have been many attempts to speed muscle healing. Traditionally, ice, rest, anti-inflammatory medications and rehabilitation have been the mainstays of treatment. The use of non-steroidal anti-inflammatory drugs (NSAIDs) has been questioned. NSAIDs increase the expression of TGF-beta1 and decrease prostaglandin E2, which has a key role in the proliferation and differentiation of satellite cells. Recent studies have shown that NSAIDs likely tip the delicate balance of regeneration versus fibrosis toward fibrosis (scar). PRP has been shown to stimulate cell migration and myofibroblastic differentiation in vitro. Today serious questions remain as when and how to use PRP in muscle injury. A muscle is an actively healing, acutely inflammatory entity, and there remains potential to affect both the timing and the quality of repair adversely. PRP is heterogenous, and it may be that certain types of PRP are more effective than others. The timing, quantity and frequency of injections required are also in question. Given the pathophysiology of muscle healing, if PRP is used, the following can be considered [10137]:

- PRP should not be administered in the first 24 h after injury. Attempts should be made to limit the secondary injury using traditional means of controlling inflammation, namely compression, elevation and ice
- a leucocyte-poor product may mitigate the potentially adverse affects of neutrophils
- a product which includes a higher proportion of plasma may have increased levels of IGF-1 and potentially enhance healing and decrease fibrosis.

The muscle healing process is defined as a complex and dynamic process resulting in the restoration of anatomic continuity and function. This process is characterized by a cascade of events triggered by the tissue injury itself. It is widely accepted that growth factors play a central role in the healing processes by modulating the recruitment, duplication, activation, and differentiation of different cell types. This observation is the basis on which the use of platelet-rich plasma in several circumstances is founded; all of them requiring the activation or the modulation of the tissue repair process. There is an extensive documentation of in vitro and in vivo studies demonstrating the safety and efficacy of growth factors in the muscle healing process. Unfortunately, the precise biological efficacy and the lack of long-term side effects have not been clearly demonstrated. With regard to sports medicine, doping-related issues are still a matter of debate, especially regarding the treatment of muscle injuries. The purpose of one review was to examine the role of growth factors during muscle healing processes and to discuss the implications of platelet-rich plasma in its therapeutic applications. Sports medicine issues are also discussed particularly with regard to antidoping regulations [10471].
GROWTH HORMONE

Overviews

Human growth hormone (GH), but also GH related growth factors like the insulin-like growth factor-1 (IGF-1) are known to be abused in sports. Although the scientific evidence supporting a distinct effect of GH on performance in healthy trained subjects is limited, it has been repeatedly found with athletes or trainers, and the recent introduction of a first test to detect GH doping has led to a number of positive cases. Currently, there is no test for the detection of IGF-1 introduced worldwide, but confiscation of the drug from sports teams can be taken as indirect evidence for its abuse. The major biochemical difficulty for the detection of GH is that the recombinant form is identical in physicochemical properties to the endogenous GH secreted by the pituitary gland. Furthermore, the very short half-life of GH in circulation inherently shortens the window of opportunity where the drug can be detected. Two strategies have been followed for more than a decade to develop a test to detect the application of recombinant GH: the marker approach, which is based on the elevation of GH-dependent markers above the level seen under physiological conditions evoked by administration of recombinant GH, and the isoform approach, which is based on a change in the pattern of GH isoforms in circulation following the injection of recombinant GH [12205]. Since 2004, the isoform test for hGH abuse has been in use in routine doping controls and has undergone fine-tuning and continuous finishing to increase its sensitivity and thus broaden the window of opportunity for detection. In 2011, a controlled administration study with two preparations of recombinant hGH (Chinese and Swiss products, 0.1 IU/kg bodyweight) was conducted and the traceability of the drug (i.e. its influence on the circulating GH isoforms) was determined using the WADA approved analytical kits. Following a single injection, detection windows between 12 and 18 h were observed, while repeated hGH application (one injection/day over 14 days) allowed for hGH abuse detection up to 21 h after cessation. In a different study, the performance of two isoform-based growth hormone detection assays, namely the above mentioned WADA approved test and a 22 kDa/20 kDa isoform immunoassay, was compared. Volunteers received recombinant hGH s.c. at 0.026 mg/kg bodyweight once daily for seven consecutive days, and collected serum samples were analyzed on both platforms. The assays demonstrated good correlation concerning the detection of abnormal isoform concentrations in serum and exhibited comparable detection windows of up to 24 h [12017].

Recombinant human growth hormone (rhGH) has been on the list of forbidden substances since availability of its recombinant form improved in the early 1990s. Although its effectiveness in enhancing physical performance is still unproved, the compound is likely used for its potential anabolic effect on the muscle growth, and also in combination with other products (androgens, erythropoietin, etc.). The degree of similarity between the endogenous and the recombinant forms, the pulsatile secretion and marked interindividual variability makes detection of doping difficult. Two approaches proposed to overcome this problem are: the indirect method, which measures a combination of several factors in the biological cascade affected by administration of GH; and the direct method, which measures the difference between the circulating and the recombinant (represented by the unique 22 kD molecule) forms of GH. One article gave an overview of what is presently known about hGH in relation to sport. The main effects of exercise on hGH production and the use and effects of rhGH in athletes are discussed. Difficulties encountered by laboratories to prove misuse of this substance by both indirect and direct analyses are emphasised. The direct method currently seems to have the best reliability, even though the time window of detection is too
short. hGH doping is a major challenge in the fight against doping. The effect of exercise on hGH and its short half-life are still presenting difficulties during doping analysis. To date the most promising method appears to be the direct approach utilising immunoassays [06135].

Doping with growth hormone (GH) is banned; however, there is anecdotal evidence that it is widely abused. GH is reportedly often used in combination with anabolic steroids at high doses for several months. Development of a robust test for detecting GH has been challenging since recombinant human 22-kDa GH used in doping is indistinguishable analytically from endogenous GH and there are wide physiological fluctuations in circulating GH concentrations. One approach to GH testing is based on measurement of different circulating GH isoforms using immunoassays that differentiate between 22-kDa and other GH isoforms. Administration of 22-kDa GH results in a change in its abundance relative to other endogenous pituitary GH isoforms. The differential isoform method is, however, limited by its short time window of detection. A second approach that extends the time window of detection is based on detection of increased levels of circulating GH-responsive proteins, such as the insulin-like growth factor (IGF) axis and collagen peptides. As age and gender are the major determinants of variability for IGF-I and the collagen markers, a test based on these markers must take these factors into account. Extensive data now validate the GH-responsive marker approach, and implementation is largely dependent on establishing an assured supply of standardized assays. It was concluded that robust tests are available to detect GH and enforce the ban on its abuse in sports. Novel approaches that include gene expression and proteomic profiling must continue to be pursued to expand the repertoire of testing approaches available and to maintain deterrence of GH doping [11160].

Human growth hormone (GH) is widely abused by athletes; however, there is little evidence that GH improves physical performance. Replacement of GH in GH deficiency improves some aspects of exercise capacity. There is evidence for a protein anabolic effect of GH in healthy adults and for increased lean body mass following GH, although fluid retention likely contributes to this increase. The evidence suggests that muscle strength, power, and aerobic exercise capacity are not enhanced by GH administration, however GH may improve anaerobic exercise capacity. There are risks of adverse effects of long-term abuse of GH. Sustained abuse of GH may lead to a state mimicking acromegaly, a condition with increased morbidity and mortality [10138].

Although there is little evidence that GH improves performance in young healthy adults, randomized controlled studies carried out so far are inadequately designed to demonstrate this, not least because GH is often abused in combination with anabolic steroids and insulin. Some of the anabolic actions of GH are mediated through the generation of insulin-like growth factor-I (IGF-I), and it is believed that this is also being abused. Athletes are exposing themselves to potential harm by self-administering large doses of GH, IGF-I and insulin. The effects of excess GH are exemplified by acromegaly. IGF-I may mediate and cause some of these changes, but in addition, IGF-I may lead to profound hypoglycaemia, as indeed can insulin. Although GH is on the World Anti-doping Agency list of banned substances, the detection of abuse with GH is challenging. Two approaches have been developed to detect GH abuse. The first is based on an assessment of the effect of exogenous recombinant human GH on pituitary GH isoforms and the second is based on the measurement of markers of GH action. As a result, GH abuse can be detected with reasonable sensitivity and specificity. Testing for IGF-I and insulin is in its infancy, but the measurement of markers of GH action may also detect IGF-I usage, while urine mass spectroscopy has begun to identify the use of insulin analogues [08239].

There is little evidence that GH improves physical performance. Replacement of GH in GH deficiency improves some aspects of exercise capacity. There is evidence for a protein
anabolic effect of GH in healthy adults and for increased lean body mass following GH, although fluid retention likely contributes to this increase. The evidence suggests that muscle strength, power, and aerobic exercise capacity are not enhanced by GH administration, however GH may improve anaerobic exercise capacity. There are risks of adverse effects of long-term abuse of GH. Sustained abuse of GH may lead to a state mimicking acromegaly, a condition with increased morbidity and mortality [10139].

Human growth hormone (hGH) is a protein endogenously produced predominantly by the anterior pituitary gland. Native hGH and, especially, its recombinant analogue (rhGH), used to treat patients with hormone deficiency, are supposed to be abused by athletes searching its anabolic and lipolytic effects. Hence, hGH use has been prohibited for a long time by the sport authorities, but until recently, hGH abuse could not be detected. Two approaches have been followed when trying to develop methods for GH abuse detection. The direct method identifies an abnormal ratio between GH isoforms – a result of hGH exogenous administration. The time window to find a cheating athlete by this approach is limited by the excretion time of the hormone. The indirect approach measures serum biomarkers directly affected by GH intake (eg, markers of released liver growth factors and of bone and collagen turnover). In this approach, the retrospective power extends further. Alternative possibilities for cheating related to hGH could be the administration of recombinant growth factors themselves, the administration of hGH metabolic precursors such as ghrelin-like GH secretagogues, or the genetic manipulation of muscle growth-related genes (gene doping) [09194].

Human growth hormone (hGH) is seen as a doping risk in sport because of its possible anabolic and lipolytic effects. Due to its considered performance enhancing effects, human growth hormone (hGH) is abused as a doping agent in sport. Its misuse also carries potentially serious side effects to a person's health. Consequently, hGH and its releasing factors are prohibited in sport, as established in the Prohibited List which is updated and published yearly by the World Anti-Doping Agency (WADA). In 2008 significant progress has been made; there is one test for detecting HGH approved for use in anti-doping and a second detection method pending. This is a strong reflection of the ongoing research efforts in anti-doping and the progress being made by the Anti-Doping Organisations in reducing the risk that doping poses to sport [09192].

In order to fight the menace that hGH doping poses to the spirit of sport and to the health of athletes, the sport movement and the anti-doping authorities, initially led by the International Olympic Committee (IOC) and later by WADA, have put substantial efforts into developing tests for its detection. Currently, a primary analytical approach, the isoform differential immunoassay, has been implemented in WADA-accredited laboratories. In parallel, a second, indirect approach for the detection of hGH abuse, based on the quantification of hGH-associated biological markers, has been developed. The final aim is to combine both methodologies to improve the sensitivity and expand the time window to detect doping with hGH. In addition, novel analytical procedures, based on proteomic and genomic technologies as well as the use of mass spectrometry-based methods of detection, are being investigated for future application in hGH anti-doping tests [09193].

People have been attracted to the tales of giants able to perform extraordinary feats of strength, like Paul Bunyan, the mythical American lumberjack. HGH, or human growth hormone, has had the promise to achieve the dreams of some people to become a real-life Paul Bunyan. This, together with the fact that HGH can't be detected, has increased the popularity of this doping agent for athletes. Recently (2007) some high-profile athletes have been identified receiving HGH. The HGH came from a shady Florida warehouse under investigation for illegally distributing prescription medications. HGH has some definite and
proven medical benefits. It is currently approved medically in the United States for 2 primary indications, short-stature in children and growth hormone deficiency in adults. All of these HGH benefits, however, are in individuals with growth hormone deficiency. In people with normal GH levels, HGH does not improve athletic performance in terms of muscle strength, flexibility, and endurance. In fact, several placebo-controlled studies have been negative. A 4-week, double-blind Swedish study using 2 doses of HGH and placebo found no differences in subjects exercising on a bicycle in terms of power output and oxygen uptake. In another study, a single injection of HGH increased plasma lactate and reduced exercise performance. Indeed, in the classic HGH excess experiment in nature, acromegalic subjects have increased muscle mass but histologic evidence of myopathy with muscle weakness and pain. In addition to the lack of effectiveness for enhancing athletic performance, HGH has a downside. It can cause dose-related side effects including diabetes, carpal tunnel syndrome, fluid retention, joint stiffness, muscle pain, and high blood pressure. It turns out that, like Paul Bunyan, the athletic benefits of HGH is a myth [07141].

Circulating microRNAs (miRNAs) in plasma are being studied for use as biomarkers of specific diseases and as markers of administration of pharmaceutical agents. Administration of recombinant human growth hormone (rhGH) is prohibited by sporting authorities, but it continues to be used by athletes attempting to gain an unfair advantage in athletic competition. Current methods for detection of rhGH use rely on immunoassay technology and are limited by a short time-frame in which detection of rhGH is possible. It was hypothesized that administration of rhGH would alter expression of circulating miRNAs and that any changes could be detected. To identify potential miRNA targets, it was utilized miRNA microarrays for screening. Confirmatory testing used real-time reverse-transcriptase (RT) quantitative PCR (qPCR) assays of selected miRNAs in 35 plasma samples obtained from individuals with no known pituitary disorders, patients with excess GH production, and patients receiving therapeutic replacement doses of rhGH. It was identified and confirmed four miRNAs that were differentially expressed in all individuals using therapeutic replacement doses of rhGH when compared to individuals with naturally high levels of GH and normal controls. This study further develops the hypothesis that circulating miRNAs may be used as biomarkers for detection of doping in sports [13356].

Beyond the discussion of supplemental GH to affect muscular adaptations, there is a consistent mistake made by athletes (and their coaches) that the natural robust exercise-induced rise in systemic GH concentrations after heavy weightlifting, is of value in augmenting skeletal muscular adaptations. There is firm evidence that refutes this “hormone hypothesis” for the support of gains in strength and lean mass after a programme of weightlifting in healthy adults. Indeed, future research will need to underpin whether there are benefits towards GH on the connective tissue and/or lipolysis. In general, the available evidence, largely derived from studies that administer rhGH, suggests that the use of peptide hormones to elevate systemic GH concentrations is unlikely to result in a competitive advantage in healthy adults. The mass availability to the athlete (via internet suppliers, nutrition stores, medical practitioners, etc) of synthetic peptides, combined with the lack of regulation of the supplement contents, underpins the important role of the sports scientist, coaches or medical practitioners in educating the athlete consumer that this doping practice is ineffective and with unknown side effects [13357].

Exercise depends on an adequate supply of nutrients and oxygen to the muscle fibers. Glucose for short-term high-intensity activity and free fatty acids (FFAs) for more prolonged activity are combusted to release kinetic energy. GH has a number of effects on protein anabolism and intermediate metabolism that may improve exercise performance through increased fuel and oxygen delivery to exercising muscle, increased muscle strength or any combination of these factors. In addition, it may improve cardiovascular function and
Thermoregulation. Athletes have been misusing growth hormone (GH) for its anabolic and metabolic effects since the early 1980s, at least a decade before endocrinologists began to treat adults with GH deficiency. Although there is an ongoing debate about whether GH is performance enhancing, recent studies suggest that GH improves strength and sprint capacity, particularly when combined with anabolic steroids. Prior to treatment, adults with GH deficiency lose lean tissue while accumulating fat, in particular visceral fat. Skeletal muscle mass and strength is reduced with a consequent impairment of physical performance, exercise capacity, and VO₂ max (aerobic capacity or the maximum ability to take in and use oxygen). Following treatment with recombinant human GH (rhGH), body composition normalizes and physical performance improves. It does not necessarily follow, however, that the administration of supraphysiological GH doses will lead to further performance advantage in healthy adults. Indeed the opposite is suggested by acromegaly, which is characterized by marked abnormalities in protein and carbohydrate metabolism, muscle weakness rather than excessive strength, and cardiomyopathy. A systematic review of 44 articles describing 27 studies reported that although GH administration was associated with increase in lean body mass and decrease in fat mass, it did not improve a number of measures of performance. The detection of GH misuse is challenging because it is an endogenous hormone. Two approaches have been developed to detect GH misuse; the first is based on the measurement of pituitary GH isoforms and the ratio of 22-kDa isoform to total GH. The second is based on the measurement of insulin like growth factor-I (IGF-I) and N-terminal propeptide of type III procollagen (P-III-NP) which increase in a dose-dependent manner in response to GH administration. Both methodologies have been approved by the World Anti-Doping Agency (WADA) and have led to the detection of a number of athletes misusing GH [13007].

Recombinant human growth hormone

Recombinant human growth hormone (rhGH), which is favoured due to its anabolic, lipolytic and post-trauma healing properties, is commonly abused in sport with the aim of improving performance. Substantial efforts have been made to develop tests for detection of doping with rhGH, but the challenge is a difficult one. Long-term efficacy of growth hormone in adults with growth hormone deficiency It is well known that GH stimulates lipolysis and lipid oxidation thereby reducing fat mass, while GH and insulinlike growth factor-I (IGF-I) increase protein synthesis and thus increase protein mass. Meanwhile, it has been shown that long-term treatment of GH deficient adults with GH, normalizes body composition by increasing lean body mass and decreasing fat mass. Exercise capacity is reduced in growth hormone deficiency (GHD) adults, while maximal oxygen consumption (VO₂ max), the aerobic capacity, is increased following GH replacement in subjects with adult GHD. However, it has not been shown that GH increases muscle strength in GHD patients. As primary impairment in the GH/IGF-I axis often leads to a high-risk cardiovascular profile which may partially be reversible during GH replacement. Benefits and harm arising from GH use in healthy subjects It has been reported that GH tends to increase whole body protein synthesis in some highly trained athletes, while in other athletes it does not. The use of rhGH in healthy adults causes a change in body composition with significant decrease in fat mass and significant increase in lean body mass. It has been suggested that hGH may be beneficial in stimulating the supporting connective tissues (strengthening of the matrix by increased tendon collagen synthesis). This may then accelerate healing of soft tissue injury and bone fractures. GH in sports is seldom used in high doses (in other words, several times higher than the daily production rate of GH). However, when high doses are involved, the many side-effects of GH abuse in sports are actually signs of acromegaly, such as swelling and arthralgias, providing ample evidence of the harmful effects of excessive GH. More serious
side-effects are diabetes and negative impact on heart function (increase in left ventricular mass and increased cardiac output). The results of most of the controlled studies in which supraphysiological dosages of hGH have been used are less impressive than the claims of those who misuse hGH, a substance whose abuse is prevalent in top-level sport. In order to detect GH abuse there are two approaches. One, the analytical method, relies on the measurement of molecular isoforms of GH, while the second relies on measurement of sensitive markers of biological activity of rhGH. A method was developed for detection of GH abuse (the GH-2000 project) based on the measurement of IGF-I and the amino-terminal pro-peptide of type III collagen (P-III-NP) by two commercial assays available for each analyte. By using this method, decision limits were developed to measure IGF-I and P-III-NP in elite athletes, thus enabling the introduction of a test for GH abuse based on the detection of GH biomarkers [12011].

**Epidemiology and demographic factors**

GH-responsive markers of the IGF system and of collagen turnover hold promise as the basis of a GH doping test. The purpose of one study was to determine the influence of age, gender, body mass index (BMI), ethnicity, and sporting type on GH-responsive serum markers in a large cohort of elite athletes from different ethnic backgrounds. The study was designed as a cross-sectional study. Participants: A total of 1103 elite athletes (699 males, 404 females), aged 22 years, from 12 countries and 10 major sporting categories participated in this study. Serum IGF-I, IGF binding protein-3 (IGFBP-3), acid labile subunit (ALS), and collagen markers [N-terminal propeptide of type I procollagen (PINP), C-terminal telopeptide of type I collagen (ICTP), N-terminal propeptide of type III procollagen (PIIINP)] were measured. There was a significant negative correlation between age and each of the GH-responsive markers. Serum IGF-I, IGFBP-3, and ALS were all lower, whereas the collagen markers PINP, ICTP, and PIIINP were higher in men than in women. Multiple regression analysis indicated that age, gender, BMI, and ethnicity accounted for 23-54 percent of total between-subject variability of the markers. Age and gender cumulatively accounted for 91 percent of the attributable variation of IGF-I and more than 80 percent for PINP, ICTP, and PIIINP. Gender exerted the greatest effect on ALS (48 %), and BMI accounted for less than 12 percent attributable variation for all markers. The influence of ethnicity was greatest for IGFBP-3 and ALS; however, for the other markers, it accounted for less than 6 percent attributable variation. Analysis of 995 athletes indicated that sporting type contributed 5-19 percent of attributable variation. It was concluded that age and gender were major determinants of variability of GH-responsive markers except for IGFBP-3 and ALS. Ethnicity is unlikely to confound the validity of a GH doping test based on IGF-I and these collagen markers [06136].

In order to verify the effects of the sporting season (entailing periods of training, competition, recovery, resting) on GH-dependent parameters in male and female athletes from different sporting disciplines, 47 male and female athletes (3 rowers, 5 swimmers, 7 alpine skiers, 3 soccer players, 7 middle distance runners, 14 sprinters, 4 triathletes, 1 road walker, 3 cyclists) were followed-up for a period of 6 months. Blood samples were taken every two months for the evaluation of IGF-I, N-terminal propeptide of type III procollagen (PIIINP) and C-terminal cross-linked telopeptide of type I collagen (ICTP). Abnormal IGF-I, PIIINP and ICTP levels were observed during the follow-up period in 7/100 (7 %), 9/100 (9 %) and 8/100 (8 %) samples of the male group, respectively, and in 9/88 (10 %), 1/88 (1 %) and 0/88 (0 %) samples of the female group, respectively. Abnormal levels appeared to be randomly distributed over the different periods of the sporting season and within male and female subjects, with the large majority of abnormal values being found in the younger athletes.
Taking into account all the tests done during the 6-month period (n=564), individual markers falling outside the normal range (for age) were observed in a small number of instances (34/564 tests done, 24/300 for males and 10/264 for females). When our method for the detection of exogenous recombinant GH (rhGH) administration, based on the concomitant determination of these three peripheral GH-dependent markers and on the attribution of specific scores, was applied in the same athlete at a given time point of the 6-month period, the prevalence of a positive score was extremely low (i.e. 3/188 samples or 1.6 %). Total positive scores were actually recorded in only three male athletes (2 swimmers and 1 skier, aged <21 yr) at one occasion during the 6-month period considered. In contrast, no total positive scores were found in female athletes (i.e. 0/88 samples). In conclusion, the concentrations of IGF-I, PIIINP and ICTP were stable and not significantly modified during 6 months of a sporting season (entailing periods of training, competition, recovery, resting) in athletes from different sporting disciplines. Therefore our method, based on the concomitant determination of three peripheral GH-dependent biomarkers appears safe, acceptable, relatively inexpensive and repeatable (in case of positive or suspected values) immediately or at different intervals of the sporting season. Further additional studies are requested to precise the cut-off values for narrower age-class subdivisions in both genders in order to improve the proposed method [06137].

**Adolescents**

The rationale for adolescent athletes taking rhGH, is that it should synergize with the physiologic (or doping) increases in testosterone to grow faster and to add additional lean body mass. It is understood that by becoming bigger and stronger the athlete will perform at a higher level. hGH is lipolytic in addition to being anabolic and thus may be considered for those in "physique" related sports such as body-building. In multiple surveys of adolescents who take anabolic agents, it is to "look good" as often as it is to augment athletic performance. As noted previously athletic performance is much more than just strength or endurance; for the athlete must produce, control and efficiently use the energy in a fashion that maximizes sport performance [10001].

Despite the paucity of data for performance enhancement, the "word on the street" is that this agent is quite actively being abused by athletes. It is unlikely that the athlete uses rhGH in isolation, thus making it quite difficult to define its exact role in athletic performance. There is however one very well controlled study that shows an effect on strength in a group of well defined abstinent steroid abusers (subject to strict drugs of abuse testing). There are no studies in child and adolescent athletes, but it may be that faster growth, weight gain and the salutary effects on body composition during adolescence could confer advantage to age-group athletes, for it has been shown at the elite level that the taller, heavier early adolescent athlete is represented out of proportion to his/her age-peers. Adolescents reach "near-adult" height later in the teen years with girls reaching this stature several years before boys; however, the adult body composition (muscle, bone and fat) and the regional distribution of body fat are not attained until early-to-middle third decade. If rhGH is effective to help one obtain these milestones early, perhaps there is some advantage; however this is quite theoretical [10001].

**Children**

There are many data for the short-term (years to more than a decade) in children with a number of conditions of childhood: GH deficiency, several genetic condition as well as "short-
normal" children. Two major data bases, the Kabi International Growth Study, now managed by Pfizer, Inc and the National Cooperative Growth Study produced and managed by Genentech, Inc show quite low incidences of scoliosis, slipped capital femoral epiphysis and rare instances of idiopathic intracranial hypertension, the one of greatest concern. Adults have noted more muscle and joint complaints than children. There is the long-term (theoretical) issue of neoplasia [10001].

The most notable effect in children and adolescents is linear growth. Growth hormone-deficient children and adolescents are likely to be 20 to 30 cm shorter than mid-parental target height if left untreated. Physiological stimuli to hGH release include: deep sleep, some amino acids and exercise. hGH is secreted in a pulsatile fashion every hour or two and has significant peaks approximately 90 min after the onset of deep sleep and within minutes of completing a bout of exercise. The main (iso)form of hGH is a 191 amino acid, 22 kD peptide with a significant amount of a 20 kD splice variant form. There are a multitude of post-translationally modified forms including those affected by metabolism and sulfation and phosphorylation. Several of the forms, predominantly the 22 kD and 20 kD forms interact with the hGH receptor, a dimeric member of the cytokine family of receptors at the cell surface to lead to the cellular actions of hGH. One of the main ones is to stimulate the hepatic synthesis of IGF-I (endocrine), but also to stimulate the paracrine and autocrine synthesis of IGF-I in various tissues, such as bone and muscle. In addition, it is lipolytic and can significantly alter body composition by directing muscle protein synthesis relative to fat. In fact, it is this action that has led to its use in animal husbandry with the "partitioning" of feed and "feed-efficiency" as the major outcomes [10001].

Native hGH and, especially, its recombinant analogue (rhGH), used to treat patients with hormone deficiency, are supposed to be abused by athletes searching its anabolic and lipolytic effects. Hence, hGH use has been prohibited for a long time by the sport authorities, but until recently, hGH abuse could not be detected. Two approaches have been followed when trying to develop methods for GH abuse detection. The direct method identifies an abnormal ratio between GH isoforms – a result of hGH exogenous administration. The time window to find a cheating athlete by this approach is limited by the excretion time of the hormone. The indirect approach measures serum biomarkers directly affected by GH intake (e.g. markers of released liver growth factors and of bone and collagen turnover). In this approach, the retrospective power extends further. Alternative possibilities for cheating related to hGH could be the administration of recombinant growth factors themselves, the administration of hGH metabolic precursors such as ghrelin-like GH secretagogues, or the genetic manipulation of muscle growth-related genes (gene doping). In parallel with the new types of abuse, which will surely emerge in the near future, the research and development for the improvement of the analytical detection of GH itself will continue [10141].

**Women**

*Prevalence*

Athletes use hGH, an endogenous hormone with an anabolic effect, to improve muscle mass and performance. The first documented use of hGH appeared in the early 1980s. A survey of adolescent boys and girls that grouped hGH use with DHEA and AAS use found that 0.2 percent of girls admitted to taking one of these substances at least once in the past year, while 0.1 percent used the supplement at least weekly. A 2001 NCAA survey found that 3.5 percent of college athletes had used hGH in the past year; however, separate data for men and women was not reported. hGH is secreted by the anterior pituitary gland. Its level peaks
during adolescence and decreases with age; production increases with exercise and decreases with starvation. hGH stimulates insulin-like growth factor (IGF-1) production in the liver. Both hGH and IGF-1 have effects of lipolysis and protein anabolism. Providing hGH to athletes was thought to have a protein-building, fat-burning effect. Athletes began using hGH instead of AAS because it was less detectable due to a shorter half-life. Athletes have also reported that hGH enhances performance, although scant scientific evidence supports its effectiveness. However, clinical trials are unable to administer the high doses that athletes may take — often in combination with the other supplements — in order to evaluate hGH efficacy. A 2002 review evaluated randomized, double-blind, placebo-controlled trials of exogenous hGH in trained adult athletes, with muscle strength or performance as outcome measures, finding few well-designed studies using performance-based outcomes in normal healthy athletes. Additionally, the studies did not consistently show any benefit in healthy athletes without growth hormone deficiencies. More recently, it was conducted a double-blind, placebo-controlled trial in 15 women and 15 men to evaluate hGH supplementation effects on endurance exercise capacity. Healthy active volunteers were given low hGH dose, high hGH dose, or placebo for 28 days, and maximal oxygen uptake during stationary cycling was measured. No effect was found in maximal oxygen uptake or maximum power output during exercise. Participants increased total body weight, but this was attributed to fluid retention, not muscle mass [07086].

Side effects

Adverse effects related to hGH range in severity. People with too much hGH develop acromegaly, an overgrowth of facial bones, tongue, nose, and jaw. These effects are thought to be nonreversible. Other effects include joint pain, muscle weakness, fluid retention, and carpal tunnel syndrome. More severe effects are impaired glucose regulation, cardiomyopathy, and hyperlipidemia. Overall, performance-enhancing benefits do not seem to outweigh risks [07086].

Physiology

The major isoform of growth hormone is made up of 191 amino acids stabilized by two disulfide bonds with a molecular weight of 22 kilodaltons. Hypothalamic secretion of human growth hormone-releasing hormone stimulates HGH release and somatostatin inhibits HGH release from the anterior pituitary. Recombinant HGH (rHGH) first became available for clinical use in 1983. rHGH has a short half life, approximately 15 minutes. It has been demonstrated that serum concentrations of growth hormone return to baseline approximately 8 to 16 hours after intramuscular injection and 11 to 20 hours after subcutaneous injection of rHGH. The usual therapeutic dose of recombinant HGH is 1-2 IU/day given either by intramuscular or subcutaneous injection at night. The doses used by athletes for performance enhancement are often 20 times the normal prescription doses and may cost from USD 3,000-5,000 [07002].

The diagnosis of growth hormone (GH) deficiency in children with short stature is complex, and in certain cases, might be very difficult. Most of the provocative tests used to evaluate GH deficiency use pharmacological agents. The artificial nature of the pharmacological tests and the possibility that these tests might not always reflect GH secretion under normal physiological conditions provides the impetus for a more physiologic test. Exercise is one of the important GH releasing physiological stimuli [09205].

The ability of the somatotroph cells in the anterior pituitary to synthesize and secrete the
polypeptide, hGH, is determined by a gene called the Prophet of Pit-1 (PROP1). When hGH is translated, 70-80 percent is secreted as a 191-amino-acid, 4-helix bundle protein, and 20-30 percent as a less abundant 176-amino-acid form. Hypothalamic-releasing and hypothalamic-inhibiting hormones acting via the hypophysial portal system control the secretion of hGH, which is secreted into the circulation. In healthy persons, the hGH level is usually <0.2 μg/L throughout most of the day. There are approximately 10-12 intermittent secretory bursts in a 24 hour period, mostly at night, when the level can rise to as much as 30 μg/L. hGH secretion declines at 14 percent per decade from the age of 20 years. hGH action is mediated by an hGH receptor, which is expressed mainly in the liver and is composed of dimers that change conformation when occupied by an hGH ligand. Cleavage of the hGH receptor provides a circulating hGH binding protein (GHBP), prolonging the half-life and mediating the transport of hGH [67]. Intracellular hGH signalling is decreased by suppressors of cytokine signaling. hGH induces the synthesis of peripheral insulin-like growth factor I (IGF-I) [ while endocrine, autocrine, and paracrine IGF-I induces cell proliferation and is thought to inhibit apoptosis [08240].

Growth hormone (GH) is secreted in a pulsatile pattern from the anterior pituitary, influenced by a variety of normal and pathophysiological conditions. Exogenous recombinant hGH is virtually indistinguishable from the predominant naturally occurring isoform and is cleared from the body within 24 hours. Although GH is on the World Anti-doping Agency list of banned substances, the detection of GH abuse remains challenging. This article gives an overview of the potential application of surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry to examine proteomic changes following GH administration, using both serum and white blood cell extracts as samples for analysis. Results to date indicate that proteomic changes observed following GH administration have the potential to yield novel biomarker sets for the detection of GH abuse [09206].

Human growth hormone (hGH) is a proteohormone secreted by the pituitary gland. It acts through binding to the hGH receptor, inducing either direct effects or initiating the production of insulin-like growth-factor I (IGF-I), the most important mediator of hGH effects. Growth hormone is primarily known to promote longitudinal growth in children and adolescents, but has also various important metabolic functions throughout adult life. Effects of hGH on the adult organism are well established from studies with recombinant growth hormone (rhGH) therapy in growth hormone deficient subjects. In this particular group of patients, replacement of hGH leads to increased lipolysis and lean body mass, decreased fat mass, improvements in VO_{2max}, and maximal power output. Although extrapolation from these findings to the situation in well trained healthy subjects is impossible, and controlled studies in healthy subjects are scarce, abuse of hGH seems to be popular among athletes trying to enhance physical performance. Detection of the application of rhGH is difficult, especially because the amino acid sequence of rhGH is identical to the major 22,000 Da isoform of hGH normally secreted by the pituitary. Furthermore, some physiological properties of hGH secretion also hindered the development of a doping test: secreted in a pulsatile manner, it has a very short half-life in circulation, which leads to highly variable serum levels. Concentration alone therefore cannot prove the exogenous administration of hGH. Two approaches have independently been developed for the detection of hGH doping: The so-called "marker approach" investigates changes in hGH-dependent parameters like IGF-I or components of bone and collagen metabolism, which are increased after hGH injection. In contrast, the so-called "isoform approach" directly analyses the spectrum of molecular isoforms in circulation: the pituitary gland secretes a spectrum of homo- and heterodimers and - multimers of a variable spectrum of hGH isoforms, whereas rhGH consists of the monomeric 22,000 Da isoform only. This isoform therefore becomes predominant after injection of rhGH. Specific immunoassays with preference for the one or the other isoform allow analysis of the relative abundance of the 22,000 Da isoform. Application of rhGH can
be proven when the ratio of this isoform relative to the others is increased above a certain threshold. Because the "marker method" and the "isoform method" have a different window of opportunity for detection, complementary use of both tests could be a way to increase the likelihood of detecting cheating athletes [10140].

One of its best-characterized effects of growth hormone is increasing levels of circulating insulin-like growth factor I (IGF-I), which is primarily of hepatic origin. It also induces synthesis of IGF-I in most non-hepatic tissues. The effects of GH in promoting postnatal body growth are IGF-I dependent, but IGF-I-independent functions are beginning to be elucidated. Although benefits of GH administration have been reported for those who suffer from GH deficiency, there is currently very little evidence to support an anabolic role for supraphysiological levels of systemic GH or IGF-I in skeletal muscle of healthy individuals. There may be other performance-enhancing effects of GH. In contrast, the hypertrophic effects of muscle-specific IGF-I infusion are well documented in animal models and muscle cell culture systems. Studies examining the molecular responses to hypertrophic stimuli in animals and humans frequently cite upregulation of IGF-I messenger RNA or immunoreactivity. The circulatory/systemic (endocrine) and local (autocrine and paracrine) effects of GH and IGF-I may have distinct effects on muscle mass regulation [08244].

Development of an assay for human GH was closely followed by the observation that plasma levels of GH increase soon after the beginning of exercise. Because of the known anabolic and lipolytic effects of GH and the observation that the exercise-associated increase in GH precedes an increase in circulating free fatty acids (FFAs), it was hypothesized that GH might play an important metabolic role during exercise. Further evidence for this notion came from the discovery in the 1980s that exercise capacity and muscle strength are impaired in GH-deficient (GHD) adults and improved by GH replacement. Reports of the use of GH by athletes as a performance-enhancing agent predated the introduction of recombinant human GH (rhGH) by at least 5 years, and there is an increasing body of evidence that GH abuse represents a significant problem in a number of sports including athletics, swimming, and cycling. The likelihood that attaining supraphysiological GH levels improves exercise performance should be considered in the context that although GH is clearly anabolic, there is no evidence that exercise capacity is enhanced by administration of GH to normal subjects, and in patients with long-standing endogenous GH excess (acromegaly), muscle strength is usually reduced. However, recent metabolic studies provide a plausible mechanistic explanation through which supraphysiological GH administration could lead to short- or medium-term improvements in exercise performance, and regular seizures of GH from athletes demonstrate an ongoing belief in sporting circles that GH is performance enhancing. Measures of exercise performance including maximal oxygen uptake and ventilatory threshold are impaired in adult GH deficiency and improved by GH replacement, probably through some combination of increased oxygen delivery to exercising muscle, increased fatty acid availability with glycogen sparing, increased muscle strength, improved body composition, and improved thermoregulation. In normal subjects, in addition to the long-term effects of GH/IGF-I status, there is evidence that the acute GH response to exercise is important in regulating substrate metabolism after exercise. Administration of supraphysiological doses of GH to athletes increases fatty acid availability and reduces oxidative protein loss, particularly during exercise, and increases lean body mass. Despite a lack of evidence that these metabolic effects translate to improved performance, GH abuse by athletes is widespread. Tests to detect GH abuse have been developed based on measurement in serum of 1) indirect markers of GH action, and 2) the relative proportions of the two major naturally occurring isoforms (20 and 22kDa) of GH. There is evidence that exercise performance and strength are improved by administration of GH and testosterone in combination to elderly subjects. The potential benefits of GH in these situations must be weighed against potential adverse effects [07142].

1069
The majority of studies reported to date have demonstrated increased maximum work rate and $\text{VO}_{2\text{max}}$ after GH replacement in subjects with both childhood- and adult-onset (AO) GH-deficient, although statistically significant improvements compared with placebo were not demonstrated in all of these studies. One study demonstrated no improvement after GH replacement, whereas another demonstrated no difference in the improvement in after exercise training in combination with GH replacement compared with after exercise training alone. Some of these studies may have been underpowered to detect between-group differences. The largest study to date addressing exercise performance in response to GH replacement included 55 patients with adult onset-GH-deficient in a placebo-controlled, crossover study in which GH therapy was individually dosed to obtain an IGF-I concentration within the normal range for age and gender. A highly significant effect of GH replacement to increase $\text{VO}_{2\text{max}}$ by approximately 6 percent was observed. The overall body of evidence therefore supports an effect of GH to improve maximum work rate, $\text{VO}_{2\text{max}}$, with changes in $\text{VO}_{2\text{max}}$ apparently accounted for by increased LBM [07142].

One study examined effects of prior sprint exercise on hormonal responses to subsequent resistance exercise with different recovery periods between exercise bouts. Nine men performed three types of exercise regimens: resistance exercise only (R), resistance exercise with prior sprint exercise and 60 min of rest (SR60), and resistance exercise with prior sprint exercise and 180 min of rest (SR180). Sprint exercises consisted of maximal sprint cycling (eight sets of 5-s sprints with 30-s rest periods between sets) with prior 10-min warm-up. Resistance exercise consisted of five exercises, each with three sets at a 10-repetition maximum with 1-min rest periods. Prior sprint exercise significantly increased blood lactate, glycerol, epinephrine, norepinephrine, growth hormone (GH), and free testosterone concentrations. Before the resistance exercise, free fatty acids concentration was higher in the SR180 trial than in the SR60 and R trials, whereas GH concentration was significantly higher in the SR60 trial. After the resistance exercise, no significant difference was found in responses of pH, epinephrine, norepinephrine, and free testosterone among trials. The SR180 trial showed a smaller GH response (peak value: $7.8 \pm 1.6$ ng/mL) than in the R trial ($12.8 \pm 3.7$ ng/mL), with no significant difference between trials. In the SR60 trial, GH response to resistance exercise was attenuated. Maximal strength and power measured immediately before the resistance exercise showed no difference among trials. These results indicate that GH response to resistance exercise was attenuated strongly when the exercise was preceded by sprint exercise and a shorter (60 min) recovery period [07143].

**Molecular and metabolic mechanisms**

Skeletal muscle is a target tissue of GH. Based on its anabolic properties, it is widely accepted that GH enhances muscle performance in sports and muscle function in the elderly. This paper critically reviews information on the effects of GH on muscle function covering structure, protein metabolism, the role of IGF1 mediation, bioenergetics and performance drawn from molecular, cellular and physiological studies on animals and humans. GH increases muscle strength by enhancing muscle mass without affecting contractile force or fibre composition type. GH stimulates whole-body protein accretion with protein synthesis occurring in muscular and extra-muscular sites. The energy required to power muscle function is derived from a continuum of anaerobic and aerobic sources. Molecular and functional studies provide evidence that GH stimulates the anaerobic and suppresses the aerobic energy system, in turn affecting power-based functional measures in a time-dependent manner. GH exerts complex multi-system effects on skeletal muscle function in part mediated by the IGF system [13364].
Promoters of secretion

The purpose of one study was to investigate the role of muscle metaboreflex on exercise-induced growth hormone (GH) secretion. In order to accumulate metabolites within exercised muscle with minimized central motor activity, electromyostimulation (EMS) was performed combined with blood flow restriction (BFR). Seven men performed one-legged isometric knee extension evoked by EMS (frequency, 20 Hz; pulse duration, 400 μs; on-off ratio, 3-1 s). Just before the exercise, proximal portion of either a stimulated thigh (ST) or a non-stimulated thigh (NT) was compressed at 150 mmHg with an air-pressure cuff for the purpose of BFR. The compression was kept throughout the exercise session, and was released 2 min after the end of the exercise. Two exercise sessions (ST(BFR), BFR for ST; NT(BFR), BFR for NT) were separated by 1 week. ST(BFR) was aimed to accumulate metabolites within exercised muscle, whereas NT(BFR) was aimed to match mechanical stress with ST(BFR) without accumulating metabolites. Blood samples for hormonal measurements were taken from the antecubital vein before and after the exercise. Blood lactate increased immediately after the exercise in the NT(BFR), whereas it increased after the cuff deflation in the ST(BFR), suggesting that locally produced metabolites were retained and accumulated within the exercised muscle in the ST(BFR). Although serum cortisol and plasma noradrenaline increased in a similar manner in two conditions, serum immunoreactive GH (irGH) increased only in the ST(BFR). These results suggest that muscle metaboreflex plays an important role in the exercise-induced GH secretion, at least in terms of irGH secretion [11470].

Influence of nutrition

Nocturnal endocrine responses to exercise performed in the evening and the potential role of nutrition are poorly understood. To gain novel insight, 10 healthy men ingested carbohydrate with (C+P) and without (C) protein in a randomized order and double-blind manner during 2 hr of interval cycling followed by resistance-type exercise and into early postexercise recovery. Blood samples were obtained hourly throughout 9 hr of postexercise overnight recovery for analysis of key hormones. Muscle samples were taken from the vastus lateralis before and after exercise and then again the next morning (7 a.m.) to calculate mixed-muscle protein fractional synthetic rate (FSR). Overnight plasma hormone concentrations were converted into overall responses (expressed as area under the concentration curve) and did not differ between treatments for either growth hormone (1,464 ± 257 vs 1,432 ± 164 pg/mL for 540 min) or total testosterone (18.3 ± 1.2 vs 17.9 ± 1.2 nmol/L for 540 min, C and C+P, respectively). In contrast, the overnight cortisol response was higher with C+P (102 ± 11 nmol/L for 540 min) than with C (81 ± 8 nmol/L for 540 min). Mixed-muscle FSR did not differ between C and C+P during overnight recovery and correlated significantly with the plasma total testosterone response. No correlations with FSR were apparent for the response of growth hormone cortisol or the ratio of testosterone to cortisol. In conclusion, protein ingestion during and shortly after exercise does not modulate the endocrine response or muscle protein synthesis during overnight recovery [11471].

Influence of testosterone on GH

Testosterone (T) supplementation increases skeletal muscle mass, circulating GH, IGF-I, and im IGF-I expression, but the role of GH and IGF-I in mediating T's effects on the skeletal muscle remains poorly understood. Here, it was shown that T administration increased body weight and the mass of the androgen-dependent levator ani muscle in hypophysectomized as well as castrated plus hypophysectomized adult male rats. T stimulated the proliferation of primary human skeletal muscle cells (hSKMCs) in vitro, an effect blocked by transfecting
hSKMCs with small interference RNA targeting human IGF-I receptor (IGF-IR). In differentiation conditions, T promoted the fusion of hSKMCs into larger myotubes, an effect attenuated by small interference RNA targeting human IGF-IR. Notably, MKR mice, which express a dominant negative form of the IGF-IR in skeletal muscle fibers, treated with a GnRH antagonist (acyline) to suppress endogenous T, responded to T administration by an attenuated increase in the levator ani muscle mass. In conclusion, circulating GH and IGF-I are not essential for mediating T's effects on an androgen-responsive skeletal muscle. IGF-I signaling plays an important role in mediating T's effects on skeletal muscle progenitor cell growth and differentiation in vitro. However, IGF-IR signaling in skeletal muscle fibers does not appear to be obligatory for mediating the anabolic effects of T on the mass of androgen-responsive skeletal muscles in mice [10475].

**Immunofunctional and traditional growth hormone**

The purpose of this study was to compare the growth hormone (GH) response, including the immunofunctional (IF) GH response, between an acute bout of aerobic and resistance exercise in the same subjects. Ten cross-trained males (24 years) performed both 30 min of continuous cycling at 70 percent of VO$_{2\text{max}}$, and intermittent free weight squatting at 70 percent of 1-RM, in a randomly assigned crossover design, separated by at least 1 week. Blood samples were collected at 10-min intervals for 2 h (30 min rest, 30 min exercise, 60 min recovery) and analyzed for total human and IF GH. After adjusting for the amount of work performed per minute of exercise, integrated GH AUC was significantly greater during the resistance session than the aerobic session as measured by both the total and IF GH assays. Peak GH concentrations were significantly greater during the resistance session than the aerobic session. A similar overall GH pattern was observed in response to both types of exercise, with peak values occurring at the end of exercise, regardless of the GH assay used. These data demonstrate that in young, cross-trained males, intermittent resistance exercise elicits a greater response of GH, including IF GH, compared to a continuous aerobic session, when controlling for the work performed per minute, intersubject variability, relative exercise intensity and session duration [07144].

**Effects on circulation**

The ability to perform exercise requires combustion of metabolic fuels, transforming chemical into kinetic and thermal energy. Glucose is the preferred fuel source for short-term high-intensity activity, whereas FFAs (derived from the circulation or from triglycerides stored in muscle or adipose tissue) become increasingly important during more prolonged activity. Oxygen delivery to muscles depends upon adequate ventilation and O$_2$ transport to hemoglobin, circulatory distribution by an adequate cardiac output (CO) and peripheral circulation, dilatation of the muscle capillary network, and extraction of O$_2$ by the muscle fibers with either storage in myoglobin or immediate combustion. GH could improve exercise performance through increased delivery of substrate and oxygen to exercising muscle, increased fat oxidation with glycogen sparing, increased muscle strength, or a combination of these variables. GH could also improve exercise performance through indirect mechanisms, including changes in body composition or more efficient thermoregulation. When pulmonary function – which does not appear to be impaired in GHD or improved by GH replacement – is adequate, delivery of O$_2$ to exercising muscle is dependent on the O$_2$-carrying capacity of the blood, CO, and regional blood flow. GH and IGF-I increase erythropoiesis in vitro, in animal models, and in growing children. It has been demonstrated reduced red cell mass and total blood volume in GHD adults, and normalization after GH replacement. Consistent with other studies, GH replacement also increased plasma volume, which by increasing preload, would be predicted to increase stroke volume (SV) and CO, the product of SV and heart rate.
Independent of effects on preload, GH could also increase cardiac contractility through an anabolic effect on the myocardium, mediated either directly or through increased IGF-I. Most, but not all studies using echocardiography or equilibrium radionuclide angiography have demonstrated reduced left ventricular (LV) mass and LV ejection fraction (EF) in GHD adults compared with normal subjects. Reports of the effects of GH replacement on cardiac structure and function are inconsistent, but a recent meta-analysis of placebo-controlled trials demonstrated a significant effect of GH replacement to increase left ventricular posterior wall thickness and SV. Of particular importance to this review is evidence from studies using radionuclide angiography that GH enhances the ability of LVEF to increase during exercise, which is necessary to provide adequate blood supply to exercising muscle. The effects of GH on SV and CO must be considered in relation to changes in systemic vascular resistance (SVR) and afterload. As described above, GH replacement increases SV, which in the absence of change in heart rate would be expected to increase mean arterial pressure. However, mean arterial pressure does not change or even decreases after GH replacement, and because it represents the product of CO and SVR, this observation can only be explained by a reduction in SVR. A mechanistic explanation for this effect is provided by a study that demonstrated increased production of nitric oxide, the key mediator of endothelial relaxation, after 3 months of GH replacement [07142].

**Fett-substrate metabolism**

It has been reported in 1965 that the exercise-induced increase in GH was followed by an increase in fatty acids and suggested that through its lipolytic effect GH could increase availability of fat as substrate during exercise. Under resting conditions, particularly when fasting, fatty acids are the predominant fuel used by skeletal muscle. Fat oxidation increases in relation to the intensity of exercise up to 65 percent of VO\(_{2\text{max}}\), when it accounts for approximately 50 percent of energy expenditure, but with increasing intensity of exercise, the reliance on glucose as fuel increases, and the relative oxidation of glucose in relation to fat increases. Whether fatty acid availability influences partitioning of substrate oxidation during exercise is unclear, some studies demonstrating increased fat oxidation and reduced muscle glycogen depletion when fatty acid availability is greater, and others demonstrating no effect. GH directly stimulates lipolysis through activation of adenyl cyclase followed by activation of cAMP-dependent protein kinase and phosphorylation and activation of hormone-sensitive lipase. Studies in fat cells and in animal models have shown that in addition to its direct lipolytic effect on adipose tissue (demonstrated by stimulation of basal lipolysis), GH also increases lipolysis indirectly by altering the effect of adipocytes to respond to lipolytic factors such as catecholamines. Already in 1959 it was also demonstrated that GH increased plasma FFA in human subjects, and that administration of GH enhanced forearm muscle uptake and oxidation of FFA and increased the release of FFA from adipose tissue. GH, administered as a bolus or by infusion, increases circulating levels of glycerol and FFA in GHD and normal subjects after a lag time of 2-3 hours. Small pulses of GH designed to mimic physiological pulses have been shown to induce a dose-dependent stimulation of lipid oxidation and increase circulating levels of FFA and glycerol. Using microdialysis techniques, it has been shown that a physiological GH pulse stimulates lipolysis in both abdominal and femoral adipose tissue, although to a greater degree in abdominal tissue [07142].

In normal subjects, the onset of exercise leads to a 3-fold increase in the rate of lipolysis and a rapid increase in uptake of FFAs into skeletal muscle. The rate of disappearance of FFA from the circulation, which during exercise is largely into skeletal muscle, was also reduced after GH withdrawal. One experimental protocol resulted in an increment in circulating GH levels during exercise that was indistinguishable from that seen in healthy normal subjects. Under resting conditions, there was no effect of GH, whereas during and after 45 min of
exercise at lactate threshold there was a greater increment in fatty acid turnover after GH administration. GH clearly increases whole-body fat oxidation under resting conditions, and an increase in maximal fat oxidation during exercise has also been demonstrated in GH-deficient adults after 6 and 12 months of GH replacement. There are also findings suggesting that under resting conditions (when the biopsies were taken) increased fat oxidation in response to GH occurs in tissues other than skeletal muscle. It is possible that different effects would occur during exercise [07142].

*Glucose-substrate metabolism*

There is less information available concerning the effects of GH-deficiency and GH replacement on glucose kinetics during exercise. Under resting conditions, GH administration results in increased hepatic glucose production, reduced glucose uptake into skeletal muscle, and increased insulin secretion. There is increasing evidence that this effect may occur secondary to the lipolytic effect of GH. In summary, therefore, there is strong evidence that GH replacement increases lipolysis, FFA availability, and uptake from the circulation more markedly during exercise compared with resting conditions. There is also preliminary evidence that GH replacement increases whole-body fat oxidation during exercise, although it is not known whether this effect occurs in skeletal muscle or in other tissues. The effects of GH replacement on glucose metabolism during exercise appear to be less marked 07[142].

*Effects on muscle mass and strength.*

There is an extensive body of literature from in vitro and animal models concerning the cellular mechanisms through which GH and IGF-I exert anabolic effects on skeletal muscle. Studies in human subjects have provided information regarding the immediate and short-term effects on gene transcription through which these processes occur. GH-induced tyrosine phosphorylation (indicating activation) of STAT5, consistent with a direct effect of GH in skeletal muscle, mediated through the Janus kinase/signal transducer and activator of transcription (JAK-STAT) signaling pathway. This finding is consistent with previous observations from regional amino acid balance studies that showed an acute effect of GH to promote protein synthesis in forearm muscle. The physiological importance of the anabolic effect of GH is apparent in GHD adults. Briefly, lean body mass (LBM) is reduced in GH-deficient adults by approximately 7-8 percent compared with age- and gender-matched normal subjects, representing similar reductions in extracellular water and body cell mass, the metabolically active component of LBM. Skeletal muscle comprises the majority of body cell mass, and studies using computed tomography and magnetic resonance imaging scanning have demonstrated a reduction in cross-sectional skeletal muscle area in GH-deficient adults that is proportional to the reduction in LBM estimated by measurement of total body potassium. Reduced muscle mass in GHD subjects is associated with reduced isometric muscle strength, whereas some but not all studies have also demonstrated reduced isokinetic strength. It remains uncertain whether reduced strength is entirely accounted for by the reduction in muscle mass or whether there is also intrinsic muscle weakness associated with GHD. In contrast to the protein anabolic effect of GH replacement, which occurs within days to weeks of initiation of treatment, the overall body of evidence suggests that long-term but not short-term GH replacement increases and normalizes muscle strength. It was carried out an extensive series of strength tests at the beginning and end of 6 months of GH replacement. Strength increased in most of the nine muscle groups that were studied, but it only reached statistical significance in one of the groups. This study and two other studies of GH replacement, lasting 12 weeks and 6 months, respectively, may not have been adequately powered to demonstrate a statistically significant effect. After 2 years of treatment with a mean daily dose of 0.62 ± 0.03 mg of GH, isometric and isokinetic
strength in GH-deficient men increased into the normal range, although a reduction was seen in muscle endurance. A later study confirmed that these effects persisted after 5 years of treatment. Like many of the clinical features of GHD, the effect of GH replacement was most pronounced in subjects in whom strength was most abnormal at baseline [07142].

**Body fat, extracellular water, and thermoregulation**

In addition to reduced muscle mass, other abnormalities of body composition and the ability to dissipate excess heat in GH-deficient individuals could contribute to impaired exercise performance. Total body and centrally distributed fat are increased in proportion to the duration of GHD, whereas extracellular water is reduced. The ability to carry out weight-bearing exercise is influenced by the quantity of body fat, which represents a mechanical limitation to exercise. The effect of reduced extracellular water on exercise capacity is less clear but might also be important. Sweating is essential for maintenance of body temperature during exercise, and thus impaired thermoregulation during exercise may also contribute to reduced exercise capacity in GHD. Using pilocarpine iontophoresis, it has been demonstrated that the sweat secretion rate is significantly lower in GHD adults than in appropriately matched control subjects and is increased during GH replacement. Despite GH replacement, sweat secretion rates were reduced, body heat storage was increased, and therefore there was a greater increase in core temperature during exercise in GHD subjects. Interestingly, five of 10 GHD patients stopped exercise prematurely because of subjective discomfort and signs of heat exhaustion [07142].

**Interactions with thyroid hormones and sex steroids**

Interactions between the GH/IGF-I axis and thyroid hormones and sex steroids may also be important. Thyroid hormone replacement in the hypopituitary patient cannot be titrated against serum TSH, the most sensitive index of tissue activity of thyroid hormones, and thus subtle degrees of over- and underreplacement with thyroid hormones likely occur in hypopituitarism. GH, through increased 5′-deiodinase activity, increases conversion of T₄ to metabolically active T₃, and it has been suggested that this effect might underlie some of the metabolic changes observed with GH replacement. Untreated testosterone deficiency in males is associated with reduced LBM, increased body fat, and reduced exercise capacity, whereas orally administered estrogen reduces fat oxidation and increases body fat in normal women. When administered together, testosterone and GH exert a combined effect on protein anabolism and body composition, and there is increasing evidence that androgen deficiency might also contribute to the phenotype of hypopituitary women [07142].

**Hypothalamo-pituitary-adrenal (HPA) axis**

Activation of the hypothalamo-pituitary-adrenal (HPA) axis and of the growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis represents a physiological response to the energetic, metabolic, vascular, and sometimes neurophysiologic or psychological needs of exercise. Long-lasting increased and (or) decreased secretion of cortisol (the end-product of the HPA axis) or of GH is detrimental to health. This suggests that the activity of these hormonal axes is finely tuned toward homeostasia, tolerating limited prolonged homeostatic disruption. However, the relationships between exercise training and cortisol and GH secretion are full of odd and controversial ideas. In one review, the relationships between HPA axis adaptation to exercise training or disadaptation with overtraining was discussed, with an emphasis on the limitation on the current measures used to profile hormonal activity. Knowledge of these relationships between cortisol and GH responses to exercise is an important tool to fight against doping with glucocorticoids and GH, and their health-damaging
consequences [07145].

**Growth hormones secretagogues**

The administration of growth-promoting agents such as human growth hormone as well as compounds with respective secretagogue activity is prohibited in sports according to the regulations of the World Anti-Doping Agency. Acetylcholine esterase inhibitors have been demonstrated to stimulate growth-hormone secretion in elderly humans, and new orally active drugs have been developed to provide alternatives to therapeutic injections of growth-hormone preparations. Preventive anti-doping strategies include method development for emerging drugs and potentially misused compounds. Hence, the mass spectrometric dissociation behavior of three acetylcholine esterase inhibitors (donepezil, galantamine and rivastigmine) and a structural analogue to the growth-hormone secretagogue SM-130686 were studied using high-resolution/high-accuracy orbitrap mass spectrometry. These data provided substantial information for screening procedures, complementing common methods of sports drug testing. Using liquid-liquid extraction and subsequent liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis, the four target analytes were determined at urinary concentrations of 15-20 ng/mL, recoveries ranged from 55-97 percent, and assay precisions were calculated at 5.2-15.8 percent (intraday) and 10.2-21.6 percent (interday) for all compounds. The applicability of the developed assay to authentic urine specimens was tested using two administration study urine samples after application of Reminyl (galantamine) and Aricept (donepezil). In both cases, the administered drug and the respective desmethylated metabolites were detected [06138].

**The GH-IGF-I axis and exercise in normal subjects**

It has been demonstrated that plasma levels of GH increase during exercise, and it was later shown that exercise is the most potent physiological stimulus to GH release. GH levels start to increase 10 to 20 min after the onset of exercise, peak either at the end or shortly after exercise, and remain elevated for up to 2 h after exercise. The neuroendocrine pathways through which GH secretion is regulated during exercise are complex and poorly understood, but there is evidence that adrenergic, cholinergic, and opioid pathways are involved. The magnitude of the GH response to exercise is influenced by age, gender, body composition, physical fitness, and the intensity, nature and duration of exercise. The impact of these variables has been more clearly defined in a recent series of meticulously carried out studies using ultrasensitive chemiluminescence GH assays and deconvolution analysis of GH secretion. A linear dose-response relationship between exercise intensity and the GH secretory response has been demonstrated, with escalating GH release across the range (25 to 175 % of lactate threshold) of exercise intensities. Later studies from the same laboratory demonstrated that GH secretion correlates positively with duration of exercise when intensity is constant, is augmented by repetitive bouts of exercise, but is not influenced by the time of day that exercise was performed. It has also been demonstrated that GH secretion rates under resting conditions were greater in women; but during exercise, although absolute GH secretion rates were also increased, the increment from baseline was similar in men and women and did not correlate with sex hormones. The GH response to exercise, like 24-hr GH secretion rates, declines with aging, and it has been demonstrated that even in early middle age (mean age, 42 years), the GH response to exhaustive exercise is greatly attenuated compared with younger (mean age, 21 years) subjects. It is, however, difficult to separate inherent effects of aging from changes in body composition, because body fat increases with aging and GH secretory rates are reduced in overweight subjects. In a study designed to separate out the effects of aging, body composition, and physical fitness, the GH response was found to be determined by age and physical fitness (VO\textsubscript{2max}) but not by body.
fat, implying that maintenance of physical fitness with increased aging is more important in determining GH release than avoidance of increased adiposity. However, training programs that improve physical fitness do not appear to increase the GH response to exercise. The physiological mechanisms through which GH secretion increases during exercise are not known, but changes in body temperature, blood lactate levels, and pH have all been postulated [07142].

Exercise exerts acute effects on other components of the GH/IGF-I axis. GH-binding protein, total IGF-I, IGF binding protein (IGFBP)-3, and acid-labile subunit increase slightly during exercise, whereas IGFBP-1 increases after exercise, and free IGF-I does not appear to change during or after exercise. These observations are not altered after adjustment for changes in hydration status during exercise. IGF-I, IGFBP-3, and acid-labile subunit circulate as a ternary complex, and the observation that all three components increase in parallel with no change in free IGF-I suggests that these effects occur due to mobilization of preformed intact complexes. Consistent with this, IGFBP-3 proteolysis has been shown not to increase during or after strenuous rowing exercise. The physiological relevance of these effects is not known, but it has been postulated that the modest increase in IGF-I might enhance postexercise reparative processes, or that increased IGFBP-1 might protect against delayed onset hypoglycemia. Twenty-four-hour GH secretion rates and plasma IGF-I levels correlate positively with VO_{2max} and leisure time physical activity whereas long-term exercise training approximately doubles integrated GH concentrations in women when measured on nonexercising days. Levels of IGFBP-3 and total and free IGF-I increase after training, increased IGF-I levels becoming detectable within 2 week of commencing training and remaining above baseline for at least 6 months. These long-term effects of exercise on the GH-IGF-I axis might also contribute to some of the effects of training, including increased muscle mass and increased CO, although evidence for this is currently lacking [07142].

**Supraphysiological GH and exercise performance**

Administration of supraphysiological GH to normal subjects under resting conditions increases insulin secretion, lipolysis, fatty acid availability, and fat oxidation, and reduces glucose uptake into skeletal muscle. More recently, the effects of administration of supraphysiological GH on intermediate metabolism during exercise have also been addressed. It was demonstrated that plasma levels of glucose, glycerol, FFA, and lactate were greater during moderate to high-intensity exercise in trained men after administration of a single dose of rhGH, 2.5 mg sc, 4 h before exercise. It was also studied glycerol and glucose turnover using stable isotope techniques in endurance-trained athletes before and during 4 wk of rhGH (0.06 mg/kg/d) administration. rhGH increased lipolysis and plasma levels of FFA at rest and during and after submaximal exercise. rhGH did not influence glucose turnover at rest but increased rates of glucose production and uptake during and after exercise. Taken together, the findings of available studies demonstrate that GH enhances lipolysis during exercise under both postabsorptive and postprandial conditions and that the lipolytic effect of GH during and after exercise does not depend on increased circulating levels of GH during exercise. Despite increased fatty acid availability, there is no effect of GH on fat oxidation during or after exercise. A number of small studies have addressed the effect of supraphysiological GH administration to normal or trained subjects on protein metabolism with some conflicting findings. Observations vary between studies of athletic and nonathletic subjects, and between those of whole-body protein turnover and muscle protein synthesis. It is apparent that studies of protein metabolism in the resting state may fail to recognize important changes occurring during or after exercise. rhGH administration has been consistently shown to increase LBM in young normal or trained subjects, but it is not known how much of this increase is secondary to protein accretion and
how much to increased total body water (TBW) secondary to the antinatriuretic effect of GH. There is evidence, therefore, that supraphysiological GH administration to trained subjects results in conservation of protein and that this effect is particularly marked during exercise. However, protein anabolic processes are influenced by fatty acid availability, and therefore it is possible that these effects are secondary to the lipolytic effect of supraphysiological GH. Although findings suggest that long-term GH excess (acromegaly) is likely to be detrimental to exercise performance, it should be noted that clinical features of acromegaly are usually present for some years before diagnosis, and that biochemical GH excess precedes the appearance of clinical signs [07142].

GH was recommended in “The Underground Steroid Handbook” in 1983 as “a new and exiting anabolic agent” approximately 7 years before any publication suggesting that this effect occurred in adults appeared in the scientific literature. Ben Johnson was disqualified from the gold medal position in the 100 m in the 1988 Olympic Games and subsequently admitted under oath to having self-administered GH as well as anabolic steroids. Although it is clear that GH abuse by athletes is widespread there is no evidence of its efficacy. The most plausible mechanisms by which administration of supraphysiological doses of GH could improve exercise performance are through increased muscle mass and strength and through increased fatty acid availability resulting in glycogen sparing and increased endurance. Only two studies, with seven and eight subjects, respectively, receiving GH, have investigated the effect of GH on strength in young normal or trained subjects. Neither demonstrated any significant improvement, although the studies were of short duration and almost certainly lacked statistical power to detect a meaningful difference. There is also no evidence that GH improves endurance. However, to put these unimpressive scientific findings into context, anabolic steroids were widely abused for more than 40 years before they were definitively shown to increase strength, and the pattern of GH abuse by athletes may differ considerably from controlled clinical trials. In particular, there is evidence of an additive effect between testosterone and GH, and trials of their combined administration to athletes have not yet been reported. Furthermore, the marginal changes that differentiate winning from losing in high-level sport are unlikely to be detected in classical clinical trials, which are usually statistically powered to distinguish much larger differences. Athletes and coaches, who meticulously monitor their own performance, can detect much smaller changes with different interventions that could not be identified in small or medium-sized clinical trials. This has been demonstrated in secret doctoral theses pertaining to the sports doping program of the German Democratic Republic, which became available in the early 1990s after German reunification. In these papers, it is clearly shown that the principal method used by doctors and coaches to evaluate the effects of anabolic steroids was by comparing performance targets in individual athletes when taking and not taking different agents [07142].

Influence of training on GH response

One study examined the effects of short-term physical training on the acute hormonal response (i.e., growth hormone [GH], total and free IGF-I, and IGFBP-1, -2, and -3) to resistance exercise (RE) in women. Forty-six women (20 years) were randomly assigned to an endurance training (E), resistance training (R), combined training (R+E), or control (C) group for 8 weeks. Subjects completed a standardized bout of resistance exercise (six sets of back squats at 10-RM) before and after training. Blood samples were obtained at rest (PRE), after the third set, immediately post-exercise (POST), and at 15 min and 30 min after exercise. Acute RE significantly increased serum GH, total IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 concentrations and decreased free IGF-I concentrations. Following 8 weeks of training, total IGF-I concentrations were significantly increased and IGFBP-1 concentrations were significantly decreased during exercise in groups that participated in resistance training;
no significant changes were seen following E or C. It was concluded that participation in resistance training increased total IGF-I and reduced IGFBP-1 concentrations during acute resistance exercise, indicating exercise mode-specific adaptations in the circulating IGF-I system [12208].

Influence of carbohydrates and proteins

Endocrine responses to repeated exercise have hardly been investigated and no data are available regarding the mediating influence of nutrition. On three occasions, participants ran for 90 min at 70 percent \(\text{VO}_{2\text{max}}\) (R1) before a second exhaustive treadmill run at the same intensity (R2). During the intervening 4 h recovery, participants ingested either: 0.8 g sucrose/kg/h with 0.3 g/kg/h whey protein isolate (CHO-PRO); (ii) 0.8 g sucrose/kg/h (CHO) or; (iii) 1.1 g sucrose/kg/h (CHO-CHO). The latter two solutions therefore matched the former for carbohydrate or for available energy, respectively. Serum growth hormone concentrations increased from 1.7 ± 0.9 micr g/L to 16.7 ± 7.8 micro g/L during R1 considered across all treatments. Concentrations were similar immediately after R2 irrespective of whether CHO or CHO-CHO was ingested, whereas ingestion of CHO-PRO produced an augmented response. Growth hormone binding protein concentrations were unaffected by R1 but increased similarly across all treatments during R2, as was the case for plasma total testosterone. There was an overall treatment effect for serum cortisol, with no specific differences at any given time-point but lower concentrations immediately after R2 with CHO-PRO than CHO or CHO-CHO. Thus, ingesting carbohydrate with added whey protein isolate during short-term recovery from 90 minutes of treadmill running increases the growth hormone response to a second exhaustive exercise bout of similar duration [12209].

Influence on glucose homeostasis

It has been demonstrated that AAS use, specifically growth hormone, can affect glucose homeostasis through increasing cellular insulin resistance and reducing glucose uptake. Excess growth hormone has been shown to cause symptoms of acromegaly which predisposes up to 40 percent of patients to diabetes. As trenbolone acetate is not indicated for human use and athletes are known to use supraphysiologic doses of this underground, performance enhancing drug, the correlation of the timing of events and the use of this veterinary growth hormone likely exacerbated an underlying condition or caused this new onset diabetes. A 33-year-old male presented to the emergency department with complaints of polydipsia, polyuria, nausea, headaches, blurry vision and malaise. Lab work revealed a serum glucose level of 1166 mg/dl (64.8 mmol/L). The patient admitted to completing a cycle of androgenic anabolic steroids (AASs) for bodybuilding. His regimen consisted of supraphysiologic intramuscular injections of a bovine growth hormone, trenbolone acetate and testosterone. The patient received intravenous fluids and insulin to restore metabolic balance. Previously healthy with a non-contributory family history, he was diagnosed with new onset diabetes. AAS have the potential to induce or exacerbate diabetic conditions due to decreased glucose tolerance and increased insulin resistance [12210].

Influence on cardiovascular system

Growth hormone (GH) exerts its effects through insulin-like growth factor-1, and although ubiquitous in human tissues, it has a significant role in cardiovascular function. In recent years, there has been a great deal of interest in GH as an etiologic factor in many cardiovascular disease states. Acromegaly, a state of endogenous GH excess, results in myocardial hypertrophy and decreased cardiac performance with increased cardiovascular mortality. Additional insight into the role of excess GH on the cardiovascular system has
been gained from data collected in athletes doping with GH. Likewise, GH deficiency is associated with increased mortality, possibly from the associated increase in atherosclerosis, lipid abnormalities, and endothelial dysfunction. However, further research is required to clarify the benefit of GH treatment in both deficient states and in heart failure patients [12211].

**Effect on IGFBP-4 and -5**

It was studied the effects on insulin-like growth factor binding proteins (IGFBP)-4 and -5 after one month's treatment with supraphysiological doses of growth hormone (GH) in healthy, active young adults with a normal GH-IGF-I axis. Furthermore, the possible use of IGFBP-4 and IGFBP-5 as markers of GH doping is discussed. Thirty healthy, physically active volunteers (15 men and 15 women), mean age 26 years (range 18-35), participated in this randomized, double-blind, placebo-controlled, parallel study with three groups (n=10; 5 men and 5 women in each group). The groups comprised the following: placebo, GH 0.1IU/kg/day (0.033mg/kg/day) and GH 0.2IU/kg/day (0.067mg/kg/day). Baseline levels of IGFBP-4 were higher (+20 %), while IGFBP-5 levels were lower (-37 %) in women than in men. IGFBP-5 levels were positively correlated to age, but no significant correlation was found for IGFBP-4. In the pooled group with active GH treatment (n=20), both IGFBP-4 and IGFBP-5 levels were significantly increased versus the placebo group from day 14 until end of treatment: day 28, IGFBP-4 (+40 %) and IGFBP-5 (+61 %). After inclusion of serum IGF-I as a covariate in the linear regression analysis, the associations between GH treatment and the IGFBP-4 and IGFBP-5 levels were not significant. The study shows that the levels of IGFBP-4 and IGFBP-5 are affected by supraphysiological GH treatment given to young, healthy, physically active adults of both genders. The present study, including relatively few subjects, does not support that IGFBP-4 and IGFBP-5 can be used as IGF-I independent markers in a forthcoming method for detecting GH doping, although, further studies are needed to investigate the potential use of IGFBP-4 and IGFBP-5 as markers of GH doping [07146].

**Influence of alcohol**

Alcohol decreases spontaneous growth hormone (GH) secretion, but the mechanism is unclear. The aim of this study was to test whether administration of alcohol (study 1) or a N-methyl d-aspartate (NMDA) receptor antagonist (study 2) attenuates the GH response to pharmacological dopaminergic stimulation. The 2-session repeated measures design was conducted at an endocrine laboratory. Twenty healthy Caucasian males aged 35 years without a history of alcohol use disorders were tested using the apomorphine (APO) challenge test. In study 1, it was injected APO (0.01 mg/kg s.c.) 1 hour after oral administration of 1 g/kg ethanol and placebo, respectively. In study 2, the APO challenge was conducted after 0.3 mg/kg dextromethorphan (DXM) and placebo. The main outcome measures were the peak serum GH concentration and area under the time/concentration curve up to 120 minutes after APO. Compared with placebo, alcohol significantly decreased the APO-induced GH release (mean and SEM peak GH concentration 20 ± 3 vs 6 ± 2 ng/mL). Dextromethorphan did not change APO-induced GH response. It was concluded that a single intermediate alcohol dose markedly reduces GH response to dopaminergic stimulation. Although alcohol is thought to stimulate dopaminergic function in certain pathways, but not necessarily in the hypothalamus, our results are in line with the alcohol effect on baseline GH secretion. Growth hormone suppression appears not to be mediated by ethanol's NMDA-antagonistic properties [07147].

**Effects on other variables**
A double blind placebo controlled study of one month's GH administration to 102 healthy non-competing but trained subjects. Blood levels of nine markers of GH action were measured throughout the study and for 56 days after cessation of GH administration. Blood samples were also taken from 813 elite athletes both in and out of competition. GH caused a significant change in the nine measured blood markers. Men were more sensitive to the effects of GH than women. IGF-I and N-terminal extension peptide of procollagen type III were selected to construct formulae which gave optimal discrimination between the GH and placebo groups. Adjustments were made to account for the fall in IGF-I and P-III-P with age and the altered distribution seen in elite athletes. Using a cut-off specificity of 1:10,000 these formulae would allow the detection of up to 86 percent of men and 60 percent of women abusing GH at the doses used in this study. This will provide the basis of a robust and enforceable test identifying those who are already cheating and provide a deterrent to those who may be tempted to do so [07148].

Effects on respiration

To determine whether 6 days recombinant human growth hormone (rhGH) administration, in an abstinent anabolic-androgenic steroid (AAS) using group had any respiratory, endurance exercise and biochemical effects compared with an abstinent AAS control group male subjects (n=48) were randomly divided, using a single blind procedure into two groups. Anthropometry, respiratory muscle function and endurance exercise were investigated. Respiratory measurements examined, were forced expiratory volume in one second, forced vital capacity, maximum inspiratory pressure and maximum expiratory pressure. Endurance exercise was assessed by measuring peak oxygen uptake (VO$_{2peak}$). Biochemical analysis included; haemoglobin, packed cell volume, glucose, sodium, urea, creatinine, total protein, albumin, testosterone and insulin like growth factor-I (IGF-I). Forced expiratory volume in one second/forced vital capacity, maximum inspiratory pressure, maximum expiratory pressure, and IGF-I significantly increased compared with the control group. Body mass index, fat free mass index, peak oxygen uptake, maximum inspiratory pressure, maximum expiratory pressure, IGF-I and serum sodium significantly increased, whilst body fat, total protein and albumin, significantly decreased within the GH group. The findings of the study indicated that short-term high dose rhGH increased aerobic performance and respiratory muscle strength in former AAS users [07149].

Side effects

The side effects associated with GH administration in GH deficiency are well-documented and may affect any athlete receiving GH; however, as anecdotal evidence suggests that many athletes are taking much higher doses than those used therapeutically, it is possible that features of acromegaly may become apparent with prolonged use. Additional long-term effects may include fluid retention, which may lead to ankle swelling, hypertension, headache, diabetes, and cardiomyopathy. There is also a potential for increased risk of certain cancers; including colorectal, thyroid, breast, and prostate cancer. Cadaveric GH, with its attendant risk of the prion-induced Creutzfeldt-Jakob disease, is still available in the black market [13007].

Clinical use

GH replacement is an effective and rational therapy for adult men and women with known pituitary disease or risk factors for hypopituitarism; for example, severe head trauma or
pituitary irradiation, provided that GHD is proven. Only at-risk adults should be tested, and 
adults with childhood GHD should be retested before prescribing GH for adult needs. A 
neuroendocrine review should be considered in all cases of TBI and SAH. The AACE,
Endocrine Society, and GRS have issued detailed GHD treatment guidelines. Diagnostic 
evaluations should follow these guidelines, and physicians should stay abreast of changing 
information about testing cut-points and the use of IGF-1 as a marker for GHD. The pros and 
cons of GH treatment must be discussed with each patient, after which GH doses should be 
individualized and titrated to maximum efficacy with minimal side effects. Off-label use of GH 
therapy as an anti-aging treatment or sports enhancement is clearly at odds with current 
guidelines and should not be prescribed under any circumstances. GH replacement therapy 
has been shown to improve the clinical features of GHD in adults, although there have yet 
been no studies on endpoints such as cardiovascular events, fractures, and death. When 
used in an approved and ethical fashion for the defined population, this treatment can 
enhance the health and well-being of the many patients who suffer from the complications of 
GHD [09200].

In recent years there have been rapid developments in the use of growth factors for 
accelerated healing of injury. Growth factors have been used in maxillo-facial and plastic 
surgery with success and the technology is now being developed for orthopaedics and sports 
medicine applications. Growth factors mediate the biological processes necessary for repair 
of soft tissues such as muscle, tendon and ligament following acute traumatic or overuse 
injury, and animal studies have demonstrated clear benefits in terms of accelerated healing. 
There are various ways of delivering higher doses of growth factors to injured tissue, but 
each has in common a reliance on release of growth factors from blood platelets. Platelets 
contain growth factors in their alpha-granules (insulin-like growth factor-1, basic fibroblast 
growth factor, platelet-derived growth factor, epidermal growth factor, vascular endothelial 
growth factor, transforming growth factor-beta1 and these are released upon injection at the 
site of an injury. Three commonly utilised techniques are known as platelet-rich plasma, 
autologous blood injections and autologous conditioned serum. Each of these techniques 
has been studied clinically in humans to a very limited degree so far, but results are 
promising in terms of earlier return to play following muscle and particularly tendon injury. 
The use of growth factors in sports medicine is restricted under the terms of the World Anti-
Doping Agency (WADA) anti-doping code, particularly because of concerns regarding the 
insulin-like growth factor-1 content of such preparations, and the potential for abuse as 
performance-enhancing agents. The basic science and clinical trials related to the 
technology are reviewed, and the use of such agents in relation to the WADA code is 
discussed [07150].

Delivery methods

In recent years there have been rapid developments in the use of growth factors for 
accelerated healing of injury. Growth factors have been used in maxillo-facial and plastic 
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autologous blood injections and autologous conditioned serum. Each of these techniques has been studied clinically in humans to a very limited degree so far, but results are promising in terms of earlier return to play following muscle and particularly tendon injury. The use of growth factors in sports medicine is restricted under the terms of the World Anti-Doping Agency (WADA) anti-doping code, particularly because of concerns regarding the insulin-like growth factor-1 content of such preparations, and the potential for abuse as performance-enhancing agents. The basic science and clinical trials related to the technology were reviewed, and the use of such agents in relation to the WADA code is discussed.[07151]

Use in weightlifters

In a study of performance-enhancing substance use among 231 experienced young male weightlifters, it was found that 27 (12%) reported illicit use of human growth hormone (HGH) or its bioactive derivative, insulin-like growth factor-1. All of these 27 men also reported use of anabolic-androgenic steroids (AAS) and 22 (81%) met criteria for current or past AAS dependence. Fifteen (56%) also reported current or past dependence on opioids, cocaine, and/or ecstasy. These findings suggest that among young male weightlifters, illicit HGH use has become a common form of substance abuse, frequently associated with both AAS dependence and classical substance dependence[11164].

Biomarkers for use (other than IGF-1)

The detection of recombinant human growth hormone (rhGH) is difficult due to its short half-life; therefore, novel and robust biomarkers of rhGH abuse are needed. In one study, serum samples derived from subjects treated with rhGH in a randomized, double blind, placebo-controlled crossover study were analyzed by 2-DE coupled with MS. Eight healthy male subjects aged 23 ± 1 years were injected with rhGH (2 mg/day) or saline for 7 days with serum samples drawn at days 0, 3, and 8. Protein intensities were quantified and analyzed for differences between rhGH and placebo treatments. Proteins that showed significant changes were identified and confirmed by Western blotting. These included specific isoforms of alpha-1 antitrypsin and transthyretin that increased; and inter-alpha-trypsin inhibitor heavy chain H4, apolipoprotein A-1, and hemoglobin beta chain that decreased. These proteins represent novel biomarkers of short-term rhGH exposure and may lead to a new method for detecting rhGH doping[11165].

GH generates the expression of both circulating and peripheral IGF-I. The GH induced circulating IGF-I, IGF binding protein (IGFBP3) and acid labile subunit (ALS) are predominantly liver derived. GH also increases bone and collagen turnover markers, with type 3 procollagen (P-III-P) being the specific marker. The biological variability of IGF-I and P-III-P has been studied longitudinally in a placebo controlled double blind manner (project GH-2000), and subsequently in project GH 2004 in amateur athletes, and longitudinally in Italian elite athletes. Some have proposed the use of these biological markers of GH action as the athlete's "passport" whereby each athlete acts as his or her own control. Another complementary GH-related biomarker is mannan-binding lectin (MBL), which is reported to increase, with a clear concentration of up to 700 %, following recombinant GH (rGH) administration while exhibiting a slow recovery time. The influence of co-administration of testosterone on the level of GH markers shows that IGF-I levels are not affected; however, the levels of P-III-P are increased[12011].
Aiming for a complementary (pre-)screening tool, the utility of DNA aptamers in an immunoassay-like scenario was reported. In a study comparing the affinity of aptamers to recombinant and natural hGH, the formation of non-covalent dimers of recombinant hGH (bridged by the aptamer DNA) was shown. The homodimeric structure was not found when applying the aptamer assay to natural hGH. A phenomenon which was suggested to provide a means to indicate the presence of non-natural hGH in doping control samples [12016].

There is growing concern about the use of recombinant human growth hormone (rhGH) by individuals taking part in competitive sports. Although rhGH is banned by the international organizations, the detection of GH doping is difficult. It was postulated that rhGH will suppress endogenous GH production, which can be assessed by the measurement of mRNA for GH and growth hormone-releasing hormone (GHRH). In order to prove this concept, it was undertaken a pilot study to examine whether circulating nucleic acids are useful in the detection of endogenous GH production. In acromegalic patients, median mRNA concentration for GHRH (corrected for beta-actin mRNA) was 30.7 times lower than in Control. There was a significant correlation between serum IGF-1 score and mRNA for GHRH. In acromegalic patients, mRNA for GH was significantly higher than in controls. As GH production is known to decline with age, it was also examined mRNA for GH and GHRH according to age subgroups. Both markers were significantly lower in the older age group (>50 years) compared to the younger age group (<34 years). These results show that mRNA for GH and GHRH can be detected in the peripheral circulation [08249].

A family of small peptides has reached the focus of doping controls representing a comparably new strategy for cheating sportsmen. These growth hormone releasing peptides (GHRP) are orally active and induce an increased production of endogenous growth hormone (GH). While the established test for exogenous GH fails, the misuse of these prohibited substances remains unrecognized. The present study provides data for the efficient extraction of a variety of known drug candidates (GHRP-1, GHRP-2, GHRP-4, GHRP-5, GHRP-6, alexamorelin, ipamorelin, and hexarelin) from human urine with subsequent mass spectrometric detection after liquid chromatographic separation. The used method potentially enables the retrospective evaluation of the acquired data for unknown metabolites by means of a non-targeted approach with high-resolution/high-accuracy full-scan mass spectrometry with additional higher collision energy dissociation experiments. This is of great importance due to the currently unknown metabolism of most of the targets and, thus, the method is focused on the intact peptidic drugs. Only the already characterised major metabolite of GHRP-2 (D: -Ala-D: -2-naphthylAla-L: -Ala, as well as its stable isotope-labelled analogue) was synthesised and implemented in the detection assay. Method validation for qualitative purpose was performed with respect to specificity, precision (<20 %), intermediate precision (<20 %), recovery (47-95 %), limit of detection (0.2-1 ng/mL), linearity, ion suppression and stability. Two stable isotope-labelled internal standards were used (deuterium-labelled GHRP-4 and GHRP-2 metabolite). The proof-of-principle was obtained by the analysis of excretion study urine samples obtained from a single oral administration of 10 mg of GHRP-2. Here, the known metabolite was detectable over 20 h after administration while the intact drug was not observed [11161].

A method to perform absolute quantification of two biomarkers (IGF-1 and IGFBP-3) of growth hormone abuse has been developed. Isotope dilution is used with synthetically labelled peptides as internal standards. Peptide selection and multiple reaction monitoring design are discussed. A simple sample preparation based on the reduction and alkylation of cysteine residues followed by tryptic digestion provides a sufficient digestion of proteins. Serum samples fortified with increasing amounts of target proteins are analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a triple quadrupole mass spectrometer. Specificity is ensured by the selection of sequences with no homology in
BLAST, as well as retention time deviation check, and ion ratio monitoring. Linearity is studied in terms of calibration curves. These curves for IGFBP-3 and IGF-1 are generated with mean slopes of 0.055 and 0.065, intercepts of 0.107 and -0.011, and with coefficients of correlation of 0.95 and 0.98, respectively. These curves result from the addition of proteins to the serum. Risks of variations related to potential matrix effects are therefore reduced, as well as probable variations related to the digestion steps. The working concentration ranges are 4-10 ng/microL for IGFBP-3 and 2-8 ng/microL for IGF-1. Preliminary data regarding repeatability show that relative standard deviations (RSDs) range between 13 and 32 percent for IGFBP-3 and between 7 and 29 percent for IGF-1 [07155].

In July 2012, the detection of growth hormone abuse by means of a biomarker-based test method was approved by WADA. This complementary assay employs the biomarkers IGF-1 and the amino-terminal pro-peptide of type-III collagen (P-III-NP) as GH-sensitive parameters increasing in response to exogenous growth hormone administration. By means of doping control serum samples collected from 404 male and 94 female elite athletes, gender-specific GH-2000 score decision limits were established using currently available commercial immunoassays. Since parameters such as serum IGF-1 and other bone remodeling markers might be influenced by circumstances other than doping, the effect of tibia fracture healing on IGF-1, C-terminal telopeptide of type-I collagen (CTX), osteocalcin, and bone-specific alkaline phosphatase was studied in a clinical setting with 406 adults. In a double-blind and placebo-controlled trial, participants received a daily dose of hGH between 0.015 mg and 0.060 mg/kg bodyweight or placebo for a period of 16 weeks and the bone turnover biomarkers were recorded. In all treatment groups, a statistically significant difference in IGF-1, CTX, and OST was observed, corroborating the utility of these markers for GH abuse detection. Besides its function as biomarker, IGF-1 itself and its synthetic derivatives are prohibited substances according to the regulations of WADA. Although IGF-1’s mechanism of action concerning improved athletic performance is yet not fully understood, epigenetic aspects have been discussed and reviewed along with serious side effects attributed to long-term abuse of hGH and IGF-1. In order to unambiguously detect at least synthetic analogs of IGF-1 in urine multiplexed with other drugs and metabolites relevant for doping controls, a multi-analyte peptide screening assay was developed, allowing for the determination of IGF-1 and long-R3-IGF-1 as well as six insulins (animal, human, and synthetic), LH releasing hormone (LH-RH), growth hormone releasing hormone (GH-RH) and its synthetic analog CJC-1295, and synacthen. From both matrices, plasma and urine, detection limits between 1 and 50 pg/mL were accomplished, enabling the unequivocal detection of these analytes in doping control samples [12017].

Human growth hormone (hGH) abuse in sport is a challenge at present. The current strategy used, known as direct method, is based on the quantification of hGH variants in serum. An alternative strategy, known as indirect method, focuses on serum markers such as insulin-like growth factor I (IGF-I) and procollagen type III N-terminal propeptide (P-III-NP). The indirect method allows a longer window of detection (WOO) of hGH abuse. To evaluate the performance of the indirect method, in parallel to the direct method, a clinical trial with recombinant hGH (rhGH) was conducted on healthy male subjects during 7 days (0.026 mg/kg/day). The data were fit to the discriminant formula proposed in the previously published GH-2000 project. The low sensitivity of the scores, judged from the high number of false negative outcomes, imposed a new discriminant analysis, standarised using local population subjects demographically similar to the ones of the study. The sensitivity of the method significantly increased, highlighting the importance of the standardisation. The indirect method allowed extended window of opportunity (WOO), although two false positive evaluations were observed derived from elevated basal IGF-I and P-III-NP concentrations stressing the need for an independent confirmation method. When direct and indirect methods were combined the best selectivity and sensitivity were achieved [12206].
GH2000

The GH-2000 project developed a method for detecting GH misuse based on the measurement of insulin-like growth factor-I (IGF-I) and the amino-terminal pro-peptide of type III collagen (P-III-NP). The objective of one study was to develop decision limits for the GH-2000 score to detect GH misuse in elite athletes using two currently available commercial assays for each analyte. There was 404 male (mean age 24 years, range 12-37 years) and 94 female elite athletes (mean age 25 years, range 18-34 years) participated. Blood samples were collected according to World Anti-Doping Agency (WADA) guidelines at various sporting events including 238 samples collected as part of the UK Anti-Doping Testing Programme. For males and females separately, the distributions of GH-2000 scores were consistent with normal distributions. Using a specificity of 99.99 percent new decision limits were determined which included an allowance for uncertainty associated with calculations based on a finite sample size. One outlier was identified with results incompatible with normal physiology and tested positive with the current isoform GH test. It was thus developed decision limits using currently available commercial assays to measure IGF-I and P-III-NP in elite athletes. This should allow the introduction of a test for GH misuse based on the measurement of these GH sensitive biomarkers [12214].

The purpose of this research is to validate the biomarker-based approach for the detection of doping with recombinant human growth hormone (rhGH) in sport. The GH-2000 project proposed an indirect method for the detection of exogenously administered growth hormone (GH) based on the measurement of the GH-dependent markers: insulin-like growth factor-I (IGF-I) and Type III pro-collagen (P-III-P). These markers rise in a dose-dependent manner after GH application. In this study, the concentrations of IGF-I, IGF-BP3, and P-III-P in serum were determined to provide further incentives for the implementation of this detection assay in modern anti-doping programmes. One paper reports on an administration study of rhGH involving 25 Chinese male volunteers at a dose of 0.1 IU/kg/day for a continuous 14-day period. We observed that the serum IGF-I concentration increased rapidly in the rhGH treatment group and showed significantly higher levels compared to baseline between days 4 and day 16 after administration. Although the response of P-III-P to rhGH administration was delayed compared to the IGF-I axis, the P-III-P concentration remained increased for a longer period (from day 4 to day 28). Statistical analysis was carried out to establish a discriminant formula with Statistical Product and Service Solutions (SPSS) concluding that the biomarker methodology is valid and universally applicable [12207].

GH-2000

The GH-2000 marker method measures two serum proteins, insulin-like growth factor-I (IGF-I) and N-terminal propeptide of type III procollagen (P-III-NP), both of which increase in a dose-dependent manner in response to GH administration and therefore act as “markers” of GH administration. This method was first conceived by Peter Sönksen who established the large multicenter GH-2000 project, with funding from the European Union under their BIOMED 2 initiative, the International Olympic Committee and GH manufacturers Novo Nordisk and Pharmacia. Initially, 25 potential markers of GH action were considered; but IGF-I and P-III-NP were chosen for the GH-2000 test because these provided the best discrimination between individuals receiving GH or placebo during a 28-day randomized controlled GH administration trial. Both IGF-I and P-III-NP have little diurnal or day-to-day variation, low intraindividual variation and only change minimally in response to exercise. The markers are used in conjunction with the use of gender specific equations, “discriminant functions”, to improve the sensitivity and specificity of the test compared with single-marker
The markers decline with age as endogenous GH secretion declines and a factor relating to the inverse of age is also included in the formulae [13007].

The GH isoform approach

Circulating GH consists of multiple isoforms all derived from one gene in pituitary somatotrophs. The main isoform is 22 kDa GH, but besides this there are others such as 20 kDa isoform and 17 kDa fragment. Some isoforms are not biologically inert. There are reports that acute exercise in trained adults changes the ratio of isoforms and that exogenous 22 kDa GH administration by athletes could alter the natural GH isoform ratio. The same authors in another study conclude that a supraphysiological dose of rhGH in trained adult males suppresses exercise-induced endogenous isoforms of GH. This occurs via the classical negative feedback by IGF-I, which suppresses endogenous GH secretion, thereby suppressing endogenous molecular isoforms. However, the suppressive effect on 20 kDa GH in males is difficult to assess because at baseline 20 kDa GH levels are already undetectable. Moreover, the high degree of identity in the amino acid sequence between recombinant and endogenous GH, the pulsatile nature of GH secretion combined with inter-individual variations, presents a challenge for determination of GH [12011].

The detection test currently in use (GH isoform test) exploits the difference between recombinant GH (pure 22K-GH) and the heterogeneous nature of endogenous GH (several isoforms). Its main limitation is the short window of opportunity for detection (about 12-24 h after the last GH dose). A second test to be implemented soon (the biomarker test) is based on stimulation of IGF-I and collagen III synthesis by GH. It has a longer window of opportunity (1-2 week) but is less specific and presents a variety of technical challenges. GH doping in a larger sense also includes doping with GH secretagogues and IGF-I and its analogs. The scientific evidence for the ergogenicity of GH is weak, a fact that is not widely appreciated in athletic circles or by the general public. Also insufficiently appreciated is the risk of serious health consequences associated with high-dose, prolonged GH use. One review discussed the GH biology relevant to GH doping; the virtues and limitations of detection tests in blood, urine, and saliva; secretagogue efficacy; IGF-I doping; and information about the effectiveness of GH as a performance-enhancing agent [12215].

Intra-individual variability

Growth Hormone is abused by athletes for its lipolytic and anabolic properties. Its use is prohibited by the World Anti-Doping Agency. The GH-2000 research group developed a methodology to detect its abuse using the concentrations of two GH-dependent biomarkers, IGF-I and type 3 procollagen (P-III-P). The sensitivity of this method may be improved by considering intra-individual variability. The aim of one study was to examine the intra-individual variability of IGF-I, P-III-P and the GH-2000 score. IGF-I, P-III-P and GH-2000 score were evaluated in four longitudinal studies involving 303 elite and 78 amateur athletes. Samples were collected over a period of up to 12 months from a total of 238 men and 143 women aged between 17 and 53 years. The four studies showed good agreement with no apparent difference in within-individual variation between amateur and elite athletes. The intra-individual variability for IGF-I ranged between 14 and 16 percent while the variability for P-III-P was 7-18 percent. No athlete tested positive for growth hormone during any of the studies. The overall mean intra-individual variability of the GH-2000 score was less than 0.6 units in all studies. The high stability of marker levels suggests that concentrations are largely genetically determined. Adopting a test based on the concept of an athlete's “passport” or “profiling” would take advantage of this and most likely increase the sensitivity of the test. These data also provide strong evidence that a positive test result for GH abuse would not occur as a result of chance variability [09198].
The GH-2000 discriminant functions, using insulin-like growth factor I (IGF-I) and the N-terminal propeptide of type III procollagen (PIIINP), enabled the detection of growth hormone (GH) doping despite the broad inter-individual normal range of both peptides. The sensitivity of the discriminant function-based methodology may perhaps be further increased in future by applying individual athlete profiles. The purpose of one study was to evaluate the intra-individual variability of IGF-I, PIIINP and the GH-2000 scores in athletes. For this purpose a total of eight blood samples were taken from each of fifty male and female elite athletes over a period of up to 18 months. The IGF-I and PIIINP levels, it was found, lay predominantly within the reference range for elite athletes. The intra-individual variability for IGF-I ranged between 6 and 26 percent, while that for PIIINP ranged between 6 and 33 percent. The intra-individual variations of both parameters were higher in female than in male subjects and were found to be mostly moderate. It was found that the intra-individual variations of the GH-2000 test scores, expressed as CV, ranged from 4 to 36 percent and were in most of the subjects markedly smaller than the inter-individual variation. Individual cut-offs for the GH-2000 scores would be lower than population based ones in most of the cases [13366].

Effect of training on the analyse results

The major objective of this study was to investigate the effects of several days of intense exercise on growth hormone (hGH) testing using the World Anti-Doping Agencies hGH isoform differential immunoassays. Additionally the effects of circadian variation and exercise type on the isoform ratios were also investigated. Fifteen male athletes performed a simulated nine day cycling stage race. Blood samples were collected twice daily over a period of 15 days (stage race + three days before and after). hGH isoforms were analysed by the official WADA immunoassays (CMZ Assay GmbH). All measured isoform ratios were far below the WADA decision limits for an adverse analytical finding. Changes in the isoform ratios could not be clearly connected to circadian variation, exercise duration or intensity. The study demonstrates that the hGH isoform ratios are not significantly affected by exercise or circadian variation. It was demonstrated that heavy, long term exercise does not interfere with the decision limits for an adverse analytical finding [13375].

Theories for detection of doping with growth hormones

Isoform approach to detection abuse
Athletes recognized the performance-enhancing potential of human growth hormone when it became available for treatment of short stature in growth-retarded children. Although no controlled clinical studies have demonstrated a significant benefit in highly trained adults with normal pituitary function, the practice of doping increased with the introduction of recombinant human growth hormone. Evidence of widespread abuse has been gathered by police and customs authorities or provided by former athletes. It has been difficult to develop a test to prove the administration of exogenous growth hormone in athletes because of its specific physiological and biochemical properties. Significant progress has only recently been made, particularly via two differing approaches. The “marker approach” utilizes characteristic changes in concentrations of pharmacodynamic end points of growth hormone action, for example serum concentrations of insulin-like growth factor I and factors related to bone and soft tissue turnover. The “isoform approach” detects changes in the molecular isoform composition of circulating growth hormone evoked by the administration of exogenous recombinant growth hormone. The isoform approach was applied at the Olympic Games in Athens in 2004 and in Turin in 2006. Used in a complementary way in an out-of-competition setting, these methods are a powerful tool with which to detect growth hormone abuse in sports [07153].

The detection of exogenously administered growth hormone (GH) poses a formidable challenge but a detection method based on the measurement of two GH-dependent markers, IGF-I and type 3 pro-collagen (P-III-P) has been proposed. The measurement of multiple markers in conjunction with discriminant functions can improve the sensitivity and specificity of detection compared with single marker analysis. GH-2000 proposed a discriminant function involving IGF-I and P-III- P while the Kreischa function involved IGF-I, P-III-P and IGFBP-3. After adjustment for assay differences the formulae were applied to the other dataset. The GH-2000 formula was able to detect 90 percent of those receiving GH in the Kreischa study at one or more time points during the study period. This sensitivity was similar to that obtained on the original GH-2000 dataset. The Kreischa formula correctly identified 41 percent of individuals receiving GH in the GH-2000 study. The study provides further validation that the test proposed by GH-2000 based on IGF-I and P-III-P concentrations can be used to detect subjects receiving exogenous GH [07154].

It is believed that athletes have been abusing growth hormone (GH) for its anabolic and lipolytic effects since the early 1980s, at least a decade before endocrinologists began to treat adults with GH deficiency. There is an on-going debate about whether GH is performance enhancing. Although many of the early studies were negative, more recent studies suggest that GH improves strength and sprint capacity, particularly when it is combined with anabolic steroids. Although use of GH is banned by the World Anti-Doping Agency (WADA), its detection remains challenging. Two approaches have been developed to detect GH abuse. The first is based on measurement of pituitary GH isoforms; after injection of recombinant human GH, which comprises solely the 22-kDa isoform, endogenous production is down-regulated leading to an increase in the 22-kDa isoform relative to other isoforms. The second is based on measurement of markers of GH action. Insulin-like growth factor-I (IGF-I) and N-terminal pro-peptide of type III collagen (P-III-NP) increase in response to GH administration in a dose-dependent manner. When combined with discriminant function analysis, use of these markers differentiates between individuals taking GH and placebo. Subsequent studies have shown that the test is applicable across different ethnicities and is unaffected by injury. WADA regulations state that when analytes are measured by immunoassay, two assays are needed. Final validation of the marker test is currently being undertaken with modern commercially available immunoassays to finalise the threshold values to be used to determine whether a doping offence has been committed [11166].
Doping with growth hormone (GH) is banned; however, there is anecdotal evidence that it is widely abused. GH is reportedly often used in combination with anabolic steroids at high doses for several months. Development of a robust test for detecting GH has been challenging since recombinant human 22-kDa GH used in doping is indistinguishable analytically from endogenous GH and there are wide physiological fluctuations in circulating GH concentrations. One approach to GH testing is based on measurement of different circulating GH isoforms using immunoassays that differentiate between 22-kDa and other GH isoforms. Administration of 22-kDa GH results in a change in its abundance relative to other endogenous pituitary GH isoforms. The differential isoform method is, however, limited by its short time window of detection. A second approach that extends the time window of detection is based on detection of increased levels of circulating GH-responsive proteins, such as the insulin-like growth factor (IGF) axis and collagen peptides. As age and gender are the major determinants of variability for IGF-I and the collagen markers, a test based on these markers must take these factors into account. Extensive data now validate the GH-responsive marker approach, and implementation is largely dependent on establishing an assured supply of standardized assays [11162].

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Although doping with growth hormone (GH) is banned, there is anecdotal evidence that it is widely abused. GH is reportedly used often in combination with anabolic steroids at high doses for several months. Development of a robust test for GH has been challenging because recombinant human 22 kDa (22K) GH used in doping is indistinguishable analytically from endogenous GH and there are wide physiological fluctuations in circulating GH concentrations. One approach to GH testing is based on measurement of different circulating GH isoforms using immunoassays that differentiate between 22K and other GH isoforms. Administration of 22K GH results in a change in its abundance relative to other endogenous pituitary GH isoforms. The differential isoform method has been implemented; however, its utility is limited because of the short window of opportunity of detection. The second approach, which will extend the window of opportunity of detection, is based on the detection of increased levels of circulating GH-responsive proteins, such as insulin-like growth factor (IGF) axis and collagen peptides. Age and gender are the major determinants of variability for IGF-I and the collagen markers; therefore, a test based on these markers must take age into account for men and women. Extensive data is now available that validates the GH-responsive marker approach and implementation is now largely dependent
on establishing an assured supply of standardized assays [08242].

Possible ethnic differences

The GH-2000 team proposed a method based on IGF1 and type III pro-collagen (P-III-P) to detect exogenously administered GH. As previous studies involved predominantly white European athletes, it is important to assess whether the response of these markers to recombinant human GH (rhGH) differs with ethnicity. In a double-blind placebo-controlled rhGH administration study the study included 31 male and 14 female amateur athletes of different ethnicities. The subjects were assigned to treatment with placebo or 0.1 IU/kg per day (low dose) or 0.2 IU/kg per day (high dose) rhGH for 28 days. Blood was collected weekly during treatment and on days 35, 42 and 84 during the washout period. Serum IGF1 and P-III-P were measured, and GH-2000 score was calculated. IGF1, P-III-P and GH-2000 score rose in response to both low- and high-dose GH in both men and women. When compared with the Caucasian volunteers of the previous GH-2000 study, mean baseline and placebo-treated P-III-P and GH-2000 score were lower in GH-2004 men and women. Post-GH, however, peak IGF1 or P-III-P did not differ between studies but the peak GH-2000 score was lower in GH-2004 men. There was no difference between studies in the maximal change in IGF1, P-III-P and GH-2000 score in response to GH in either gender. These data do not support a significant ethnic effect on the peak or maximal response to rhGH [10362].

A method based on the two GH dependent markers, IGF-I and type 3 pro-collagen (P-III-P) has been proposed to detect exogenously administered GH. As previous studies involved predominantly white European elite athletes, it is necessary to validate the method in other ethnic groups. Serum IGF-I and P-III-P were measured and GH-2000 discriminant function score was calculated. Effect of ethnicity was assessed. In men, IGF-I was 21.7 ± 2.6 percent lower in Afro-Caribbeans than white Europeans but there were no differences between other ethnic groups. In women, IGF-I was 14.2 ± 5.1 percent lower in Afro-Caribbeans and 15.6 ± 7.0 percent higher in Orientals compared with white Europeans. P-III-P was 15.2 ± 3.5 percent, 26.6 ± 6.6 percent and 19.3 ± 5.8 percent lower in Afro-Caribbean, Indo-Asian and Oriental men respectively compared with white European men. In women, P-III-P was 15.7 ± 4.7 percent lower in Afro-Caribbeans compared to white Europeans but there were no differences between other ethnicities. Despite these differences, most observations were below the upper 99 percent prediction limits derived from white European athletes. All GH-2000 scores lay below the cut-off limit proposed for doping [08246].

Pharmacokinetics and pharmacodynamics of GH

In growth hormone (GH)-deficient adult patients, subcutaneous (s.c.) administration of recombinant human GH (rhGH) at a fixed dose results in less pronounced effects in females than in males. This is thought to be at least partly due to modulation of hepatic insulin-like growth factor-I (IGF-I) generation by endogenous and exogenous estrogens. In addition, male and female patterns of fat distribution differ substantially and could potentially be associated with differences in absorption from the injection depot of rhGH. The administration route in early studies of rhGH was by intramuscular (i.m.) injection; sex differences were not noted but these studies were in children who would not have developed adult differences between the sexes. Exogenously administered rhGH is structurally identical to endogenous 22 kDa GH, which is the isoform predominantly secreted in humans. The most commonly used GH immunoassays recognize equally the 22 kDa isoform and the 20 kDa GH, which results from alternative splicing. It was suggested that development of
immunoassays that could differentiate between the isoforms could be used to assess misuse of rhGH. Ten males and ten females were selected from a cohort of 50 healthy young adults based on the level of sport activities. Inclusion criteria were: aged 18-35 years, body mass index (BMI) 19–27 kg/m², regular physical exercise at least three times per week and, in females, continuous use of oral contraceptives. Subjects were excluded if they had any chronic illness, took any medications known to interfere with endocrine function or reported any previous use of rhGH. The study used a randomized crossover design. Subjects were admitted to our clinical research unit for the three study periods, each starting at 0600 h after an overnight fast. Intravenous catheters were inserted in an antecubital vein and blood samples were drawn at 60 and 0 min (baseline) before rhGH administration then at 2-h intervals for the following 36 h. At 0 h, rhGH (Humatrope, Eli Lilly) was administered as a bolus of either 0.033 mg/kg body weight s.c., 0.083 mg/kg s.c. or 0.033 mg/kg i.m., according to a previously defined randomization scheme. Over the three study periods, each patient received each of the three rhGH doses in randomized order; patients were blinded regarding the low and high s.c. doses. Study periods were separated by a washout of 4 weeks to synchronize with the menstrual cycle in females. Serum GH concentration was assayed by two sandwich immunoassays. Assay 1 (mAb 3B4/biotinylated mAb 10A7) utilized a capture antibody, which preferentially recognizes the monomeric 22 kDa isoform of GH, which is identical to rhGH and the lower detection limit was 0.1 microg/L. Both 22 and 20 kDa GH at baseline were significantly higher in females than in males. In contrast, baseline serum IGF-I levels were significantly lower in females than in males. Differences between females studied in the follicular phase and in the luteal phase were not significant. When the same rhGH dose (0.033 mg/kg) was administered, a significantly higher 22 kDa GH peak maximal concentration ($C_{\text{max}}$) and AUC were observed with the i.m. compared with s.c. route in males but not females. There was no difference between males and females for $C_{\text{max}}$ and AUC with s.c. rhGH, irrespective of the dose. In contrast, after i.m. administration mean $C_{\text{max}}$ and AUC were significantly higher in males than females, with a concomitantly lower CL/F in males. MRT was shorter in males than females in the low-dose group, irrespective of route of administration. Subjects with higher baseline IGF-I concentrations showed a greater response to rhGH than those with a lower baseline concentration; this association was observed at all three study periods. The increase from baseline integrated over time (delta AUC 0–36) was higher with the high dose for both IGF-I and IGFBP-3. There were no significant differences for IGF-I or IGFBP-3 parameters between s.c. and i.m. routes with the same rhGH dose. $T_{\text{max}}$ for serum IGF-I differed between males and females in the high-dose group. IGF-I delta AUC 0–36 showed a clear sex difference at the low dose, with higher values in males compared with females; this was independent of the route of administration. At the high dose, the difference between the sexes was not significant. IGFBP-3 delta AUC 0–36 was significantly higher in males than females at the low s.c. dose, while at the high s.c. dose similar values were observed. At baseline, 20 kDa GH was detectable in all women in all three study periods; rapid suppression occurred after injection of rhGH. In females, mean 20 kDa GH levels decreased from 0.4 at baseline to below 0.2 μ g/l within 2 h after injection of rhGH. Duration of 20 kDa GH suppression in females was dose dependent; reoccurrence of 20 kDa GH secretion was observed in the low-dose s.c. group after 26 h, in the low-dose i.m. group after 28 h, and in the high dose s.c. group after 34 h. In contrast, in males 20 kDa GH levels were close to or below the lower limit of quantification (0.05 microg/L) of the assay at baseline and throughout the observation period. The most frequent adverse event was diarrhea occurring within 24 h after rhGH in six subjects receiving high dose and two subjects receiving low-dose s.c. injections. In four of the six subjects from the high-dose group, diarrhea was accompanied by moderate dizziness. Symptoms spontaneously ceased by the end of the study period (36 h). These episodes of diarrhea were not related to any identifiable causes such as dietary issues or gastrointestinal infections. Three subjects experienced enhanced sweating without obvious relation to the dose. One subject presented with decreased blood pressure, dizziness and vomiting 24 h
after administration of the high dose; the symptoms resolved within 6 h. No edema was observed, and neither arthralgia nor headache was reported. It was concluded that after rhGH administration, pharmacokinetic parameters are mainly influenced by route of administration, whereas pharmacodynamic variables and 20 kDa GH concentrations are determined mainly by gender. The present data demonstrate that gender, dose and route of administration specifically alter bioavailability of and response to exogenous rhGH in healthy young adults. Pharmacokinetic variables were mainly influenced by the route of administration, whereas pharmacodynamic responses were primarily determined by sex. Furthermore, suppression of the 20 kDa GH isoform after injection of rhGH could be demonstrated only in women; 20 kDa GH levels in males were already low at baseline [07152].

With no exogenous rhGH, reduced serum IGF-I and IGFBP-3 concentrations have been reported during intense training. The increase in IGF-I, but not the increase in IGFBP-3, shows a marked sexual dimorphism. It has been proposed that use of oral estrogens interferes with hepatic IGF-I production, but women not using estrogen supplementation also exhibit a lower IGF-I response than males. Studies in animals indicate that complex mechanisms, including modification of hepatic GH receptor expression, lead to the sexual dimorphism in the somatotropic axis. In contrast to serum GH concentrations, IGF-I and IGFBP-3 concentrations did not return to pre-treatment levels within the observation period, supporting the idea of use of these markers to detect doping with rhGH. The existing studies on the relationship between 22 kDa and 20 kDa isoforms suggest that the secretion is a part of constant percentage of total GH. Therefore, the lower 20 kDa level and the long-term suppression in males seem to be a consequence of the lower total GH concentration. The 20 kDa GH isoform was also suppressed in females after administration of rhGH, consistent with a negative feedback of exogenous rhGH on pituitary GH secretion; the duration of suppression was dose dependent and re-occurrence of 20 kDa in the circulation was seen 26–28 h after low-dose rhGH and 34 h after high dose rhGH. The prolonged changes provide further evidence that the GH isoform pattern can be used to detect the administration of rhGH in females [07152].

**Metabolism of GH**

Growth hormone (GH) can generate insulin-like growth factor-I production, provided that the liver encounters portal insulin as a permissive factor that switches on the liver sensitivity for GH. This phenomenon is important for a proper insight into the pathophysiology of diseases as type 1 and 2 diabetes which differ in portal insulin levels. Also, acromegaly and obesity can be better understood when this effect of insulin on liver sensitivity for GH is taken into account. Moreover, as all of these factors seem to influence activity of the 11beta-hydroxysteroid dehydrogenase (11beta-HSD) type 1 (and 2), an extensive knowledge on the interplay between them is crucial as nowadays treatment options for obesity using the 11beta-HSD1 are emerging [11167].

**Effect on red blood cells**

It has been shown that growth hormone (GH) exerts regulatory effects on hemorheology and other metabolic functions. GH stimulates the production of insulin-like growth factor I (IGF-I) and GH-IGF-I system has profound effects on body fluid status. There are speculations that GH has become widely used as a performance enhancing drug among athletes of various sports. One study evaluated the possible hemorheological effects of short term
administration of human recombinant growth hormone (rhGH) in healthy young males. Thirty young healthy males (27 ± 9) participated in a 29 days study where it was administered either 0.9 percent sodium chloride or 1 mg of human rhGH from day 1 to day 7. The participants were randomly assigned into either placebo (n=15) or rhGH 1 mg/day (rhGH) group (n=15). This study evaluated plasma fibrinogen levels, red blood cell (RBC) aggregation, deformability and serum IGF-I levels between and within the groups along 29 days. There was a significant increase in erythrocytes aggregation index post injection (day 8), in accordance to an increase in serum IGF-I [11168].

**Positive association with estrogens in men**

Growth hormone (GH) and insulin-like growth factor I (IGF-I) receptors are present on pituitary gonadotrophs and on testicular Leydig and Sertoli cells. Thus, the GH/IGF-I system may modulate the pituitary-gonadal axis in males. It was now performed a randomized cross-over study. Eight healthy male volunteers (mean age 35, range 29-46 years) were treated with GH for 3 weeks (1st week 0.01, 2nd week 0.02, 3rd week 0.03 mg/day/kg) or a GH receptor antagonist (Pegvisomant) (1st week 10, last 2 weeks 15 mg/day), separated by 8 weeks of washout. Before and after the two treatment periods, concentrations of luteinizing hormone (LH), follicle-stimulating hormone, testosterone, oestradiol, sex hormone-binding globulin, inhibin B and Anti-Müllerian Hormone (AMH) were measured. During GH treatment, IGF-I increased together with oestradiol, and the oestradiol/testosterone ratio. By contrast, AMH, Inhibin B, and LH decreased. During pegvisomant treatment IGF-I and oestradiol (86 ± 28 v. 79 ± 25 pm) decreased. No significant changes or trends in the other reproductive hormones occurred during the two treatment regimens. GH/IGF-I activity was positively associated with serum oestradiol, suggesting that GH/IGF-I stimulates aromatase activity in vivo. As a novel observation, it was found that high GH activity was associated with reduced levels of the Sertoli cell marker AMH. Further studies are needed to evaluate possible effects of GH on Sertoli cell function and/or spermatogenesis [13358].

**Negative effects on collagen synthesis**

The use of recombinant human form GH (rhGH) as an anabolic aid for clinically deficient individuals is well described. It has been reported that GH administration has an important role in the regulation of body composition by decreasing fat mass, with no effect on the net gain in muscle protein, in GH deficient men. However, the evidence is lacking for rhGH administration alone or together with anabolic steroids or weightlifting programmes to enhance gains in muscle mass and strength in healthy adults. There appears to be rhGH-induced stimulation of collagen protein synthesis in tendon and skeletal muscle in healthy adults as well as increased body water retention. The anabolic effect on connective tissue may, at least partly, explain the use of GH by the athlete to accelerate recovery and/or injury prevention. Again, there is no firm evidence to support this claim. It is noteworthy that there are documented adverse consequences of GH therapy (e.g. carpal tunnel syndrome, fluid retention, diabetic-like symptoms). These harmful effects of GH administration are usually attributed to the high doses that are given to the patient. The high-dose GH regimen is undoubtedly often used by the athlete as well. Interestingly, it was demonstrated that a single dose of GH administered prior to a bout of endurance exercise resulted in considerably higher blood lactate concentrations during an exercise bout in young adults. This outcome would not favour acute performance enhancement for an athlete wishing to gain a competitive advantage [13357].
Idiopathic adult growth hormone deficiency

GH secretion is controlled by hypothalamic as well as intrapituitary and peripheral signals, all of which converge upon the somatotroph, resulting in integrated GH synthesis and secretion. Enabling an accurate diagnosis of idiopathic adult GH deficiency (IAGHD) is challenged by the pulsatility of GH secretion, provocative test result variability, and suboptimal GH assay standardization. The spectrum between attenuated GH secretion associated with the normal aging process and with obesity and truly well-defined IAGHD is not distinct and may mislead the diagnosis. Adult-onset GHD is mainly caused by an acquired pituitary deficiency, commonly including prior head/neck irradiation, or an expanding pituitary mass causing functional somatotroph compression. To what extent rare cryptic causes account for those patients seemingly classified as IAGHD is unclear. About 15 percent of patients with adult GHD and receiving GH replacement in open-label surveillance studies are reported as being due to an idiopathic cause. These patients may also reflect a pool of subjects with an as yet to be determined occult defect, or those with unclear or incomplete medical histories (including forgotten past sports head injury or motor vehicle accident). Therefore, submaximal diagnostic evaluation likely leads to an inadvertent diagnosis of IAGHD. In these latter cases, adherence to rigorous biochemical diagnostic criteria and etiology exclusion may result in reclassification of a subset of these patients to a distinct known acquired etiology, or as GH-replete. Accordingly, rigorously verified IAGHD likely comprises less than 10 percent of adult GHD patients, an already rare disorder. Regardless of etiology, patients with adult GHD, including those with IAGHD, exhibit a well-defined clinical phenotype including increased fat mass, loss of lean muscle mass, decreased bone mass, and enhanced cardiac morbidity. Definition of unique efficacy and dosing parameters for GH replacement and resultant therapeutic efficacy markers in true IAGHD requires prospective study [13359].

Effect on memory

GH has previously been shown to promote cognitive functions in GH-deficient rodents. In this study we report the effects of GH on learning and memory in intact rats pretreated with the anabolic androgenic steroid nandrolone. Male Wistar rats received nandrolone decanoate (15 mg/kg) or peanut oil every third day for 3 weeks and were subsequently treated with recombinant human GH (1.0 IU/kg) or saline for 10 consecutive days. During the GH/saline treatment spatial learning and memory were tested in the Morris water maze. Also, plasma levels of IGF1 were assessed and the gene expression of the GH receptors, IGF-1 and IGF-2, in hippocampus and frontal cortex was analyzed. The results demonstrated a significant positive effect of GH on memory functions and increased gene expression of Igf1 in the hippocampus was found in the animals treated with GH. In addition, GH was demonstrated to increase the body weight gain and was able to attenuate the reduced body weight seen in nandrolone-treated animals. In general, the rats treated with nandrolone alone did not exhibit any pronounced alteration in memory compared with controls in the Morris water maze, and in many cases GH did not induce any alteration. Regarding target zone crossings, considered to be associated with spatial memory, the difference between GH- and steroid-treated animals was significant and administration of GH improved this parameter in the latter group. In conclusion, GH improves spatial memory in intact rats and can reverse certain effects induced by anabolic androgenic steroid [13368].

Growth hormone (GH) replacement unequivocally benefits growth, body composition, cardiovascular risk factors and quality of life. Less is known about the effects of GH on
learning and memory. A recent paper on “early onset” – GH deficiency (GHD) results in spatial memory impairment in mid life – and is prevented by GH supplementation’ by Nieves-Martinez importantly adds to this literature. Other data suggest that GH beneficially affects cognitive function in rats. In man, treatment of GHD has been associated with improvements in measures of memory and attention. There are also differences in verbal memory of patients with childhood onset GHD. Further questions remain, and the beneficial effects or otherwise of treating GHD in different age groups remain to be better defined. Certainly for reasons of maturation of neural connections and their development to young adulthood contemporaneous with rises in GH and IGF1 make these important areas for further study in man. Lastly because of what is already known in terms of cognitive effects of GHD, it is important to replace GH when studying other potential causes of adverse effects on cognition, for example, with radiotherapy [10478].

GH levels increase to high concentrations immediately before puberty then progressively decline with age. GH deficiency (GHD) originating in childhood is treated with GH supplementation to foster somatic development during adolescence. It is not clear if or how early GH replacement affects memory in adulthood, or whether it can prevent the cognitive deficits commonly observed in adults with childhood-onset GHD. Rats homozygous for the Dw-4 mutation (dwarf) do not exhibit the normal increase in GH at 4 weeks of age when GH levels normally rise and are used to model childhood or early-onset GHD (EOGHD). One group of these rats was injected with GH from 4 to 14 weeks of age to model GH supplementation during adolescence with GHD beginning in adulthood (adult-onset GHD; AOGHD). Another group received GH from 4 weeks throughout the lifespan to model normal lifespan GH (GH-replete). Age-matched, Dw-4 heterozygous rats (HZ) do not express the dwarf phenotype and were used as controls. At 8 and 18 months of age, spatial learning in the water maze was assessed. At 8 months of age all experimental groups were equally proficient. However, at 18 months of age, the EOGHD group had poor spatial learning compared to the AOGHD, GH-replete, and HZ groups. The data indicate that GHD during adolescence has negative effects on learning and memory that emerge by middle-age unless prevented by GH supplementation [09202].

**Gene expression in peripheral blood**

The objective of one study was to evaluate gene expression profiling in peripheral blood leukocytes in-vivo as a test for GH doping in humans. Seven men and thirteen women were administered GH, 2 mg/d sc for 8 wk. Blood was collected at baseline and at 8 wk. RNA was extracted from the white cell fraction. Microarray analysis was undertaken using Agilent 44K G4112F arrays using a two-color design. Quantitative RT-PCR using TaqMan gene expression assays was performed for validation of selected differentially expressed genes. GH induced an approximately 2-fold increase in circulating IGF-I that was maintained throughout the 8 wk of the study. GH induced significant changes in gene expression with 353 in women and 41 in men detected with a false discovery rate of less than 5 percent. None of the differentially expressed genes were common between men and women. The maximal changes were a doubling for up-regulated or halving for down-regulated genes, similar in magnitude to the variation between individuals. Quantitative RT-PCR for seven target genes showed good concordance between microarray and quantitative PCR data in women but not in men. Gene expression analysis of peripheral blood leukocytes is unlikely to be a viable approach for the detection of GH doping [10153].

**GH-receptor antagonists**
To examine the effects of GH receptor (GHR) antagonist treatment on exercise performance, 20 healthy males were treated with the GHR antagonist pegvisomant or placebo for 16 days. After the treatment period, they exercised to determine exercise performance, hormonal and metabolic responses. Subjects were treated with the GHR antagonist (n=10, 10 mg/d) or placebo (n=10). After treatment period, they performed a maximal oxygen uptake (\(\text{VO}_{2\text{max}}\)) test, and prolonged exercise test, consisting of 60 min submaximal cycling followed by exercise to fatigue at 90 percent of \(\text{VO}_{2\text{max}}\). \(\text{VO}_{2\text{max}}\) was measured before and after the treatment period. Hormonal and metabolic responses, and time to exhaustion during prolonged exercise were determined. Resting serum IGF-I concentration decreased by 20 percent in the GHR antagonist treated group, which was a significant difference, whereas no change was observed in the placebo group. Conversely, resting serum GH concentration was significantly higher in the treatment group compared with the placebo group. \(\text{VO}_{2\text{max}}\) did not change significantly in either group after the treatment period. Time to exhaustion at 90 percent of \(\text{VO}_{2\text{max}}\) was significantly shorter in the treatment group. No significant differences were observed between the groups in terms of changes in serum free fatty acids, glycerol, \(\text{VO}_2\), or relative fat oxidation. It was concluded that GH might be an important determinant of exercise capacity during prolonged exercise, but GHR antagonist did not alter fat metabolism during exercise [09211].

The objective of one study was to evaluate gene expression profiling in peripheral blood leukocytes in-vivo as a test for GH doping in humans. Seven men and thirteen women were administered GH, 2 mg/d sc for 8 weeks. Blood was collected at baseline and at 8 weeks. RNA was extracted from the white cell fraction. Microarray analysis was undertaken using Agilent 44K G4112F arrays using a two-color design. Quantitative RT-PCR using TaqMan gene expression assays was performed for validation of selected differentially expressed genes. GH induced an approximately 2-fold increase in circulating IGF-I that was maintained throughout the 8 wk of the study. GH induced significant changes in gene expression with 353 in women and 41 in men detected with a false discovery rate of less than 5 percent. None of the differentially expressed genes were common between men and women. The maximal changes were a doubling for up-regulated or halving for down-regulated genes, similar in magnitude to the variation between individuals. Quantitative RT-PCR for seven target genes showed good concordance between microarray and quantitative PCR data in women but not in men. Gene expression analysis of peripheral blood leukocytes is unlikely to be a viable approach for the detection of GH doping [09212].

**Growth hormone releasing peptides**

New, potentially performance enhancing compounds have frequently been introduced to licit and illicit markets and rapidly distributed via worldwide operating Internet platforms. Developing fast analytical strategies to follow these new trends is one the most challenging issues for modern doping control analysis. Even if reference compounds for the active drugs are readily obtained, their unknown metabolism complicates effective testing strategies. Recently, a new class of small C-terminally amidated peptides comprising four to seven amino acid residues received considerable attention of sports drug testing authorities due to their ability to stimulate growth hormone release from the pituitary. The most promising candidates are the growth hormone releasing peptide (GHRP)-1, -2, -4, -5, -6, hexarelin, alexamorelin, and ipamorelin. With the exemption of GHRP-2, the entity of these peptides represents nonapproved pharmaceuticals; however, via Internet providers, all compounds are readily available. To date, only limited information on the metabolism of these substances is available and merely one metabolite for GHRP-2 is established. Therefore, a
A comprehensive in vivo (po and iv administration in rats) and in vitro (with human serum and recombinant amidase) study was performed in order to generate information on urinary metabolites potentially useful for routine doping controls. The urine samples from the in vivo experiments were purified by mixed-mode cation-exchange solid-phase extraction and analyzed by ultrahigh-performance liquid chromatography (UHPLC) separation followed by high-resolution/high-accuracy mass spectrometry. Combining the high resolution power of a benchtop Orbitrap mass analyzer for the first metabolite screening and the speed of a quadrupole/time-of-flight (Q-TOF) instrument for identification, urinary metabolites were screened by means of a sensitive full scan analysis and subsequently confirmed by high-accuracy product ion scan experiments. Two deuterium-labeled internal standards (triply deuterated GHRP-4 and GHRP-2 metabolite) were used to optimize the extraction and analysis procedure. Overall, 28 metabolites (at least three for each GHRP) were identified from the in vivo samples and main metabolites were confirmed by the human in vitro model. All identified metabolites were formed due to exopeptidase- (amino- or carboxy-), amidase-, or endopeptidase activity [12212].

Legal framework

The world of doping and anti-doping in sport is an ongoing race between those who want to gain a performance advantage by using prohibited substances or methods and those who want to keep the sport clean. Sophisticated doping often involves substances that are difficult to detect, which presents both scientific and legal challenges for implementing new detection methods. One article provides an overview of legal considerations in anti-doping procedures that may apply to the use of the marker method for the detection of Human Growth Hormone. In cases where the blood markers IGF-1 and P-III-P indicate use of exogenous growth hormone, reference to positivity criteria and laboratory uncertainty measurements would assist anti-doping adjudicative bodies in concluding, to their comfortable satisfaction, that a positive test has been established. Use of established positivity criteria and laboratory uncertainty measurements place the application of the marker method for growth hormone within the existing, accepted legal framework for evaluating an Adverse Analytical Finding [09199].

Effect of dietary supplements on GH levels

Intramuscular carnosine buffers protons (H⁺) in skeletal muscle. It was examined the effects of supplementation with chicken breast meat extract (CBEX) containing carnosine and anserine on hormonal responses to resistance exercise. Twenty-two men were assigned to a CBEX drink group (CBEX containing total 2 g of carnosine and anserine) (n=14) or a placebo drink group (n=8). The subjects ingested the prescribed drink (100 mL) twice daily for 30 days without physical training. Before and after the supplementation period, the subjects
completed 5 sets of bilateral knee extension exercises (with a 90-s rest between sets). The magnitude of the increase in exercise-induced free testosterone did not change significantly after supplementation in either group. The blood lactate response to exercise was significantly attenuated after supplementation in both groups. In the CBEX group, the plasma epinephrine and norepinephrine concentrations after exercise were significantly lower after supplementation. The serum growth hormone response to exercise was also reduced in the CBEX group after supplementation. No significant differences in exercise-induced strength reduction (fatigue index) were observed in the 2 groups after supplementation. These results suggest that short-term supplementation with CBEX attenuates the exercise-induced epinephrine, norepinephrine, and growth hormone responses [11169].

**Effects of hypertemia on GH levels**

The aim of one study was to differentiate the effects of hyperthermia and physical activity on circulating growth hormone (GH) secretion. Nine healthy volunteer adults performed two 40 min exercise trials and two 50 min passive standing trials. The exercise was performed in either thermo-neutral (N-Ex: air temperature 18 degrees C, air humidity 40 %, and wet bulb globe temperature (WBGT) 17.7 degrees C) or hot environmental conditions (H-Ex: air temperature 33 degrees C, air humidity 30 %, and WBGT 34.6 degrees C). The passive exposure trials were also performed in either a comfortable (N-P: air temperature 18 degrees C, air humidity 40 %, and WBGT 17.7 degrees C) or a hot climatic chamber (H-P: air temperature 40 degrees C, air humidity 100 %, and WBGT 97.1 degrees C). Plasma GH, plasma volume (PV), tympanic temperature (Tty), and body mass loss (BML) were measured before and after each trial. The decrease in PV was significantly higher during H-Ex and H-P sessions than during N-Ex and N-P sessions. Comparisons showed significantly lower BML in the thermo neutral session than in the hot climatic sessions. The rise in Tty was significantly higher during the hot climatic session when compared with the other sessions. Plasma GH concentration increased significantly during all the trials, particularly during the hot environmental session. Both exercise and heat exposure, separately, are sufficient to increase significantly the plasma GH concentration, and their combined effect induced a highly synergistic rise in growth hormone [08250].

**Effects of exercise on GH levels**

Physical activity plays an important role in tissue anabolism, growth and development, but the mechanisms that link patterns of exercise with tissue anabolism are not completely understood. The effectiveness of physical training depends on the training load and on the individual ability to tolerate it, and an imbalance between the two may lead to under or over-training. Therefore, many efforts have been made to find objective parameters to quantify the balance between training load and the athlete's tolerance. One of the unique features of exercise is that it leads to a simultaneous increase of antagonistic mediators. On the one hand, exercise stimulates anabolic components of the growth hormone (GH) → IGF-1 (insulin-like growth factor-1) axis. On the other hand, exercise elevates catabolic pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1 and tumor necrosis factor-α (TNF-α). This emphasizes probably the importance of optimal adaptation to exercise in particularly during adolescence. The very fine balance between the anabolic and inflammatory/catabolic response to exercise will determine the effectiveness of exercise training and the health consequences of exercise. If the anabolic response is stronger, exercise will probably lead ultimately to increased muscle mass and improved fitness. A greater catabolic response, in particularly if persists for long duration, may lead to overtraining. Therefore, changes in the
anabolic-catabolic hormonal balance and in circulating inflammatory cytokines can be used by adolescent athletes and/or their coaches to gauge the training intensity in individual and team sports [10476].

In-flight muscle mass and strength losses are likely exacerbated by low growth hormone (GH) concentrations. Factors associated with exercise may foretell resultant GH levels and thereby help blunt future mass and strength losses. To assess the ability of variables to predict GH variance from resistive exercise done on a flywheel ergometer designed for in-flight exercise, subjects (n=17) performed three types of workouts on the device. With a randomized design, subjects performed the workouts with the intent to determine if changes in post-exercise GH concentrations are impacted by contractile mode and workload. Body mass, blood lactate concentrations, and peak angular velocity (PAV), average power (AP), and total work (TW) from workouts attempted to predict GH variance. Pre-exercise blood draws, and at 1 and 30 min after workouts, were used to determine GH concentrations. BLac levels were measured before workouts and at 5 min post-exercise. Delta (8, post-pre) and 30-min post-workout GH levels served as criterion variables. Univariate correlations show body mass, delta-blood lactate, and total work were the best predictors of deltaGH variance [09197].

High intensity versus high volume endurance training

The purpose of one study was to examine the acute hormonal response of a short term high-intensity training (HIT) versus a high volume endurance training (HVT) and to determine the contribution of the metabolic acidosis as a stimulus for possibly different reactions of circulating hGH, IGF-1, IGFBP-3 and cortisol. Eleven subjects participated in three experimental trials separated by one week. Two times subjects performed four 30s maximal effort exercise bouts on a cycle ergometer separated by 5 min rest each. Before the exercise subjects either received (single-blinded) bicarbonate (HIT (B)) or a placebo (HIT (P)). The third exercise trail consisted of a constant load exercise for 1h at 50 percent VO₂max (HVT). Venous blood samples were taken under resting conditions, 10 min, 60 min and 240 min after each exercise condition to determine hGH, IGF-1, IGFBP-3 and cortisol concentrations. Capillary blood samples were taken to determine lactate concentrations and blood gas parameters. Power output, mean lactate concentrations and mean pH values were significantly higher during HIT (B) compared to HIT (P). Serum cortisol and hGH concentrations were significantly increased 10 min post exercise in both HIT interventions. IGFBP-3 was only significantly increased after HIT (P), whereas IGF-1 was not affected by any of the interventions. HVT showed no significant effects on cortisol, hGH, IGF-1 and IGFBP-3 levels. Additionally it was shown that the diminished acidosis during HIT (B) attenuates the cortisol and hGH response. It was concluded that the present study suggests that HIT/acidosis is a stimulus for exercise-induced cortisol/hGH secretion, but not for IGF-1 and IGFBP-3 under these experimental conditions. These findings might be relevant for arrangements of interval training, due to the fact that active or passive recovery during rest periods influence the acid base status and may therefore influence the hormonal response [10479].

Effects in immobilisation

It was examined the effect of growth hormone (GH) on connective tissue of tendon and skeletal muscle during immobilisation and re-training in humans. Young men (20-30 years; n=20) were randomly assigned to daily recombinant human GH (rhGH) (33-50 microg/kg/day) or placebo (Plc), and had one leg immobilised for 2 weeks, followed by 6 weeks of strength training. The cross-sectional area (CSA), maximal muscle strength
(maximal voluntary contraction, MVC) and biomechanical properties of the quadriceps muscle and patellar tendon were determined. Muscle and tendon biopsies were analysed for mRNA of collagen (COL1A1/3A1), insulin-like growth factors (IGF-1Ea/Ec), lysyl oxidase (LOX), matrix metalloproteases (MMP-2 and MMP-9), decorin and tenascin-C. Fibril morphology was analysed by transmission electron microscopy (TEM) to detect changes in the fibril diameter distribution. In muscle, CSA and MVC declined with immobilisation and recovered with rehabilitation similarly in both groups. Likewise, both groups showed increased IGF-1Ea/Ec and COL1A1/3A1 expression in muscle during re-training after immobilisation compared with baseline, and the increase was more pronounced when subjects received GH. The tendon CSA did not change during immobilisation, but increased in both groups during 6 weeks of rehabilitation (14%). A decline in tendon stiffness after immobilisation was observed only in the Plc group, and an increase during 6 weeks of rehabilitation was observed only in the GH group. IGF-1Ea and COL1A1/3A1 mRNA increased with immobilisation in the GH group only, and LOX mRNA was higher in the GH group than in the Plc group after immobilisation. Both groups showed an increase in MMP-2 with immobilisation, whereas no changes in MMP-9, decorin and tenascin-C were observed. The tendon fibril diameter distribution remained unchanged in both groups. In conclusion, GH stimulates collagen expression in both skeletal muscle and tendon, abolishes the normal inactivity-related decline in tendon stiffness and LOX, and results in increased tendon CSA and stiffness during rehabilitation. GH has a matrix-stabilising effect during periods of inactivity and rehabilitation in humans [13365].

Effects of GH on performance in sports

The major objective of one study was to investigate the effects of several days of intense exercise on growth hormone (hGH) testing using the World Anti-Doping Agencies hGH isoform differential immunoassays. Additionally the effects of circadian variation and exercise type on the isoform ratios were also investigated. Fifteen male athletes performed a simulated nine day cycling stage race. Blood samples were collected twice daily over a period of 15 days (stage race + three days before and after). hGH isoforms were analysed by the official WADA immunoassays (CMZ Assay GmbH). All measured isoform ratios were far below the WADA decision limits for an adverse analytical finding. Changes in the isoform ratios could not be clearly connected to circadian variation, exercise duration or intensity. The study demonstrates that the hGH isoform ratios are not significantly affected by exercise or circadian variation. It was demonstrated that heavy, long term exercise does not interfere with the decision limits for an adverse analytical finding [13360].

Endurance

Serum insulin-like growth factor-I (IGF-I) and procollagen type III peptide (P-III-P) have been proposed as indirect biomarkers of rhGH misuse in sports. The purpose of one study was to investigate concentrations of these biomarkers in athletes at different levels of physical fitness and endurance exercise. Serum total IGF-I and P-III-P were measured in 96 elite athletes of various sports along the training season; in 21 recreational athletes at baseline non-exercising conditions and in another 129 recreational athletes before and after long-distance races (10 and 21 km). No differences were evidenced for IGF-I concentrations, but statistically higher values of serum P-III-P were found in elite athletes compared to recreational ones. Among elite athletes, the specific sport did not affect serum IGF-I. However, P-III-P was statistically higher in the sport performed by the youngest athletes (rhythmic gymnastics), even after correction of the logarithm of the concentration by the reciprocal of age. Over the training season, the within-athlete variabilities of IGF-I and P-III-P
in elite athletes were low (23 % and 22 %, respectively). Recreational athletes taking part in a 21 km competition race showed a significant increase in serum values of IGF-I and P-III-P immediately after the event. Exercise workload and age had a significant effect on serum concentration of P-III-P, while age alone affected IGF-I serum concentrations. Therefore, athlete's reference concentration ranges for doping detection should include subjects from as many different ages and sports as possible [06139].

The growth hormone (GH)/ insulin-like growth factor-I (IGF-I) axis exerts short-and long-term metabolic effects that are potentially important during exercise. Exercise is a potent stimulus to GH release and there is some evidence that the acute increase in GH is important in regulating substrate metabolism post-exercise. Regular exercise also increases 24-hour GH secretion rates, which potentially contributes to the physiologic changes induced by training. The effects of GH replacement in GH-deficient adults provide a useful model with which to study the effects of the more long-term effects of the GH/IGF-I axis. There is convincing evidence that GH replacement increases exercise capacity. Measures of exercise performance including maximal oxygen uptake (VO$_{2\text{max}}$) and ventilatory threshold are impaired in GH deficiency and improved by GH replacement, probably through some combination of increased oxygen delivery to exercising muscle, increased fatty acid availability with glycogen sparing, increased muscle strength, improved body composition and improved thermoregulation. Administration of supraphysiologic doses of GH to athletes increases fatty acid availability and reduces oxidative protein loss particularly during exercise, and increases lean body mass. It is not known whether these effects translate to improved athletic performance, although recombinant human GH is known to be widely abused in sport. The model of acromegaly provides evidence that long-term GH excess does not result in improved performance but it is possible that a "window" exists in which the protein anabolic effects of supraphysiologic GH might be advantageous [09207].

**Resistance exercise**

Several recent studies have shown that resistance exercise combined with vascular occlusion effectively causes increases in muscular size and strength. Researchers speculated that the vascular occlusion-induced local hypoxia may contribute to the adaptations via promoting anabolic hormone secretions stimulated by local accumulation of metabolic subproducts. Now it was examined whether acute systemic hypoxia affects metabolic and hormonal responses to resistance exercise. Twelve male subjects participated in two experimental trials: 1) resistance exercise while breathing normoxic air (normoxic resistance exercise, NR), 2) resistance exercise while breathing 13 percent oxygen (hypoxic resistance exercise, HR). The resistance exercises (bench-press and leg-press) consisted of 10 repetitions for five sets at 70 % of maximum strength with 1-min rest between sets. Blood were measured before normoxia and hypoxia exposures, 15-min after the exposures, and at 0, 15, 30, 60 min after the exercises. Lactate significantly increased after exercises in both trials. In the HR trial, growth hormone and cortisol significantly increased after the exercise, but not in the NR trial. The epinephrine, nor-epinephrine, IGF-1, and testosterone significantly increased after the exercises in both trials. The mean values of lactate, GH, epinephrine, and norepinephrine after exercises were significantly higher in the HR trial than that in the NR trial. These findings suggest that resistance exercise in hypoxic condition caused greater accumulation of metabolites, and strong anabolic hormone response [09201].

The aim of one study was to compare the acute hormonal responses following two different eccentric exercise velocities. Seventeen healthy, untrained, young women were randomly placed into two groups to perform five sets of six maximal isokinetic eccentric actions at slow (30°/s ) and fast (210°/s ) velocities with 60-s rest between sets. Growth hormone, cortisol, free and total testosterone were assessed by blood samples collected at baseline,
immediately postexercise, 5, 15 and 30 min following eccentric exercise. Changes in hormonal responses over time were compared between groups, using a mixed model followed by a Tukey’s post hoc test. The main findings of the present study were that the slow group showed higher growth hormone values immediately and 15 min posteccentric exercise compared with the fast group and other hormonal responses were not different between groups. In conclusion, slow eccentric exercise velocity enhances more the growth hormone (GH) response than fast eccentric exercise velocity without cortisol and testosterone increases [13361].

Sprint

Growth hormone is widely abused by athletes, frequently with androgenic steroids. Its effects on performance are unclear. To determine the effect of growth hormone alone or with testosterone on body composition and measures of performance a randomized, placebo-controlled, blinded study of 8 weeks of treatment followed by a 6-week washout period. Randomization was computer-generated with concealed allocation. Ninety-six recreationally trained athletes (63 men and 33 women) with a mean age of 28 years. Men were randomly assigned to receive placebo, growth hormone (2 mg/d subcutaneously), testosterone (250 mg/wk intramuscularly), or combined treatments. Women were randomly assigned to receive either placebo or growth hormone (2 mg/d). Body composition variables (fat mass, lean body mass, extracellular water mass, and body cell mass) and physical performance variables (endurance: maximum oxygen consumption; strength: dead lift; power: jump height; and sprint capacity: Wingate value). Body cell mass was correlated with all measures of performance at baseline. Growth hormone significantly reduced fat mass, increased lean body mass through an increase in extracellular water, and increased body cell mass in men when coadministered with testosterone. Growth hormone significantly increased sprint capacity, by 0.71 kJ (95 % confidence interval 0.1 to 1.3 kJ; relative increase 3.9 %) in men and women combined and by 1.7 kJ (confidence interval 0.5 to 3.0 kJ; relative increase 8.3 %) when coadministered with testosterone to men; other performance measures did not significantly change. The increase in sprint capacity was not maintained 6 weeks after discontinuation of the drug. Growth hormone dosage may have been lower than that used covertly by competitive athletes. The athletic significance of the observed improvements in sprint capacity is unclear, and the study was too small to draw conclusions about safety. It was concluded that the growth hormone supplementation influenced body composition and increased sprint capacity when administered alone and in combination with testosterone [10142].

Exercise stimulates growth hormone (GH) release, but there are conflicting reports regarding the acute effects of exercise on circulating ghrelin and insulin-like growth factor (IGF) concentrations. This investigation examined the effect of a single sprint on circulating GH, ghrelin and IGF concentrations as well as a marker of IGF-I bioactivity, and whether the number of muscle actions performed during a sprint influences these responses. Seven healthy men completed 3 trials in a random order. In two exercise trials they performed a single 30-s sprint on a cycle ergometer against a resistance equivalent to either 7 percent (FAST) or 9 percent (SLOW) of their body mass. In the other they rested in the laboratory (CON). Blood samples were taken pre-, immediately post-, 10 and 30 min post-exercise, and at equivalent times in the CON trial. Total ghrelin concentrations declined after the sprint and were significantly lower after 30 min of recovery than they were pre-exercise (pre-exercise vs 30 min; FAST, 0.62 vs 0.49 mug/L; SLOW, 0.59 vs 0.47 mug/L). GH concentrations increased in both exercise trials and were greater in the FAST than the SLOW trial. Serum concentrations of total IGF-I, free IGF-I, total IGF-II, and IGF-I bioactivity did not change after sprinting. In conclusion, sprint exercise suppresses total ghrelin concentrations and stimulates GH release but does not alter IGF concentrations or bioactivity [10143].
**Muscle strength**

Olympic, professional and weekend athletes abuse hGH because of unsubstantiated reports that it is as effective as anabolic steroids with fewer side effects. They often abuse growth hormone as a steroid substitute to prevent loss of muscle after discontinuing the use of steroids. According to some controlled scientific studies, hGH does not increase muscle strength [08006].

Recombinant human growth hormone (rhGH) as opposed to cadaver pituitary GH is misused for physical improvement. Six days' rhGH administration, in abstinent anabolic-androgenic steroid dependents, was compared with controls. Male subjects (n=48) were randomly divided into two groups: control group, n=24; rhGH-using group (0.058 IU/kg/day GH; n=24). Strength, peak power output and IGF-I significantly increased and total protein, albumin and free tetra-iodothyronine significantly decreased compared to controls and within the GH group. Fat-free mass index and VO₂ peak significantly increased, while body fat and thyroid-stimulating hormone significantly decreased within the GH group. It was concluded that short-term rhGH increased strength and power [08251].

One article discussed the inevitable use of growth factors for enhancing muscle strength and athletic performance. Much effort has been expended on developing a treatment of muscle wasting associated with a range of diseases and aging. Frailty in the aging population is a major socioeconomic and medical problem. Emerging molecular techniques have made it possible to gain a better understanding of the growth factor genes and how they are activated by physical activity [10144].

**With and without carbohydrate + protein**

Endocrine responses to repeated exercise have barely been investigated, and no data are available regarding the mediating influence of nutrition. On 3 occasions, participants ran for 90 min at 70 percent VO₂max (R1) before a second exhaustive treadmill run at the same intensity (R2). During the intervening 4-hr recovery, participants ingested either 0.8 g sucrose/kg/h with 0.3 g/kg/h whey-protein isolate (CHO-PRO), 0.8 g sucrose/kg/h (CHO), or 1.1 g sucrose/kg/h (CHO-CHO). The latter 2 solutions therefore matched the former for carbohydrate or for available energy, respectively. Serum growth-hormone concentrations increased from 2 ± 1 microg/L to 17 ± 8 microg/L during R1 considered across all treatments (M ± SD; p ≤ .01). Concentrations were similar immediately after R2 irrespective of whether CHO or CHO-CHO was ingested (19 ± 4 microg/L and 19 ± 5 microg/L, respectively), whereas ingestion of CHO-PRO produced an augmented response (31 ± 4 microg/L). Growth-hormone-binding protein concentrations were unaffected by R1 but increased similarly across all treatments during R2 (from 414 ± 202 pmol/L to 577 ± 167 pmol/L), as was the case for plasma total testosterone (from 9.3 ± 3.3 nmol/L to 14.7 ± 4.6 nmol/L). There was an overall treatment effect for serum cortisol, with no specific differences at any given time point but lower concentrations immediately after R2 with CHO-PRO (608 ± 133 nmol/L) than with CHO (796 ± 278 nmol/L) or CHO-CHO (838 ± 134 nmol/L). Ingesting carbohydrate with added whey-protein isolate during short-term recovery from 90 min of treadmill running increases the growth-hormone response to a second exhaustive exercise bout of similar duration [13362].

**Slow eccentric exercise velocity**
The aim of one study was to compare the acute hormonal responses following two different eccentric exercise velocities. Seventeen healthy, untrained, young women were randomly placed into two groups to perform five sets of six maximal isokinetic eccentric actions at slow (30°/s) and fast (210°/s) velocities with 60-s rest between sets. Growth hormone, cortisol, free and total testosterone were assessed by blood samples collected at baseline, immediately postexercise, 5, 15 and 30 min following eccentric exercise. Changes in hormonal responses over time were compared between groups, using a mixed model followed by a Tukey's post hoc test. The main findings of the present study were that the slow group showed higher growth hormone values immediately (5.1 ± 2.9 ng/mL, 5 (5.5 ± 3.0 ng/mL) and 15 min (4.3 ± 2.9 ng/mL) posteccentric exercise compared with the fast group (1.4 ± 2.4 ng/mL, 1.3 ± 2.0 ng/mL and 1.2 ± 1.9 ng/mL, respectively), and other hormonal responses were not different between groups. In conclusion, slow eccentric exercise velocity enhances more the growth hormone (GH) response than fast eccentric exercise velocity without cortisol and testosterone increases [13363].

**Tendons**

In skeletal muscle and tendon the extracellular matrix confers important tensile properties and is crucially important for tissue regeneration after injury. Musculoskeletal tissue adaptation is influenced by mechanical loading, which modulates the availability of growth factors, including growth hormone (GH) and insulin-like growth factor-I (IGF-I), which may be of key importance. To test the hypothesis that GH promotes matrix collagen synthesis in musculotendinous tissue, it was investigated the effects of 14 day administration of 33-50 microg/kg per day recombinant human GH (rhGH) in healthy young individuals. rhGH administration caused an increase in serum GH, serum IGF-I, and IGF-I mRNA expression in tendon and muscle. Tendon collagen I mRNA expression and tendon collagen protein synthesis increased by 3.9-fold and 1.3-fold, respectively, and muscle collagen I mRNA expression and muscle collagen protein synthesis increased by 2.3-fold and 5.8-fold, respectively. Myofibrillar protein synthesis was unaffected by elevation of GH and IGF-I. Moderate exercise did not enhance the effects of GH manipulation. Thus, increased GH availability stimulates matrix collagen synthesis in skeletal muscle and tendon, but without any effect upon myofibrillar protein synthesis. The results suggest that GH is more important in strengthening the matrix tissue than for muscle cell hypertrophy in adult human musculotendinous tissue [10145].

**No effect on tendon healing**

There have been few scientific studies that have examined usage of human growth hormone to accelerate recovery from injury. The hypothesis of this study was that human growth hormone would accelerate tendon-to-bone healing compared with control animals treated with placebo in a rat model of acute rotator cuff injury repair. Seventy-two rats underwent repair of acute rotator cuff injuries and were randomized into the following postoperative dosing regimens: placebo, and human growth hormone at 0.1, 1, 2, 5, and 10 mg/kg/day, administered subcutaneously once per day for fourteen days (Protocol 1). An additional twenty-four rats were randomized to receive either (1) placebo or (2) human growth hormone at 5 mg/kg, administered subcutaneously twice per day for seven days preoperatively and twenty-eight days postoperatively (Protocol 2). All rats were killed twenty-eight days postoperatively. Mechanical testing was performed. Ultimate stress, ultimate force, stiffness, energy to failure, and ultimate distension were determined. For Protocol 1, analysis of variance testing showed no significant difference between the groups with regard to ultimate stress, ultimate force, stiffness, energy to failure, or ultimate distension. In Protocol 2, ultimate force to failure was significantly worse in the human growth hormone group compared with the placebo group. Failure was more likely to occur through the bone than the tendon-bone interface in the human growth hormone group compared with the placebo.
group. No significant difference was found for ultimate stress, ultimate force, stiffness, energy to failure, or ultimate distension between the groups in Protocol 2. In this rat model of acute tendon-bone injury repair, daily subcutaneous postoperative human growth hormone treatment for fourteen days failed to demonstrate a significant difference in any biomechanical parameter compared with placebo. Furthermore, subcutaneous administration of 5 mg/kg of human growth hormone twice daily from seven days preoperatively until twenty-eight days postoperatively demonstrated lower loads to ultimate failure and a higher risk of bone fracture failure compared with placebo [13367].

Meta-analysis

To evaluate evidence about the effects of growth hormone on athletic performance in physically fit, young individuals randomized, controlled trials that compared growth hormone treatment with no growth hormone treatment in community-dwelling healthy participants between 13 and 45 years of age were reviewed articles. There were 44 articles describing 27 study samples that met inclusion criteria; 303 participants received growth hormone, representing 13.3 person-years of treatment. Participants were young (mean age, 27 years), lean (mean body mass index, 24 kg/m²), and physically fit (mean maximum oxygen uptake, 51 mL/kg of body weight per minute). Growth hormone dosage (mean, 36 microg/kg per day) and treatment duration (mean, 20 days for studies giving growth hormone for >1 day) varied. Lean body mass increased in growth hormone recipients compared with participants who did not receive growth hormone (increase, 2.1 kg; 95 % confidence interval 1.3 to 2.9 kg]), but strength and exercise capacity did not seem to improve. Lactate levels during exercise were statistically significantly higher in 2 of 3 studies that evaluated this outcome. Growth hormone-treated participants more frequently experienced soft tissue edema and fatigue than did those not treated with growth hormone. However, few studies evaluated athletic performance. Growth hormone protocols in the studies may not reflect real-world doses and regimens. This means that claims that growth hormone enhances physical performance are not supported by the scientific literature. Although the limited available evidence suggests that growth hormone increases lean body mass, it may not improve strength; in addition, it may worsen exercise capacity and increase adverse events. More research is needed to conclusively determine the effects of growth hormone on athletic performance [08252].

In women

The purpose of one study was to examine the effects of combined exercise training on growth hormone (GH), insulin-like growth factor-1 (IGF-1), and metabolic-syndrome factors and determine whether the changes in GH and/or IGF-1 induced by exercise correlate to the metabolic-syndrome factors in healthy middle-aged women (50-65 years of age). The participants were randomly assigned into an aerobic-exercise training (walking + aerobics) group (AEG; n=7), a combined-exercise training (walking + resistance training) group (CEG; n=8), or a control group (CG; n=7). Exercise sessions were performed 3 times per wk for 12 wk. The aerobic-exercise training consisted of walking and aerobics at 60-80 percent of heart-rate reserve, and the combined-exercise training consisted of walking and resistance exercise at 50-70 percent of 1-repetition maximum. Growth hormone, percentage body fat, fasting glucose, systolic blood pressure, and waist circumference were significantly improved in CE. However, GH induced by exercise training showed no correlation with metabolic-syndrome factors. IGF-1 was not significantly increased in either AEG or CEG compared with CG. These results indicate that the combined-exercise training produced more enhancement of GH, body composition, and metabolic-syndrome factors than did aerobic-exercise training [10146].
Impact of injuries

Growth-promoting agents are purported to increase the size of existing and newly regenerating muscle fibres and, therefore, could be used to improve muscle function if administered at appropriate times during the repair process. One review provided an update on the efficacy of some growth-promoting agents, including anabolic steroids, insulin-like growth factor-I (IGF-I) and beta₂-adrenoceptor agonists, to improve muscle function after injury. Although these approaches have clinical merit, a better understanding of the androgenic, IGF-I and beta-adrenoceptor signalling pathways in skeletal muscle is important if it may be devised safe and effective therapies to enhance muscle regeneration and function after injury [08255].

The objective of the study was to assess the effect of musculoskeletal or soft tissue injury on IGF-I and P-III-P concentrations in amateur and elite athletes and assess the effect of injury on the proposed GH detection method. There was no change in IGF-I concentration after an injury. By contrast, P-III-P concentrations rose by 41 ± 17 percent, reaching a peak around 14 days after an injury. The rise in P-III-P varied according to injury type and severity. This rise had a trivial effect on the GH-2000 discriminant function score, and no subject reached the threshold needed for a doping offense. The authors concluded that although there was a rise in P-III-P after injury, this was insufficient to invalidate the GH-2000 detection method based on IGF-I and P-III-P concentrations [08256].

Boxers

To investigate the pituitary function in retired or active amateur boxers 61 actively competing (n=44) or retired (n=17) male boxers of the Turkish National Boxing Team were investigated. Nine of 61 boxers (15 %) had growth hormone (GH) deficiency and 5 of 61 boxers (8 %) had adrenocorticotropic hormone deficiency. All boxers with GH deficiency except 1 were retired from boxing. Of 17 retired boxers, 8 (47 %) had GH deficiency. Retired boxers with GH deficiency had significantly lower pituitary volume than retired boxers with normal GH. This study suggests that retired boxers have a high rate of pituitary dysfunction [08257].

It has been recently reported that boxing and kickboxing may cause pituitary dysfunction, GH deficiency in particular. The strong link between poor cognitive performance and GH deficiency due to causes other than head trauma and the improvement of cognitive function after GH replacement therapy have been previously shown. P300 auditory event-related potential (ERP) measure is widely used to evaluate cognitive performance. In one study, it was investigated the relation between the GH-IGF-I axis and cognitive performance in boxers and kickboxers. Forty-one actively competing or retired male boxers (n=27) and kickboxers (n014) with a mean age of 29 year and 14 age- and education-matched healthy male controls were included in the study. For neuropsychological tests, the mini-mental state examination (MMSE) and Quality of Life Assessment of GH Deficiency in Adults (QoL-AGHDA) questionnaires were administered. Moreover, cognitive performance was evaluated according to P300 ERPs. Nine of 41 (22 %) athletes had GH deficiency. P300 amplitudes were lower at all electrode sites in the GH-deficient group than in controls, and the differences were statistically significant at Fz and Oz electrode sites. When GH-deficient athletes were compared with GH-sufficient athletes, the P300 amplitudes were lower at all electrode sites in the GH-deficient group; these differences were statistically significant at Fz, Pz and Cz electrode sites. In all athletes, there were significant negative correlations between IGF-I levels vs P300 latencies, and there were significant positive correlations...
between IGF-I levels vs P300 amplitudes. The study provides the first electrophysiological evidence for the close relation between the P300 ERPs and the GH-IGF-I axis in boxers and kickboxers [13369].

**Horse growth hormones**

Since the Australian commercialisation of the recombinant equine growth hormone (reGH) in 1998 (EquiGen-5), this reGH, which differs only from eGH by an additional methionine at the N-terminal end (met-eGH), is worldwide suspected to be administered to racehorses as a doping agent. Indeed, the use of this biological drug is considered as a threat to horseracing since it acts both on growth, development or reproductive functions, and on the improvement of performances. It was now described two reliable techniques based on surface plasmon resonance biosensor immunoassay and solid-phase enzyme-linked immunosorbent assay (ELISA) as new, rapid and efficient long-term screening methods applicable to horseracing antidoping analysis. The first kinetic study of serum/plasma antibodies raised as a consequence of recombinant equine growth hormone administrations, which allows the detection from eight days up to 200 days after the beginning of the treatment, was performed. In order to trace the occurrence of anti-reGH antibodies in routine analysis and to monitor the animal level exposure to this forbidden molecule, a random population study was conducted on 233 post-race horses [08258].

Equine growth hormone (eGH) has been available since 1998 as an approved drug (EquiGen-5, Bresagen) containing recombinant eGH (reGH). It is suspected of being illegally administered to racehorses in order to improve physical performance and to speed-up wound healing. Thus it may be considered a doping agent which would require a sensitive and reliable method of identification and confirmation in order to regulate its use in racehorses. reGH differs from the native eGH by an additional methionine at the N-terminal (met-eGH) and has never been unambiguously detected in any type of biological matrix at trace concentrations (1-10 mug/L). A plasma sample (4 mL) was treated with ammonium sulfate at the reGH isoelectric point and the pellet was purified by solid-phase extraction. Specific peptides were generated by trypsin digestion and analyzed by LC-MS/MS. The detection limit was 1 mug/L. Furthermore, it was successfully applied to determining the plasma concentrations of reGH with time using linear ion trap mass analyzer. The presence of the prohibited hormone was also successfully detected by triple quadrupole mass spectrometry up to 48 h postadministration of reGH to a horse [08259].

**Detection of different brands**

The detection of recombinant human growth hormone (rhGH) doping using the World Anti-Doping Agency (WADA) approved kits is reported in this research. Twenty-five young male students were selected and divided randomly into two groups with six belonging to the placebo and nineteen to the administration group. Thirteen volunteers in one group were administered with a Chinese preparation of rhGH while six volunteers included in the other group were given rhGH made in Switzerland. Both preparations were administered at a dose of 0.1 IU/kg body weight, one injection per day for 14 consecutive days. Blood samples were collected using WADA guidelines and all blood samples were analyzed with WADA-approved Kits 1 and 2. The time window for detection of rhGH doping using WADA-approved kits and criteria are discussed. Based on the comparison of the data obtained from this excretion study and from our routine (Chinese population as reference), consideration of the recent WADA criteria for rhGH AAF (Analytical Adverse Findings) is reported statistically. A
comparison of data obtained from the two sample groups administered with pharmaceutical preparations, one Chinese rhGH obtained from prokaryotic cells and the other from eukaryotic cells is reported and did not show any significant difference for the detection of doping with rhGH [11472].

**Laboratory techniques**

Recombinant human growth hormone (rhGH) is used for the treatment of several disorders. Structural integrity of rhGH is of critical importance for its clinical use and modifications thereof may act as markers in situations such as rhGH doping, as illegal rhGH-abuse in sports is of increasing interest. In the current study we investigated homogeneity of Norditropin, a recombinant human growth hormone frequently used in medicine, expressed in E. coli, strain MC1061. The most recent proteomics technologies including 2-DE, MALDI-MS followed by MALDI-MS/MS and LC-MS followed by LC-MS/MS were used for the characterisation of rhGH. MALDI-TOF-TOF and electrospray LC-MS analysis revealed one major protein with an average molecular mass of 22 126.0 Da and some additional minor components. Electrospray LC-MS/MS of the enzymatically digested Norditropin sample showed deamidation of N(12)N(149) and N(159), oxidation of M(14), M(125) and M(170) and one amino acid exchange V(14) for M(14) present in <1% of Norditropin. While deamidation and oxidation may be due to technical reasons, the single amino acid exchange may reflect infidelity of translation rather than codon usage and copy editing by E. coli [06140].

Growth hormone is abused by athletes for its lipolytic and anabolic properties. Its use is prohibited by the World Anti-Doping Agency. The GH-2000 project developed a methodology to detect its abuse using the concentrations of two GH-dependent biomarkers, IGF-I and type 3 procollagen (P-III-P). The sensitivity of this method may be improved by considering intra-individual variability. The aim of one study was to examine the intra-individual variability of IGF-I, P-III-P and the GH-2000 score. IGF-I, P-III-P and GH-2000 score were evaluated in four longitudinal studies involving 303 elite and 78 amateur athletes. Samples were collected over a period of up to 12 months from a total of 238 men and 143 women aged between 17 and 53 years (mean 24). The four studies showed good agreement with no apparent difference in within-individual variation between amateur and elite athletes. The intra-individual variability for IGF-I ranged between 14-16 percent while the variability for P-III-P was 7-18 percent. No athlete tested positive for growth hormone during any of the studies. The overall mean intra-individual variability of the GH-2000 score was less than 0.6 units in all studies. The high stability of marker levels suggests that concentrations are largely genetically determined. Adopting a test based on the concept of an athlete's “passport” or “profiling” would take advantage of this and most likely increase the sensitivity of the test. These data also provide strong evidence that a positive test result for GH abuse would not occur as a result of chance variability [10147].

Detection of athletes who use synthetic human growth hormone (hGH; or somatotropin) to enhance physical strength and obtain an advantage in competitive sports is a formidable problem, as rhGH is virtually identical to the natural pituitary hormone. However, some post-translational and other modifications have been documented by chromatographic separation and mass spectrometry (MS) in a small percentage of rhGH. In the present work, development of DNA aptamers against research-grade rhGH and natural hGH with adsorption of the rhGH aptamers against natural hGH was shown to produce a small family of aptamer sequences that bound consistently with greater affinity to rhGH over a low nanogram-to-microgram range in ELISA-like microplate assays. This collection of rhGH discriminatory aptamer sequences shared some short sequence segments and secondary
Emerging molecular techniques have made it possible to gain a better understanding of the growth factor genes and how they are activated by physical activity [10149].

Detection of athletes who use synthetic human growth hormone (hGH; or somatotropin) to enhance physical strength and obtain an advantage in competitive sports is a formidable problem, as rhGH is virtually identical to the natural pituitary hormone. However, some post-translational and other modifications have been documented by chromatographic separation and mass spectrometry (MS) in a small percentage of rhGH. In the present work, development of DNA aptamers against research-grade rhGH and natural hGH with adsorption of the rhGH aptamers against natural hGH was shown to produce a small family of aptamer sequences that bound consistently with greater affinity over a low nanogram-to-microgram range in ELISA-like microplate assays. This collection of rhGH discriminatory aptamer sequences shared some short sequence segments and secondary structural features. The top rhGH discriminatory aptamers also appeared to cross-react with human myoglobin and BSA but not with bone collagen peptides and an unrelated viral envelope peptide. The cross-reactivity results suggested several strings of up to five consecutive amino acids that might serve as common epitopes for aptamer binding. SDS-PAGE revealed that the rhGH existed largely as a 45-kDa dimer, and the natural hGH was almost exclusively monomeric. The existence of the rhGH dimer suggests that a discontinuous "bridge" epitope may exist on the rhGH, which spans the subunits, thereby accounting somewhat for the difference in detection. Overall, these results suggest that aptamers might be useful for routine, presumptive laboratory screening to identify athletes who are potentially cheating by administration of rhGH [11170].

A method based on IGF1 and type III pro-collagen (P-III-P) to detect exogenously administered GH was proposed. As previous studies involved predominantly white European athletes, it is important to assess whether the response of these markers to recombinant human GH (rhGH) differs with ethnicity. The study included 31 male and 14 female amateur athletes of different ethnicities. The subjects were assigned to treatment with placebo or 0.1 IU/kg per day (low dose) or 0.2 IU/kg per day (high dose) rhGH for 28 days. Blood was collected weekly during treatment and on days 35, 42 and 84 during the washout period.
Serum IGF1 and P-III-P were measured, and GH-2000 score was calculated. IGF1, P-III-P and GH-2000 score rose in response to both low- and high-dose GH in both men and women. When compared with the Caucasian volunteers of the previous GH-2000 study, mean baseline and placebo-treated P-III-P and GH-2000 score were lower in GH-2004 men and women. Post-GH, however, peak IGF1 or P-III-P did not differ between studies but the peak GH-2000 score was lower in GH-2004 men. There was no difference between studies in the maximal change in IGF1, P-III-P and GH-2000 score in response to GH in either gender. These data do not support a significant ethnic effect on the peak or maximal response to rhGH [10150, 10151].

Mass spectrometric approaches have been used to determine various peptide hormones in sports drug testing. While insulin-like growth factor-1 (IGF-1) and its synthetic analogs are qualitatively and/or quantitatively measured by liquid chromatography-tandem mass spectrometry after immunoaffinity purification, methods of uncovering doping rule violations with illegal applications of human growth hormone (hGH) have not yet been established using mass spectrometry-based assays. However, substantial information on the heterogeneity of hGH, splice variants and post-translational modifications with respective locations as elucidated by mass spectrometry are of utmost importance for improving currently employed immunological procedures [10152].

Application of methods for detecting GH doping depend on being able to discriminate between abnormal levels due to doping and normal physiological levels of circulating proteins that change in response to exogenous administration. Constituents of the IGF and collagen systems have been shown to be promising markers of GH abuse. Their ultimate utility, however, depends on identification of the factors that regulate their concentrations in blood. Among these are demographic factors that are known to influence these markers in the general population. In a large cross-sectional study of the GH-responsive markers in over 1000 elite athletes from 12 countries representing 4 major ethnic groups and 10 sport types, it was shown that there is a significant negative correlation between age and all the IGF and collagen markers that was studied, with a rapid decrease in early adolescence. Age was the major contribution to the variability, equivalent to >80 percent of the attributable variation in IGF-I and the collagen markers. The IGF axis markers were all significantly higher in women, and the collagen markers significantly higher in men, however, the contribution of gender was smaller than that of age, except for IGFBP-3 and ALS. BMI had a minor contribution to variability of the GH-responsive markers. After adjustment for the confounding influences of age, gender and BMI, the effect of ethnicity in elite athletes was trivial except for IGFBP-3 and ALS, which were both lower in Africans and higher in Caucasians. Compared to age and gender, the contribution of sport type was also modest. The findings on the influence of age, gender, BMI and sport type have also been confirmed in a study of mostly Caucasian elite athletes in the post-competition setting. It was concluded that age and gender are the major determinants of variability for IGF-I and the collagen markers, whereas ethnicity and sport type have a minor influence. Therefore, a test based on IGF-I and the collagen markers must take age into account for men and women, and ethnicity and sport type are unlikely to be confounders for these markers [09208].

Following the successful production of recombinant 20K-GH, several studies investigating the physiology of this GH isoform have been undertaken. In one report, it was reviewed studies of its biological effect, measurement and secretion. To use serum 20K-GH level in detecting GH abuse, new method has been established and serum 22K-GH, 20K-GH were measured in normal subjects and athletes, and no abnormal results were found among athletes. Another study confirmed that serum 22K-GH increased remarkably and 20K-GH decreased following the exogenous administration of 22K-GH. The duration was relatively short, approximately 24-36h in several studies. The increase of the ratio, 22K-GH/20K-GH...
was the most suitable indicator of GH abuse. Studies supported by the WADA were undertaken in collaboration with an Australian Group. A new approach for the GH isoform assay by beads assay platform is being developed. It is concluded that the direct measurement of 20K-GH is a valid scientific approach, for detecting GH abuse, although the duration of the positive results is short. The method will be useful in combination with the marker method, an out-of-competition test or test for target cases [09209].

It was developed a semiquantitative method for the analysis of main growth hormone isoforms. The use of immunoaffinity sorbents, two-dimensional electrophoresis, and immunoblotting allows detection of more than 90 percent circulating growth hormone. It was demonstrated that the proportion of growth hormone isoforms in human serum before and after strenuous exercise remained unchanged [09210].

The utility of insulin-like growth factor (IGF) axis and collagen markers for a growth hormone (GH) doping test in sport depends on their stability and reproducibility. It was sought to determine short-term within-subject variability of these markers in a large cohort of healthy individuals by measuring IGF-I, IGF binding protein 3 (IGFBP-3), acid labile subunit (ALS), and the collagen markers N-terminal propeptide of type I procollagen (PINP), C-terminal telopeptide of type I collagen (ICTP), and N-terminal propeptide of type III procollagen (PIIINP) in serum samples obtained on multiple occasions (median 3 per participant) over a 2- to 3-week period from 1103 elite athletes (699 men, 404 women) aged (mean) 22 years. Within-subject variance accounted for 32-36 percent and 4-13 percent of the total variance in IGF markers and collagen markers, respectively. The within-subject CV ranged from 11 to 21 percent for the IGF axis markers and from 13 to 15 percent for the collagen markers. The index of individuality for the IGF axis markers was 0.66-0.76, and for the collagen markers, 0.26-0.45. For each marker, individuals with initial extreme measured values tended to regress toward the population mean in subsequent repeated measurements. These results indicate that in healthy individuals the within-subject variability was greater for IGF-I than for the collagen markers, and that where a single measurement is available, it is possible to estimate the long-term probable value of each of the markers by applying the Bayesian approach. Such an application can increase the reliability and decrease the cost of detecting GH doping [08253].

IGF axis proteins and collagen peptides are promising markers of GH abuse. The objective was to investigate whether responses of serum IGF axis and collagen markers to GH differ between men and women, and are influenced by testosterone in a randomized, double-blind, placebo-controlled study of 8-week treatment followed by 6-week washout. A total of 96 recreationally trained healthy athletes (63 men, 33 women), aged 18-40 year, were studied. All subjects received GH (2 mg/d sc) or placebo for 8 weeks; men also received testosterone (250 mg/week im) or placebo for 5 weeks. Serum IGF axis proteins (IGF-I, IGF binding protein-3, and acid labile subunit) and collagen peptides (N-terminal propeptide of type I procollagen, C-terminal telopeptide of type I collagen, and N-terminal propeptide of type III procollagen) were measured. GH induced significant increases in IGF axis and collagen markers that were greater in men than women. Of the IGF axis markers, IGF-I showed the greatest increase. The relative incremental responses of the collagen markers in general were greater than the IGF markers, especially for PIIINP. The collagen markers increased and decreased more slowly with most remaining elevated after 6 weeks, in comparison to IGF markers, which returned to baseline within 1 week. Addition of T to GH amplified the response of PIIINP by more than 1.5-fold but did not affect any other marker. Testosterone alone did not affect IGF axis markers but modestly increased collagen markers. These markers of GH abuse are less responsive in women. The increases in collagen markers have a different time course to the IGF markers and extend the window of detection in both sexes. The response of PIIINP is increased by coadministration of testosterone [08254].

1112
It has been argued that increased levels of bone remodelling markers are not suitable indicators of GH abuse, as bone injuries per se increase the expression levels of these markers. To investigate the impact of a recovering tibia fracture on circulating bone markers in subjects receiving placebo or GH treatment in a randomised, double-blind, placebo-controlled trial of up to 16 weeks GH treatment, followed by a 16-week washout was conducted. Subjects (406 adult males and females) with a tibia fracture were randomly allocated within three days after surgery, to either placebo or GH treatment (15, 30 or 60 microg/kg daily) until fracture healing or 16 weeks after treatment initiation. IGF-I, serum C-terminal telopeptide of type I collagen (CTX), osteocalcin (OST) and bone-specific alkaline phosphatase (BAP) were measured during and after treatment. Dose-dependent increases were observed in groups receiving GH, and mean levels in the highest GH dose group peaked at eight (IGF-I, CTX) or 12 weeks (OST) after treatment initiation. Statistically significant differences between GH treatment and placebo were seen for IGF-I, CTX and OST in all GH dose groups throughout the treatment period, and persisted until eight (CTX) or 12 (OST) weeks after cessation of treatment. It was concluded that IGF-I, CTX and OST are suitable candidate markers of prolonged, illicit administration of GH. Furthermore, CTX and OST have potentials to serve as markers also after cessation of GH administration [11473].

GH is believed to be widely employed in sports as a performance-enhancing substance. Its use in athletic competition is banned by the World Anti-Doping Agency, and athletes are required to submit to testing for GH exposure. Detection of GH doping is challenging for several reasons including identity/similarity of exogenous to endogenous GH, short half-life, complex and fluctuating secretory dynamics of GH, and a very low urinary excretion rate. hGH has been widely abused as a doping agent in sports for many years. There are some important approaches for the detection of hGH doping, and the ratio of 22:20 kDa GH was considered one of the most suitable detection indicators of GH abuse. Currently, effective anti-GH antibodies and related reagents are needed to develop a detection method, in particular, highly specific anti-20 kDa hGH monoclonal antibodies are a prerequisite. Herein we constructed the expression vector of 20 kDa hGH and prepared the corresponding antibodies by the immunization of the recombinant human 20 kDa into mice. Positive clones that can specifically recognize 20 kDa hGH were screened and characterized by enzyme immunoassay, Dot-ELISA and surface plasmon resonance. In total, 14 specific monoclonal cell lines were screened out. By a series of characterization, it was found that the 6C8, 44H3, 12G7 and 33Y19 clones were showing much higher specificity and affinity to 20 kDa hGH, and P3H9 could recognize both 20 and 22 kDa hGH isoforms. 6C8 and 44H3 matched well with P3H9 in the surface plasmon resonance testing. The 12G7 clone had the best surface properties with an association constant of $3.4 \times 10^9$ M$^{-1}$ and a dissociation constant of $2.95 \times 10^{10}$ M. Highly specific monoclonal antibodies against 20 kDa hGH were generated, and also two paired antibodies (P3H9 and 6C8 or P3H9 and 44H3) were characterized, which can serve as the potential components for 22:20 kDa detection kit [12213].

It was implemented a surface plasmon resonance (SPR) immunosensor based on a sandwich assay for the simultaneous detection of the two main hGH isoforms, of 22 kDa (22K) and 20 kDa (20K). An oriented-antibody sensor surface specific for both hormone isoforms was assembled by using the biotin-streptavidin system. The immunosensor functionality was checked for the direct detection of the 22K hGH isoform in buffer, which gave high specificity and reproducibility (intra and inter-assay mean coefficients of variation of 8 % and 9 % respectively). The selective determination of the 22K and 20K hGH isoforms in human serum samples in a single assay was possible by using two specific anti-hGH monoclonal antibodies. The detection limit for both hormone isoforms was 0.9 ng mL$^{-1}$ and the mean coefficient of variation was below 7.2 percent. The excellent reproducibility and
sensitivity obtained indicate the high performance of this immunosensor for implementing an anti-doping test [130370].

Growth hormone abuse in sports has been suspected and purported for decades and also occasionally been proven in the last years. As the major temptations the assumed ergogenic activity of hGH, accelerated recovery (e.g. after injury) and also its former ‘stealth’ and undetectable nature have been mentioned. In a comprehensive overview concerning health risks associated with hGH (and IGF-1) abuse, detection strategies (GH isoform and biomarker test) and their advantages as well as limitations is presented. Despite the substantial knowledge concerning adverse effects of GH abuse, incidences and case reports with bovine growth hormone self-administrations have been reported. Since 2004, the isoform test for hGH abuse has been in use in routine doping controls and has undergone fine-tuning and continuous finishing to increase its sensitivity and thus broaden the window of opportunity for detection. In 2011, a controlled administration study with two preparations of recombinant hGH (Chinese and Swiss products, 0.1 IU/kg bodyweight) was conducted and the traceability of the drug (i.e. its influence on the circulating GH isoforms) was determined using the WADA-approved analytical kits. Following a single injection, detection windows between 12 and 18 h were observed, while repeated hGH application (one injection/day over 14 days) allowed for hGH abuse detection up to 21 h after cessation. In a different study, the performance of two isoform-based growth hormone detection assays, namely the above mentioned WADA-approved test and a 22 kDa/20 kDa isoform immunoassay, was compared. Volunteers received recombinant hGH subcutaneously at 0.026 mg/kg bodyweight once daily for seven consecutive days, and collected serum samples were analyzed on both platforms. The assays demonstrated good correlation concerning the detection of abnormal isoform concentrations in serum and exhibited comparable detection windows of up to 24 h [13012].

In July 2012, the detection of growth hormone abuse by means of a biomarker-based test method was approved by WADA. This complementary assay employs the biomarkers IGF-1 and the amino-terminal pro-peptide of type-III collagen (P-III-NP) as GH-sensitive parameters increasing in response to exogenous growth hormone administration. By means of doping control serum samples collected from 404 male and 94 female elite athletes, gender-specific GH-2000 score decision limits were established using currently available commercial immunoassays. Since parameters such as serum IGF-1 and other bone remodeling markers might be influenced by circumstances other than doping, the effect of tibia fracture healing on IGF-1, C-terminal telopeptide of type-I collagen (CTX), osteocalcin, and bone-specific alkaline phosphatase was studied in a clinical setting with 406 adults. In a double-blind and placebo-controlled trial, patients received a daily dose of hGH between 0.015 mg and 0.060 mg/kg bodyweight or placebo for a period of 16 weeks and the bone turnover biomarkers were recorded. In all treatment groups, a statistically significant difference in IGF-1, CTX, and OST was observed, corroborating the utility of these markers for GH abuse detection [13012].

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sensitivity obtained indicate the high performance of this immunosensor for implementing an anti-doping test [13371].

Successful application clinical-grade human growth hormone (hGH) immunoassays to the discovery of illegal doping cases has been rare. Indeed, the preferred biological matrix in doping control is urine, where the estimated baseline concentration of hGH falls well below the linear range and sensitivity threshold of all commercially available immunoassays, including hGH isoform differential immunoassays which can discriminate pituitary endogenous hGH from recombinant hGH. It was employed hydrogel nanoparticles as a pre-processing step that concentrates urinary hGH into the linear range of isoform differential immunoassays. It was explored the characteristics of immunoassays in urine spiked with both phGH or rhGH, after pre-treatment with the nanoparticles. Subsequently, pre-treatment was applied to urine obtained from 3 healthy volunteers administered during three days with daily subcutaneous injections of 0.026 mg/kg/day rhGH, Genotonorm®). Linearity between both rhGH and phGH concentrations in urine measured by a chemoluminescent assay (Immulfite) and in the particle eluate was evident for differential immunoassays (R square higher than 0.999). In case of treated individuals the recombinant/pituitary concentration ratios remained above the established World Anti-Doping Agency (WADA) criterion for hGH misuse up to 24h after the last administration dose, using both assays for volunteer 1 and 2 while in case of volunteer 3 results were inconclusive. The use of nanoparticles appears to open the possibility of assessing rhGH misuse in urine [13372].

The isoform methods

Endogenous pituitary GH occurs in multiple isoforms of which 70 percent is in the form of a 22 kDa polypeptide. By contrast, rhGH comprises solely the 22-kDa isoform. When rhGH is administered, endogenous pituitary secretion is downregulated through negative feedback and the concentration of non-22-kDa isoforms is suppressed. The isoform method relies on the measurement of the ratio of 22 kDa GH to total GH. The proportions of GH isoforms are unaffected by age, sex, sporting discipline, and pathological states, but exercise causes a transient relative increase in the 22-kDa isoform, thereby reducing the sensitivity of the test if samples are taken immediately after competition. This method was introduced prior to the 2004 Athens Olympic Games and subsequently improved when the assays used for the method were commercialized. The Achilles’ heel of the isoform method is its short window of detection. Recombinant GH, even when injected subcutaneously, is cleared rapidly and GH is frequently undetectable in a blood sample taken the morning after an injection, while spontaneous GH secretion returns to normal within 48 h of the last dose of rhGH. Consequently, any athlete who ceases GH several days prior to a competition will not be detected. A further disadvantage of this method is that it will not detect the use of cadaveric GH or GH secretagogues as these do not alter the isoform profile [13007].

With nano-technology

Successful application clinical-grade human growth hormone (hGH) immunoassays to the discovery of illegal doping cases has been rare. Indeed, the preferred biological matrix in doping control is urine, where the estimated baseline concentration of hGH falls well below the linear range and sensitivity threshold of all commercially available immunoassays, including hGH isoform differential immunoassays which can discriminate pituitary endogenous hGH from recombinant hGH. It was employed hydrogel nanoparticles as a pre-processing step that concentrate urinary hGH into the linear range of isoform differential immunoassays. It was explored the characteristics of immunoassays in urine spiked with both phGH or rhGH, after pre-treatment with the nanoparticles. Subsequently, pre-treatment...
Stability of GH during testing procedures

IGF-I and type III procollagen (P-III-P) have been proposed as markers to detect GH abuse. One study aimed to determine whether the pre-analytical storage temperature or delayed centrifugation affect the measured IGF-I and P-III-P concentrations. The storage temperature or timing of centrifugation did not appear to affect IGF-I concentration. In contrast, the measured P-III-P concentration rose by 6.5-7 percent per day in clotted and lithium heparin samples when stored as whole blood or serum (6.2-6.5 % per day) at room temperature. P-III-P did not change when the samples were stored at 4 degrees C. Although collection into EDTA inhibited the rise in P-III-P, the baseline measured values were significantly higher than in other media and spiking experiments demonstrated that EDTA exerted a significant matrix effect on the assay. While the optimum collection method is immediate centrifugation and storage at -80 degrees C, it would seem acceptable to store serum or clotted blood samples at 4 degrees C, but not ambient temperature, for up to five days [08247].

Monoclonal antibodies

Recombinant human growth hormone (rhGH) is abused in sports, but adequate routine doping tests are lacking. Analysis of serum hGH isoform composition has been shown to be effective in detecting rhGH doping. It was developed and validated selective immunoassays for isoform analysis with potential utility for screening and confirmation in doping tests. Monoclonal antibodies with preference for pituitary hGH (phGH) or rhGH were used to establish 2 pairs of sandwich-type chemiluminescence assays. It was analyzed specimens from volunteers before and after administration of rhGH. Functional sensitivities were <0.05 microg/L, with intra- and interassay imprecision ≤ 8.4 percent and ≤ 13.7 percent, respectively. In 20 recreational athletes, ratios (median) increased after a single injection of rhGH, reaching 350 percent and 400 percent of baseline ratios. At a moderate dose (0.033 mg/kg), mean ratios remained significantly increased for 18 h (men) and 26 h (women). After high-dose rhGH (0.083 mg/kg), mean ratios remained increased for 32-34 h in men and were still increased after 36 h in women. It was concluded that using sensitive chemiluminescence assays with preferential recognition of phGH or rhGH, detection of a single injection of rhGH was possible for up to 36 h [09195].

Electrophoresis and mass spectrometry

The heterogeneity of human endogenous growth hormone (GH) is used in doping control analysis to distinguish it from the homogeneous recombinant analogue in plasma samples. Pituitary GH variants were characterized by gel electrophoresis and mass spectrometry. Besides 22 and 20 kDa isoforms, fragments of 9 and 12 kDa were identified and a glycosylated 23 kDa GH variant was elucidated to bear a HexHexNac 2 NeuAc modification presumably located at Thr 60 [09196].
Immunoaffinity purification

Human growth hormone (GH) has several central metabolic functions including bone growth in childhood, and its anabolic and lipolytic effects in particular are assumed reasons for the abuse of GH by athletes. Human endogenous GH consists of a main 22 kDa variant and several isoforms. In contrast, recombinant GH consists of only one variant being identical to the main endogenous isoform. The method presented here separates different isoforms by 2-D PAGE after isolation of GH from plasma using an immunoaffinity purification system. While samples containing endogenous GH yield up to four isoforms, samples with recombinant GH contain the main 22 kDa spot only. Normalized spot volumes (NSV) are calculated after addition of an internal standard and a discrimination limit was determined at 0.52 for the NSV of the main 22 kDa spot. Above this value, samples containing endogenous GH show at least the main 22 kDa isoform and the 20 kDa splice variant. In contrast, samples with a NSV >0.52 and only one spot are suspicious to contain recombinant GH. This method detects discrete isoforms of GH from plasma and discriminates endogenous GH from its recombinant analog, which makes it useful for doping control purposes [08241].

Immunoassays

Detecting recombinant human growth hormone (rhGH) abuse in sport remains one of the major challenges in doping control. It was compared two different approaches to detect the hGH (human growth hormone) abuse. The first measures the concentrations of the 22 kDa hGH isoform (rec assay) and pituitary derived isoforms (pit assay) and a ratio rec/pit is obtained. The second measures the concentrations of 22 and 20 kDa hGH isoforms and also a ratio 22/20 kDa is derived. Using a single set (nine healthy male subjects, 7 days, 0.026 mg/kg/day of rhGH, 2 week wash out period) both approaches were compared. To quantify the agreement between the immunoassays, B.A. (Bland-Altman) analysis and P.r. (Pearson correlation) were used. To fully understand the assay readings, all relevant antibodies were characterised by surface plasmon resonance (SPR). In either approach the ratio numerator produces similar results and the denominator determines both signal-amplitude and time-frame of possible application. The rec vs pit approach displays a higher distinctive capacity to detect hGH abuse but the complex binding properties of the capture antibodies make it very difficult to evaluate the precise contributions of the individual hGH variants to the assay result. In the 22 vs 20 approach, the 20 kDa hGH concentration measures determine its applicability. Both approaches are based on a different principle, should be preferably applied within 24 h after rhGH administration, and are perfectly comparable given the results obtained. The reduced time frame of application indicates that their principle application should be preferably in an out-of-competition setting [12216].

Freeze-thaw cycling

A method based on two serum biomarkers – insulin-like growth factor-I (IGF-I) and pro-collagen type III N-terminal propeptide (P-III-NP) – has been devised to detect growth hormone (GH) misuse. The aims of this study were to determine the stability of IGF-I and P-III-NP concentrations in serum stored at -20°C and to establish the effects of one freeze-thaw cycle. Blood was collected from 20 healthy volunteers. Serum aliquots were analyzed after storage for one day at 4°C and one day, one week, five weeks, and three months at -20°C. IGF-I and P-III-NP results were combined to calculate a GH-2000 discriminant function score for each volunteer. Inter-assay precision was determined by analysing one quality control sample at each time-point. A single freeze-thaw cycle, storage of serum at 4°C for one day and at -20°C for up to three months had no significant effect on IGF-I or P-III-NP concentration. Intra-sample variability for IGF-I was 7 percent (Immunotech assay) and 13
percent (DSL assay). Intra-sample variability for P-III-NP was 11 percent (Cisbio assay) and 14 percent (Orion assay). When IGF-I and P-III-NP results were combined, intra-sample variability of the GH-2000 score expressed as a standard deviation varied between 0.31 and 0.50 depending on the assay combination used. Variability in IGF-I and P-III-NP results of stored samples is largely determined by the characteristics of the assays. A single freeze-thaw cycle, storage of serum at 4°C for one day or at -20°C for up to 3 months does not result in a significant change in GH-2000 score [12217].

Intra- and inter-laboratory validation

Insulin-like growth factor-II (IGF-II), insulin-like growth factor binding proteins (IGFBPs) -2 and -3 and C-terminal telopeptide of type I collagen (ICTP) have been proposed, among others, as indirect biomarkers of the recombinant human growth hormone misuse in sport. An extended intra- and inter-laboratory validation of commercially available immunoassays for biomarkers detection was performed. Although the majority of evaluated assays showed an overall reliability not always suitable for antidoping control analysis, relatively high concordances between laboratory results were obtained for all assays. Evaluated immunoassays were used to measure serum concentrations of IGF-II, IGFBP-2 and -3 and ICTP in elite athletes of various sport disciplines at different moments of the training season; in recreational athletes at baseline conditions and finally in sedentary individuals. Serum IGF-II was statistically higher both in recreational and elite athletes compared to sedentary individuals. Elite athletes showed lower IGFBP-2 and higher IGFBP-3 concentration with respect to recreational athletes and sedentary people. Among elite athletes, serum IGFBP-3 (synchronized swimming), and ICTP (rhythmic gymnastics) concentrations were sport-dependent. Over the training season, within athlete variability was observed for IGFBP-2 in case of taekwondo and IGFBP-2 and -3 in case of weightlifting. Variations due to those aspects should be taken in careful consideration in the hypothesis of setting reference concentration ranges for doping detection [08245].

Tests for GH deficiency

The diagnostic accuracy of tests used to diagnose GH deficiency (GHD) in adults is unclear. It was conducted a systematic review and meta-analysis of studies that provided data on the available diagnostic tests. It was searched electronic databases (MEDLINE, EMBASE, Cochrane CENTRAL, Web of Sciences, and Scopus) through April 2011. Review of reference lists and contact with experts identified additional candidate studies. Reviewers, working independently and in duplicate, determined study eligibility. Reviewers, working independently and in duplicate, determined the methodological quality of studies and collected descriptive, quality, and outcome data. Twenty-three studies provided diagnostic accuracy data; none provided patient outcome data. Studies had fair methodological quality, used several reference standards, and included over 1100 patients. Several tests based on direct or indirect stimulation of GH release were associated with good diagnostic accuracy, although most were assessed in one or two studies decreasing the strength of inference due to small sample size. Serum levels of GH or IGF1 had low diagnostic accuracy. Pooled sensitivity and specificity of the two most commonly used stimulation tests were found to be 95 and 89 percent for the insulin tolerance test and 73 and 81 percent for the GHRH plus arginine test respectively. Meta-analytic estimates for accuracy were associated with substantial heterogeneity. It was concluded that several tests with reasonable diagnostic accuracy are available for the diagnosis of GHD in adults. The supporting evidence, however, is at high risk of bias (due to heterogeneity, methodological limitations, and imprecision) [11474].
Ethics of use of growth hormone in sports

Athletes have enjoyed almost a thirty year amnesty of rhGH abuse, which they consider has contributed to the winning of medals and the breaking of world records. Such a reprieve is almost at an end, since WADA have identified a method to detect rhGH abuse. The anecdotal word "on the street" is that rhGH is still undetectable and athletes believe that the benefits, at the dosages they administer, far outweigh the risks! Scientists are aware that in a hormone deficiency condition, replacement can halt and in certain situations reverse some of the adverse effects. Growth hormone deficiency can lead to a loss of skeletal muscle mass and an increase in abdomino-visceral obesity, which is reversed on replacement with rhGH. Since the availability of GH, athletes have been trying to extrapolate these effects from the deficiency state to the healthy corpus and increase their sporting prowess. Past confessions from athletes, such as Ben Johnson, Kelly White, Tim Montgomery, Marion Jones and currently Dwain Chambers have demonstrated that they are prepared to tread the very fine lines that separate the "men from the boys". Rewards are so great, that anonymous surveys have identified that athletes will risk ill health, if they believe they can cheat, win and not get caught. The question that still needs to be answered is, "does growth hormone enhance performance"? Recent research suggests that it could. There is also a suspicion that in "cycled" low supraphysiological doses, it is no where near as harmful as WADA claim it to be [09203].

The hype about human growth hormone (hGH) goes far beyond the available data. The issue of administration of recombinant rhGH to adolescents, whether for athletic performance or for esthetic purposes adds another wrinkle because normal pubertal growth and the acquisition of adult body composition depends upon the GH/IGF-I and the hypothalamic-pituitary-gonadal axes. The range of normal is wide and it is more difficult to determine the natural evolution of pubertal development from that fueled by rhGH (or anabolic steroids). There are no compelling data based on clinical trials to indicate enhanced athletic performance (young adults); however, one cannot be sure how rhGH is used in combination with other agents, including anabolic steroids and anabolic "supplements". There are no studies in adolescent athletes. The detection of administration of rhGH to adolescents using the analytes of the GH/IGF-I and of collagen turnover (bone modeling and remodeling) is complicated by the physiologic increases in these parameters during normal puberty. It should be noted that many older adolescents are physiologically younger, especially in those aesthetic sports that demand the thin (linear) physique [09204].
The recent introduction of IGF-I to clinical practice is likely to increase its availability and abuse. Insulin and IGF-I work together with GH to control the supply of nutrients to tissues in the fasted and fed state. The actions of insulin and IGF-I that may enhance performance include increased protein anabolism and glucose uptake and storage. The detection of IGF-I and insulin abuse is challenging. There are established mass spectrometry methods for insulin analogs [10155].

There is significant evidence that athletes are using recombinant human growth hormone (rhGH) to enhance performance, and its use is banned by the World Anti-Doping Agency and professional sports leagues. Insulin-like growth factor-1 (IGF-1) is the primary mediator of growth hormone action and is used as a biomarker for the detection of rhGH abuse. The current biomarker-based method requires collection and expedited shipment of venous blood which is costly and may decrease the number of tests performed. Measurement of GH biomarkers in dried blood spots (DBS) would considerably simplify sample collection and shipping methods to allow testing of a greater number of samples regardless of location. A method was developed to quantify intact IGF-1 protein in DBS by liquid chromatography-tandem mass spectrometry. A step-wise acid-acetonitrile extraction was optimized to achieve a sensitive assay with a lower limit of quantification of 50 ng/mL. IGF-1 remained stable at room temperature for up to 8 days, which would allow shipment of DBS cards at ambient temperature. In a comparison between plasma concentrations of IGF-1 and concentrations measured from venous and finger prick DBS, there was good correlation and agreement, r (2) of 0.8551 and accuracy of 86-113 percent for venous DBS and r (2) of 0.9586 and accuracy of 89-122 percent for finger prick DBS. The method is intended for use as a rapid screening method for IGF-1 to be used in the biomarker method of rhGH abuse detection [12218].

It is believed that insulin and insulin-like growth factor I (IGF-I) are abused by professional athletes, either alone or in combination with growth hormone (GH) and anabolic steroids. The recent introduction of IGF-I to clinical practice is likely to increase its availability and abuse. Insulin and IGF-I work together with GH to control the supply of nutrients to tissues in the fasted and fed state. The actions of insulin and IGF-I that may enhance performance include increased protein anabolism and glucose uptake and storage. The detection of IGF-I and insulin abuse is challenging [10156].

The rationale for using rhIGF-I as an ergogenic aide differs little from that of rhGH. This compound has only recently become more available and its use in athletes will only increase. The potential benefits include increased muscle protein synthesis and the sparing of glycogenolysis with glycogen synthesis and increased fatty acid availability. IGF-I is the main effector for the action of hGH. Whereas hGH is insulin antagonistic several hours after ingesting a meal, the main effect of hIGF-I is to reduce glucose levels (insulin-like) and it is this effect that has been noted clinically. It is strongly anabolic in muscle, but has a very much diminished effect in comparison to hGH on lipids. In fact, children with virtually no IGF-I (growth hormone receptor deficiency, Laron-type) gain a disproportionate amount of fat when treated for many years with rhIGF-I [10001].

Performance-enhancing substances are illicitly used in elite or amateur sports and may be obtained from the black market due to a cheaper and easier availability. Although various studies have shown that black market products frequently do not contain the declared substances, enormous amounts of illegally produced and/or imported drugs are confiscated.
from athletes or at customs with alarming results concerning the outcome of the analyses of the ingredients. This case report describes the identification of His-tagged Long-R3-IGF-I, which is usually produced for biochemical studies, in an injection vial. The ingredients were isolated by immunoaffinity purification and identified by nano-UPLC, high-resolution/high accuracy mass spectrometry of the intact and trypsinated substance and by an enzyme-linked immunosorbent assay. (Tandem) mass spectra characterized the protein as Long-R3-IGF-I with a His(6)-tag attached to the C-terminus by the linker amino acids Leu-Glu was used. It was concluded that His-tags are commonly added to proteins during synthesis to allow a convenient and complete purification of the final product and His-tags are subsequently removed by specific enzymes when being attached to the N-terminus. The effects of His-tagged Long-R3-IGF-I in humans have not been elucidated or described and the product may rather be a by-product from biochemical studies than synthesized for injection purposes [10364].

Exercise stimulates growth hormone (GH) release, but there are conflicting reports regarding the acute effects of exercise on circulating ghrelin and insulin-like growth factor (IGF) concentrations. One investigation examined the effect of a single sprint on circulating GH, ghrelin and IGF concentrations as well as a marker of IGF-I bioactivity, and whether the number of muscle actions performed during a sprint influences these responses. Seven healthy men completed 3 trials in a random order. In two exercise trials they performed a single 30-s sprint on a cycle ergometer against a resistance equivalent to either 7 percent (FAST) or 9 percent (SLOW) of their body mass. In the other they rested in the laboratory (CON). Blood samples were taken pre-, immediately post-, 10 and 30 min post-exercise, and at equivalent times in the CON trial. Total ghrelin concentrations declined after the sprint and were significantly lower after 30 min of recovery than they were pre-exercise. GH concentrations increased in both exercise trials and were greater in the FAST than the SLOW trial. Serum concentrations of total IGF-I, free IGF-I, total IGF-II, and IGF-I bioactivity did not change after sprinting. In conclusion, sprint exercise suppresses total ghrelin concentrations and stimulates GH release but does not alter IGF concentrations or bioactivity [10365].

Possible performance enhancement is unknown at present as there just haven’t been studies in adults or children. rhIGF-I has been used as a growth promoting agent in children with both primary IGF-I deficiency and in a few genetic condition which are associated with short stature. It is quite early for the “efficacy” results, but growth rates have increased and it is the only presently available drug for those with complete growth hormone insensitivity. The major adverse event in children with growth hormone insensitivity has been hypoglycemia; however, most of the instances can be overcome with food ingestion around the time of the administration. Some unique side effects include jaw pain, headache, fluid retention and myalgia. As with rhGH there has been an incidence of idiopathic intracranial hypertension. Similar to the findings with rhGH stopping the drug for several days and perhaps restarting at one-half dose seems prudent in those who have experienced this adverse event. In the longer term there is the theoretical aspect of tumorigenesis, although no data exist, other than associative ones for patients with certain cancers (for example, breast, prostate, and colon) have had higher IGF-I levels in the years before their cancers became detected [10001].

Insulin like growth factor-I (IGF-I), the prime target of growth hormone action, is one candidate gene for improving performance. In recent years a number of transgenic and somatic gene transfer studies on animals have shown that upregulation of IGF-I stimulates muscle growth and improves function. This increase in muscle IGF-I is not reflected in measurable increases in circulating IGF-I. Whilst the responses obtained in the animal studies would appear to give clear benefits for performance, the transfer of such techniques
to humans still presents many technical challenges. Further challenges will also be faced by the anti doping authorities in detecting the endogenously produced products of enhanced gene expression [10157].

As the tests for detecting growth hormone (GH) abuse develop further, it is likely that athletes will turn to doping with insulin-like growth factor-I (IGF-I). IGF-I mediates many of the anabolic actions of growth hormone. It stimulates muscle protein synthesis, promotes glycogen storage and enhances lipolysis, all of which make IGF-I attractive as a potential performance-enhancing agent. Pharmaceutical companies have developed commercial preparations of recombinant human IGF-I (rhIGF-I) for use in disorders of growth. The increased availability of rhIGF-I increases the opportunity for athletes to acquire supplies of the drug on the black market. The long-term effects of IGF-I administration are currently unknown but it is likely that these will be similar to the adverse effects of chronic GH abuse. The detection of IGF-I abuse is a challenge for anti-doping organisations. Research has commenced into the development of a test for IGF-I abuse based on the measurement of markers of GH action. Simultaneously, the effects of rhIGF-I on physical fitness, body composition and substrate utilisation in healthy volunteers are being investigated [09213].

Human insulin-like growth factor-1 (IGF-1) is a peptide hormone that acts as a mediator of most of the somatotropic effects of growth hormone (GH). Therefore, it is supposed to be a biomarker indicating GH abuse in sports as well as diseases associated with a change in IGF-1 plasma concentration. It can be applied locally by injection to increase total protein and DNA content in tissues such as skeletal muscle – a highly desirable effect in various sports disciplines. In order to improve its growth-promoting properties, the primary structure of IGF-1 has been modified, yielding analogues such as des(1-3)IGF-1 and LONGR3IGF-1, which show a considerably reduced affinity to the respective binding proteins in plasma and, thus, an increased bioavailability at target tissues. Due to their capability to enhance performance, IGF-1 and its analogues belong to the prohibited list of the World Anti-Doping Agency. Hence, it was necessary to develop a reliable assay for the quantification of human IGF-1 as well as the detection of its derivatives. Immunoaffinity isolation and purification from 60 microL of plasma followed by liquid chromatography/electrospray ionisation tandem mass spectrometry enabled the unequivocal determination of all target analytes. Diagnostic product ions were characterised utilising an Orbitrap mass spectrometer with high resolution/high accuracy properties and employed for triple quadrupole MS/MS analysis. The described assay provided lower limits of detection (LLODs) between 20 and 50 ng/mL, recovery rates between 34-43 percent and a precision <15 percent at the lower limits of detection as well as higher concentration levels. In order to prove the applicability of the developed assay, human plasma samples were analysed and the results were compared with the values obtained from a commercially available immunoradiometric assay (IRMA). Four of six samples resulted in concentration ratios with good correlation between both assays, whereas the absolute concentrations were lower for the presented procedure [08260].

Skeletal muscle is highly adaptive to environmental stimuli and can alter its mass accordingly. This tissue is almost unique in that it can increase its size through two distinct mechanisms. It can grow through a cellular process mediated by cell fusion, or it can increase its size simply by increasing its protein content. Understanding how these processes are regulated is crucial for the development of potential therapies against debilitating skeletal muscle wasting diseases. Two key signalling molecules, Insulin like Growth Factor (IGF) and GDF-8/myostatin, have emerged in recent years to be potent regulators of skeletal muscle size. In this review we bring together recent data highlighting the important and novel aspects of both molecules and their signalling pathways, culminating in a discussion of the cellular and tissue phenotypic outcomes of their stimulation or
antagonism. It was emphasise the complex regulatory mechanisms and discuss the temporal and spatial differences that control their action, understanding of which is crucial to further their use as potential therapeutic targets [10480].

The effect of exercise training on anabolic hormones and inflammatory mediators is particularly important during childhood and puberty, since during this period there is a spontaneous increase in anabolic hormones that leads to the marked puberty-related growth spurt. Therefore, any training-associated hormonal and/or inflammatory effect during this critical period may have profound consequences on growth and development, especially if the effect is maintained for long periods. Several studies suggest the hypothesis that a sudden imposition of a training program which is associated with substantial increase in energy expenditure leads initially to an increase in pro-inflammatory cytokines, and as a consequence, to decreases in IGF-1 levels. Further, if the training adaptation is successful, the proinflammatory cytokines fall, and with that decrease, the suppression of IGF-1 diminishes, an anabolic ‘rebound’ in the GH-IGF-1 axis may ensue, and IGF-1 level exceed the pretraining level. Exactly how and when this switch takes place, and whether the initial catabolic-type stage is necessary for the ultimate anabolic adaptation remains unknown. Consistent with the two phases hypothesis, longer periods of training were indeed associated with stable or with increases in circulating GH and IGF-1 levels. Despite the early training-associated decrease in circulating IGF-1 levels, there is an increase in muscle mass and fitness may improve, suggesting that the local tissue effect of exercise on growth factors differ from systemic effects. Total caloric intake as well as macronutrient content, consumption and timing influence the anabolic and inflammatory response to training. Finally, changes in the balance of anabolic and catabolic hormones and inflammatory mediators during the training season may help elite athletes and their coaches in their training and preparation for competition [10481].

Growth hormone (GH) regulates important physiological processes, including somatic growth and development as well as muscle protein synthesis and carbohydrate and lipid metabolism, directly by binding to and activation of its receptors or indirectly by stimulation of IGF1. Several pathways contribute to initiate myogenesis in response to different extracellular signals. The PI3K/Akt pathway, activated by IGF-1, the stress activated MKK6/p38 MAPK pathway and the calcium/calmodulin-dependent kinase (CaMK) pathway promote muscle-specific gene expression. In myogenesis, IGF-1 and myostatin are key signalling molecules and control skeletal muscle mass. IGF-1 is a positive and myostatin is a negative regulator. IGF-1 induces satellite cell proliferation, myoblast differentiation and subsequent myoblast fusion into myotubes. Knock-out mice of IGF-1 or its GH receptor show increased growth retardation and reduced organ and skeletal muscle mass, while overexpression of IGF-1 within skeletal muscle causes significant myofibre hypertrophy and myoblast proliferation. Investigational studies have demonstrated that acute exercise increases circulating IGF-1 levels in blood. On the other hand, mice that lacked myostatin, a member of the transforming growth factor beta superfamily of secreted growth and differentiation factors, were found not only to be stronger and more muscular than their counterparts with normal myostatin levels, but also to have reduced fibrosis and fatty remodelling, suggesting improved regeneration of muscle. Abuse of GH is widespread; overdose of GH leads to increased muscle protein synthesis along with increased fatty acid availability and sparing of glycogen stores. Most of these anabolic effects of GH are mediated by IGF-1. Thus, based on their attributes which make GH and IGF-1 attractive as potential performance-enhancing agents, they are often used as doping substances by athletes. Approximately every fourth sportsman who uses anabolic androgenic steroids also takes these both drugs. As measured by maximal oxygen uptake, the combination of GH and testosterone improves the fitness, which is larger than with one compound alone. Anabolic androgenic steroids stimulate hypertrophy and mass of muscle fibres by regulation of
satellite cell proliferation, the number of myonuclei and the balance of muscle proteins. The exact biochemical mechanisms responsible for increased skeletal muscle mass and strength by anabolic androgenic steroids are partly unknown. In myoblast culture systems, testosterone has been shown to be able to stimulate the mitotic activity of satellite cells, and IGF-1 signalling pathway plays an important role in mediating these effects of testosterone on skeletal muscle cell growth and differentiation. Following anabolic steroid administration, increased expression of IGF-1 together with alterations in expression of several IGF-1 binding proteins has been observed in muscle. Hence, at least in part the muscle IGF-1 seems to mediate the growth-promoting influences of anabolic steroids. Up-regulation of GH in hepatic chromatin induced rapid and dramatic changes at the IGF-1 locus and activated IGF-1 gene transcription by distinct promoter-specific mechanisms. Alterations in the IGF-1 axis, which predispose individuals to diseases, have been reported to be due to an altered epigenetic regulation that can modify IGF-1 transcript levels. Systemic GH treatment caused an approximately robust increase in transcription from each IGF-1 promoter within an hour of hormone administration and led to a sustained accumulation of IGF-1 mRNA. The coordinated induction of both IGF-1 promoters by GH was accompanied by hyperacetylation of histones H3 and H4 in promoter-associated chromatin, a decline in monomethylated lysine 4 of histone H3 (H3K4), and recruitment of RNA polymerase II to IGF-1 promoter 2. These findings show that GH may regulate the transcript levels of IGF-1 by modifying the chromatin architecture. The molecular mechanism underlying the improved muscle performance by the action of IGF-1 is not well understood. It is speculated that IGF-1 may modulate genomic methylation to promote normal development of muscles. Investigations on cell cultures showed that after a 6-hour exposure of cells, IGF-1 increased the global DNA methylation. This change in the nuclear methylation pattern is based on the fact that IGF-1 is able to stimulate the activity of methionine synthase via a mechanism that requires the activity of both PI3 kinase and MAP kinase pathways. Moreover, during early embryonic development the occurrence of IGF-1 may affect the DNA methylation pattern allowing the activation of expression of particular genes important for embryonic survival. Consequently, the administration of IGF-1 as a doping agent might also affect DNA methylation in athletes. However, the long-term intakes of GH or IGF-1, along with their effects on DNA methylation signal transductions or modulations of gene expression, are currently unknown. Due to the consensus statement of the Growth Hormone Research Society there seems to be no increased risk of cancer when GH is given at physiological replacement doses, but there is mounting evidence that the intake of GH higher than physiological doses for many years may be associated with increased incidences of colorectal, thyroid, breast, and prostate cancers. In these cancer patients, high levels of circulating IGF-1 were detected in serum and have been associated with cancer risk and cancer prognosis. Drug candidates that target IGF1 signal transduction have revealed anti-neoplastic activity. Moreover, the signalling pathway of IGF-1 is involved in tumour development and progression. Experiments have shown that cancer cells have an increased expression of IGF-1 receptors. Activation of IGF-1 receptor, an inducer of Akt and MAPK signalling networks, by IGF-1 has mitogenic and anti-apoptotic effects in neoplastic tissue. Thus, IGF-1 may provide an anti-apoptotic environment that may favour survival of cancer cells. Regarding the global chromatin changes during the malignant transformation of cells and the involvement of IGF-1 in DNA methylation, it is equitable to speculate that the increased abuse of GH and IGF-1 may at least partly contribute to the tumour-associated epigenetic changes. However, too little data are available to sustain this theory. Future investigations are warranted to clarify the implication of DNA methylation in doping increasing cancer risk. Recent findings have indicated that IGF-1 may play a role in neoplasia. Although the extent of DNA methylation of IGF-1 gene was highly variable in most tumours as its mRNA expression levels, a relationship could be detected between IGF-1 overexpression and gene demethylation in hepatocarcinomas and tumours associated with hypoglycaemia. In multiple myeloma IGF-1 mediated down-regulation of the proapoptotic BH3-only protein Bim by the activation of Akt and MAPK pathway. It has been reported that
IGF-1 is involved in epigenetic regulation of the Bim promoter. Chromatin immunoprecipitation of IGF-1 showed both a reduced acetylation of lysine 9 of histone H3 (H3K9) and an increased H3K9 dimethylation, which contributed actively to the silencing of BIM. These data identified a new mechanism in the IGF-1 dependent survival of multiple myeloma cells. Using chromatin immunoprecipitation assays, histone methyltransferase RIZ1 was shown to bind to the IGF-1 promoter and to increase histone H3 lysine 9 methylation. In chronic myelogenous leukemia (CML), blastic transformation is associated with decreased expression and activity of RIZ1. Overexpression of RIZ1 in model CML blast crisis cell lines decreased proliferation, increased apoptosis and supported differentiation which was accompanied with a reduced IGF-1 receptor activation and activation of downstream signalling components ERK1/2 and AKT. These data highlight the therapeutic potential of inhibiting IGF-1 signalling pathway by modulating the histone pattern at IGF-1 promoters [11475].

There is significant evidence that athletes are using recombinant human growth hormone (rhGH) to enhance performance, and its use is banned by the World Anti-Doping Agency and professional sports leagues. Insulin-like growth factor-1 (IGF-1) is the primary mediator of growth hormone action and is used as a biomarker for the detection of rhGH abuse. The current biomarker-based method requires collection and expedited shipment of venous blood which is costly and may decrease the number of tests performed. Measurement of GH biomarkers in dried blood spots (DBS) would considerably simplify sample collection and shipping methods to allow testing of a greater number of samples regardless of location. A method was developed to quantify intact IGF-1 protein in DBS by liquid chromatography-tandem mass spectrometry. A step-wise acid-acetonitrile extraction was optimized to achieve a sensitive assay with a lower limit of quantification of 50 ng/mL. IGF-1 remained stable at room temperature for up to 8 days, which would allow shipment of DBS cards at ambient temperature. In a comparison between plasma concentrations of IGF-1 and concentrations measured from venous and finger prick DBS, there was good correlation and agreement. The method is intended for use as a rapid screening method for IGF-1 to be used in the biomarker method of rhGH abuse detection [13376].

To athletes, insulin-like growth factor-1 (IGF-I) is an attractive performance-enhancing drug, particularly as an alternative to growth hormone (GH) because IGF-I mediates many of the anabolic actions of GH. IGF-I has beneficial effects on muscle protein synthesis and glycogen storage that could enhance performance in several sporting disciplines. Recombinant human IGF-I (rhIGF-I) is used in clinical practice, but a variety of IGF-I compounds and IGF-I analogues are also advertised on the internet and many have been available on the black market for several years. Although methods for detecting GH misuse are now well established and there have been several cases in which athletes have tested positive for GH, no test is yet in place for detecting IGF-I misuse. The GH-2004 research group has been investigating methods for detection of IGF-I misuse and a test is being developed on the basis of the principles of the successful GH-2000 marker method, in which markers from the IGF axis and markers of collagen and bone turnover are used to detect GH misuse. Commercial immunoassays for these markers have been validated for anti-doping purposes but new methods, including IGF-I measurement by use of mass spectrometry, should improve the performance of the tests and help in the detection of athletes who are doping with these peptide hormones [13377].

Besides its function as biomarker, IGF-1 itself and its synthetic derivatives are prohibited substances according to the regulations of WADA. Although IGF-1's mechanism of action concerning improved athletic performance is yet not fully understood, epigenetic aspects
have been discussed and reviewed along with serious side effects attributed to long-term abuse of hGH and IGF-1. In order to unambiguously detect at least synthetic analogs of IGF-1 in urine multiplexed with other drugs and metabolites relevant for doping controls, a multi-analyte peptide screening assay was developed, allowing for the determination of IGF-1 and long-R3-IGF-1 as well as six insulins (animal, human, and synthetic), LH releasing hormone (LH-RH), growth hormone releasing hormone (GH-RH) and its synthetic analog CJC-1295, and synacthen. From both matrices, plasma and urine, detection limits between 1 and 50 pg/ml were accomplished, enabling the unequivocal detection of these analytes in doping control samples. A consideration that at least synacthen and long-R3-IGF-1 have demonstrated limited stability (approx. 24 h) in urine samples stored at +4°C; here, frozen conditions are highly recommended. The relevance of growth hormone releasing peptides (GHRPs) as well as new analytical techniques including ion mobility have further been reviewed in the context of sports drug test protocols; the use of ion mobility particularly can complement existing strategies in terms of substantiated analytical results (by adding another characterizing dimension, e.g. drift time of the compound of interest) as well as speed of analysis [13012].

To athletes, insulin-like growth factor-I (IGF-I) is an attractive performance-enhancing drug, particularly as an alternative to growth hormone (GH) because IGF-I mediates many of the anabolic actions of GH. IGF-I has beneficial effects on muscle protein synthesis and glycogen storage that could enhance performance in several sporting disciplines. Recombinant human IGF-I (rhIGF-I) is used in clinical practice, but a variety of IGF-I compounds and IGF-I analogues are also advertised on the internet and many have been available on the black market for several years. Although methods for detecting GH misuse are now well established and there have been several cases in which athletes have tested positive for GH, no test is yet in place for detecting IGF-I misuse. The GH-2004 research group has been investigating methods for detection of IGF-I misuse and a test is being developed on the basis of the principles of the successful GH-2000 marker method, in which markers from the IGF axis and markers of collagen and bone turnover are used to detect GH misuse. Commercial immunoassays for these markers have been validated for anti-doping purposes but new methods, including IGF-I measurement by use of mass spectrometry, should improve the performance of the tests and help in the detection of athletes who are doping with these peptide hormones [13378].

In the absence of analytical proof, speculations as to the prevalence of IGF-1 as doping agent have arisen, particularly with regard to elite sprinters, and also deer antler velvet-derived nutritional supplements enriched with IGF-1 were recently reported. In order to provide a platform for the generation of factual information on peptide hormone-based therapeutics and drug candidates, metabolism studies and method development/expansion for substances such as growth hormone releasing peptides (GHRPs, including GHRP-1, -2, -4, -5, and 6, as well as hexarelin, alexamorelin, and ipamorelin), luteinizing hormone releasing hormone (LHRH), and desmopressin were conducted. In a comprehensive rat in vivo study, the metabolic fate of eight GHRPs was investigated following oral as well as intravenous administration. Urinary metabolites were identified by UHPLC-high resolution/high accuracy (tandem) mass spectrometry, and three metabolites per GHRP were characterized being potential target compounds for routine doping controls. In subsequent human serum in vitro incubations, the production of the main metabolites was also observed, corroborating the utility of these analytes as viable target compounds in sports drug testing. With the increasing number of potential peptidic drugs, detection assays have to be extended and updated to cover these additional substances [13009].
Polymorphism

Insulin-like growth factor-I (IGF-I) plays a key role in exercise-associated muscle growth and development. The regulatory region of the promoter of the IGF-I gene is labile, but changes in this region were studied mostly in the elderly and in relation to pathological states. C-1245T (rs35767) is a genetic variation in the promoter region of the IGF-I gene. The minor allele T was found to be associated with higher circulating IGF-I levels, and possibly with increased muscle mass. The aim of one study was to analyze the frequency distribution of C-1245T SNP in athletic and nonathletic Israeli populations. One hundred and sixty-five athletes (78 endurance-type athletes, and 87 power-type athletes) and 159 nonathletic healthy individuals participated in the current study. Genomic DNA was extracted from peripheral EDTA treated anti-coagulated blood using a standard protocol. Genotyping of the IGF1 C-1245T polymorphism was performed using polymerase chain reaction (PCR). It was found that the endurance and power athletes’ allele and genotype frequencies were significantly different from those of the control group. Only 5 percent of the athletes were TT carriers, but none of the controls carried this genotype. The T allele was found to be more frequent in the top-level power athletes (international and Olympic level) compared to national level athletes, but such a difference was not found in endurance athletes. The findings suggest a possible contribution for the relatively rare IGF-I TT genotype to endurance performance, and in particular to power sport excellence in Israeli athletes [13379].

Effect of substitution with GH

Indirect biomarkers of recombinant human growth hormone (rhGH), insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), insulin-like growth factor binding proteins (IGFBP-2 and IGFBP-3) and insulin (C-peptide) were measured together with urinary parameters of renal damage (beta2-microglobulin and proteinuria) by immunoassays, in house validated for the purpose, in 61 subjects (36 elite athletes, 18 recreational athletes and 7 sedentary individuals) with different levels of physical fitness and endurance exercise. Validation parameters were good for the evaluated assays, excluding a high inter-assay imprecision and inaccuracy of 24 and 26 percent obtained for GH assay. The range of
concentrations found in urine samples under investigation was generally covered by the calibration curves of the studied immunoassays. However, for the samples below or above the calibration curve, opportune dilution or concentration were performed. Particularly, C-peptide samples had to be diluted 1:5 and beta2-microglobulin ones assayed using a triple sample volume, to fall within the calibration range. Urinary C-peptide was the only biomarker statistically higher in samples of elite athletes when compared to recreational athletes and sedentary individuals. Among elite athletes, tae-kwon-do athletes showed the highest IGF-II basal values while weightlifting athletes showed the lower IGF-I and IGFBP-3 basal values. The trend observed in weightlifters' basal samples was confirmed in their training samples: IGF-I, IGF-II, IGFBP-3 and beta2-microglobulin were lower in with respect to those from synchronised swimming. Over the training season, within athlete variability was observed for IGFBP-3 for weightlifting athletes. In the studied subjects, no direct associations were found between biomarkers of GH or insulin misuse and urinary parameters of renal damage, eventually due to high-workload endurance training. The variations observed in different biomarkers should be taken in consideration in the hypothesis of setting reference concentration ranges for doping detection [10361].

**Physiology**

The offer of human saliva IGF-I (sIGF-I) measurement in athletes investigation is a new proposal. The aim was now to investigate the physical exercise effect on sIGF-I and explore plasma free IGF-I relation. Saliva and blood were collected from well-trained athletes, investigated immediately before and at the end of a physical exercise test. sIGF-I was significantly increased at the end of the physical exercise. The plasma free IGF-I concentrations did not demonstrate any difference. The saliva total protein level (sTP) was also significantly increased. A positive correlation between sTP and sIGF-I, was observed, both before and after physical exercise, and between salivary and plasma free IGF-I only after physical exercise. The salivary free IGF-I level significantly increased after physical exercise, moreover a correlation with the plasma levels exists in post-exercise condition. The physical exercise affects sIGF-I as well as the sTP. The correlation between plasma and salivary free IGF-I levels only in post-exercise condition suggests further studies to investigate the effects of different type and duration of physical exercise. The comparison with other salivary biochemical parameter investigation would also further increase comprehension on the role of salivary IGF-I [09214].

The aim of one study was to investigate possible relationships between different right-hand finger-length ratios and different fasting hormone concentrations in young swimmers. Fifty-five young swimmers participated in this study (26 boys and 29 girls, aged 10-17 years). All finger-length ratios were significantly higher in girls compared with boys. Ghrelin, leptin, testosterone in boys, estradiol in girls, insulin-like growth-factor I (IGF-I), IGFBP-3, and insulin were analyzed. Leptin and insulin concentrations were lower in boys compared with girls. In both groups, the relationships between finger-length ratios and basic anthropometric parameters were not significant. In conclusion, ghrelin appears to be a further biochemical parameter in addition to the sex steroids which correlated with different digit-length ratios at least in boys [08243].

**In peripubertal females**

Intense physical activity in peripubertal girls may delay menarche and cause menstrual disorders and estrogen deficiency, particularly in sport disciplines that require strict weight
control. It may also have a deleterious effect on bone mass acquisition. The aim of one study was to determine the time-course of bone mass accretion in peripubertal elite female rhythmic gymnasts over a 1-year period, as well as the anthropometric and hormone parameters that could be helpful for predicting bone mineral density (BMD) gain. It was conducted a 1-year follow-up study in 29 female rhythmic gymnasts (11-16 years old). Whole body composition and BMD of the whole body, proximal femur, lumbar spine, mid-radius, and skull were measured by dual energy X-ray absorptiometry (DXA). Moreover, baseline growth- and bone metabolism-related hormones such as IGF1, IGF-binding protein 3 (IGFBP3), leptin, and bone markers were measured. BMD increased significantly at all bone sites throughout puberty, particularly between Tanner stages II and IV-V. The IGF1 level, IGF1/IGFBP3 ratio, and leptin level were higher in late pubertal stages (i.e. IV-V) compared with early stage (i.e. I). In simple and multivariate analyses, only the IGF1/IGFBP3 ratio was strongly correlated with the BMD change at all bone sites. It was concluded that the plasma IGF1/IGFBP3 ratio was associated with bone mass acquisition in this period, and it may thus serve as a surrogate marker of bone mass gain in this population [10158].

**Effects of training**

Increased concentrations of circulating insulin-like growth factor-I (IGF-I) or IGF-I relative to IGF-binding proteins (IGFBPs) are associated with increased risk of developing several forms of cancer. Conversely, exercise is linked with reduced risk. This study aims to investigate the effect of a low-intensity exercise program on circulating levels of IGF-I, IGFBP-1, and IGFBP-3, in previously sedentary males. Fourteen healthy men participated in cycle ergometer training at lactate threshold intensity for 60 min/day, 5 days/week for 6 weeks. After aerobic training, insulin sensitivity improved by 20 percent, while fasting insulin levels decreased by 13 percent. Simultaneously, low-intensity aerobic training decreased the circulating levels of IGF-I by 9 percent, while IGFBP-1 levels increased by 16 percent. An interesting finding was that higher pretraining level of IGF-I was associated with greater decline in IGF-I with training. Insulin-sensitizing low-intensity aerobic exercise is thus considered to be an effective method for downregulating IGF-I and upregulating IGFBP-1 levels [10366].

One study examined the impact of 4 weeks of either complete cessation of training (DTR) or a tapering period (TAP; short-term reduction of the strength training volume, while the intensity is kept high), subsequent to 16 weeks of periodized heavy resistance training (PRT) on strength/power gains and the underlying physiologic changes in basal circulating anabolic/catabolic hormones in strength-trained athletes. Forty-six physically active men were matched and randomly assigned to a TAP (n=11), DTR (n=14), or control group (C; n=21), subsequent to a 16-week PRT program. Muscular and power testing and blood draws to determine basal hormonal concentrations were conducted before the initiation of training (T0), after 16 weeks of training (T1), and after 4 weeks of either DTR or TAP (T2). Short-term DTR (4 weeks) results in significant decreases in maximal strength (-6 to -9 %) and muscle power output (-17 and -14 %) of the arm and leg extensor muscles. However, DTR had a significant larger effect on muscle power output more than on strength measurements of both upper and lower extremity muscles. Short-term (4 weeks) TAP reached further increases for leg (2 %) and arm (2 %) maximal strength, whereas no further changes were observed in both upper and lower muscle power output. Short-term DTR resulted in a tendency for elevation resting serum insulin-like growth factor (IGF)-1 concentrations, whereas the corresponding TAP experienced elevation in resting serum insulin-like binding protein-3 (IGFBP-3). These data indicated that DTR may induce larger declines in muscle power output than in maximal strength, whereas TAP may result in further strength enhancement.
(but not muscle power), mediated, in part, by training-related differences in IGF-1 and IGFBP-3 concentrations [07156].

The purpose of the research was to compare changes in circulating levels of total IGF-I and IGFBP-3 during continuous, moderate-intensity exercise (CE) and high-intensity interval exercise (IE) of equal duration. Ten healthy males completed 2 exercise sessions and a resting control session (R) in random order. The CE was 20 minutes of cycling at 60-65 percent of VO$_{2\text{max}}$. During IE, subjects cycled at 80-85 percent of VO$_{2\text{max}}$ for 1 minute followed by 40 seconds of active recovery, with the cycle repeated for a total of 20 minutes. In each session blood samples were drawn at -10, 0, 5, 10, 20 and 30 minutes. Both IGF-I and IGFBP-3 increased significantly during exercise and repeated measures ANOVA revealed a significant effect for session. Area under the curve (AUC) analyses showed no difference in IGF-I between sessions, however, the IGFBP-3 AUC was significantly greater during IE than R. These results suggest interval and continuous exercise will result in similar changes in circulating IGF-I and IGFBP-3. This could be beneficial to individuals who can exercise longer and at a higher intensity in intervals than would be possible using a continuous protocol [07157].

**Resistance exercise**

Does resistance exercise order affect hormone availability? Participants performed arm exercise before and after leg exercise. Hormone delivery was estimated by multiplying brachial artery blood flow and hormone concentrations. Blood flow increased after arm (276 %) and leg (193 %) exercise. Testosterone, growth hormone, and insulin-like growth factor 1 showed with distinct delivery patterns between conditions; however net exposure was similar. The anabolic potential of postexercise hormones was not affected by exercise order [13381].

One study examined the effects of short-term physical training on the acute hormonal response (i.e. growth hormone, total and free insulin-like growth factor I [IGF-I], and IGF binding proteins [IGFBP]-1, IGFBP-2, and IGFBP-3) to resistance exercise (RE) in women. Forty-six women (20) were randomly assigned to an endurance training (E), resistance training (R), combined training (R + E), or control (C) group for 8 weeks. Subjects completed a standardized bout of RE (six sets of back squats at 10 repetition maximum) before and after training. Blood samples were obtained at rest (PRE), after the third set, immediately postexercise (POST), and at 15 min and 30 min after exercise. Acute RE significantly increased serum growth hormone, total IGF-I, IGFBP-1, IGFBP-2 and IGFBP-3 concentrations and decreased free IGF-I concentrations. After 8 weeks of training, total IGF-I concentrations were significantly increased, and IGFBP-1 concentrations were significantly decreased during exercise in groups that participated in resistance training (R and R + E); no significant changes were seen after E or C. In conclusion, participation in resistance training increased total IGF-I and reduced IGFBP-1 concentrations during acute RE, indicating exercise mode-specific adaptations in the circulating IGF-I system [13382].

One study was designed to determine endocrine responses during 2 days of strenuous resistance training. Ten healthy men performed resistance training twice a day for two successive days to induce acute fatigue (excessive physical stress). The resistance training consisted of four exercises for the lower body in the morning and seven exercises for the upper body in the afternoon. Maximal isometric and isokinetic strengths were measured from day 1 (before the training period) to day 3 (after the training period). Fasting blood samples were taken on days 1-3. Maximal isometric and isokinetic strengths significantly decreased with two successive days of training, with significant increases in serum creatine phosphokinase and myoglobin concentrations. Significant reductions in the fasting
concentrations of serum insulin-like growth factor-1, free testosterone, insulin and high-molecular-weight adiponectin were observed on day 3, whereas there were no changes in the serum cortisol concentration or the free testosterone/cortisol ratio. Plasma active ghrelin and serum leptin concentrations decreased by -20.7 ± 2.8 and -29.6 ± 4.1 percent, respectively. Two days strenuous resistance training significantly affects the profiles of anabolic hormone and endocrine regulators of appetite and energy balance, such as ghrelin and leptin. The present findings suggest that decreased ghrelin and leptin concentrations might reflect excessive physical stress and may be early signs of accumulated fatigue [13383].

Training with calorie restriction and sleep deprivation

The pulsatile release of growth hormone (GH) and luteinizing hormone (LH) from the anterior pituitary gland is integral for signaling secretion of insulin-like growth factor (IGF)-I and testosterone, respectively. One study examined the hypothesis that 84 h of sustained physical exertion with caloric and sleep restriction alters the secretion of GH and LH. Ten male soldiers (22 years 183 cm, 87 kg) had blood drawn overnight from 1800 to 0600 every 20 min for GH, LH, and leptin and every 2 h for IGF-I (total and free), IGF binding proteins-1 and -3, testosterone (total and free), glucose, and free fatty acids during a control week and after 84 h of military operational stress. Time-series cluster and deconvolution analyses assessed the secretion parameters of GH and LH. Significant results were as follows: body mass (-3 %), fat-free mass (-2 %), and fat mass (-7 %) declined after military operational stress. GH and LH secretion burst amplitude (approximately 50 %) and overnight pulsatile secretion (approximately 50 %), IGF binding protein-1 (+67 %), and free fatty acids (+33 %) increased, whereas leptin (-47 %), total (-27 %) and free IGF-I (-32 %), total (-24 %) and free testosterone (-30 %), and IGF binding protein-3 (-6 %) decreased. GH and LH pulse number were unaffected. Because GH and LH positively regulate IGF-I and testosterone, these data imply that the physiological strain induced a certain degree of peripheral resistance. During periods of energy deficiency, amplitude modulation of GH and LH pulses may precede alterations in pulse numbers [06141].

The endocrine response to sustained physical exertion superimposed on caloric and sleep restriction serves to provide adequate fuels to meet metabolic needs for such physiological processes as tissue repair, regeneration, and recovery. In particular, the somatotrophic (i.e. growth hormone and insulin-like growth factor) and pituitary-testicular (i.e. luteinizing hormone and testosterone) axes mediate many metabolic and anabolic processes during altered energy states. The pulsatile release of GH and LH from the anterior pituitary gland is integral for the trophic effects that these hormones exert on IGF-I and testosterone, respectively. The manner in which anterior pituitary hormones are released and delivered to target tissues is more biologically significant than the overall mean hormonal concentrations. It was demonstrated that GH administered in a pulsatile manner elicited greater increases in IGF-I than GH administered in a continuous manner. Because of the episodic release pattern of GH and LH, multiple time-point measures are necessary to fully characterize the trophic effects these hormones may have. Although alterations in IGF-I and testosterone are known to be mediated by fluctuations in GH and LH, few studies have concomitantly measured these hormones, especially in healthy subjects exposed to multiple physiological stressors. Additionally, recent work has suggested that leptin may also serve as an integrator of neuroendocrine secretion of GH and LH. Information concerning the pulse parameters (i.e. frequency, amplitude, etc) of GH and LH is important to understand when delineating central vs. peripheral mechanisms underlying alterations in circulating endogenous hormones. Although prior pulsatility programs were limited in that they provided only descriptive information about hormone concentration peaks, recent advances in time series software analysis now allow accurate determination of underlying glandular hormonal secretion rate
and halflife from measurements obtained from the systemic circulation [06141].

Of the IGF-I system components measured, IGFBP-1 demonstrated the largest magnitude change at training. It has been speculated that the IGFBP-1 increase may be viewed as a protective mechanism by neutralizing the insulin-like hypoglycemic activity of IGF-I and serving to mobilize energy from fats and protein. Furthermore, it has been suggested that low levels of liver glycogen are linked to an increased secretion of IGFBP-1. IGFBP-1 remains an important regulatory BP to study relative to physiological stress as increased levels may be one mechanism whereby IGF-I bioactivity is adjusted to meet metabolic needs [06141].

In Kenyans

Overnight fasting blood plasma insulin-like growth factor-I (IGF-I), insulin-like growth factor binding protein-1 (IGFBP-1), coenzyme Q10, (CoQ) vitamin E and plasma lipids were compared between a semi-nomadic Samburu population and relatively urbanized cohorts from Nairobi, Kenya. 143 middle aged subjects without known diabetes were included. IGF-I and IGFBP-1 were analyzed by RIA, and CoQ and vitamin E by HPLC. Plasma lipid levels were analyzed by standard laboratory methods routinely used in the clinics. The age adjusted IGF-I serum levels were low in the Samburu male and female populations, ranging from 0 to -4 IGFSD-score, and a minor part of the investigated population reaching as low as -5 and -7 SDS. The Nairobi cohorts showed significantly higher values reaching from -2.5 to +1 SDS. The nomadic Samburu population showed fasting IGFBP-1 values ranging from 30-100 microg/L, while that of the urbanized Nairobi cohorts was considerably lower (25-60 microg/L) (P<0.0001). CoQ concentrations of the Nairobi cohorts were 1.5-2.0 nmol/mL similar to the levels found in several European countries. The Samburu population on the other hand showed extremely high CoQ values ranging from 2 to 9 nmol/mL. Vitamin E levels of the Nairobi group were low (5-20 nmol/mL), but the Samburu population had even lower levels ranging from 3 to 15 nmol/mL. Plasma lipid levels such as cholesterol, triglycerides, LDL/HDL, ApoB/ApoA ratios as well as BMI and weight were significantly higher in the Nairobi population. It was concluded that low IGF-I and high IGFBP-1 levels of the Samburu cohorts indicate malnutrition. High lipid levels of the Nairobi cohorts indicate that these groups have several risk factors for cardiovascular diseases and diabetes type 2 [13384].

Local administration

Collagen is the predominant structural protein in tendons and ligaments, and can be controlled by hormonal changes. In animals, injections of insulin-like growth factor I (IGF-I) has been shown to increase collagen synthesis in tendons and ligaments and to improve structural tissue healing, but the effect of local IGF-I administration on tendon collagen synthesis in human has not been studied. The purpose of one study was to study whether local injections of IGF-I would have a stimulating effect on tendon collagen synthesis. Twelve healthy nonsmoking men (age 62 ± 1 years, BMI 27 ± 1) participated. Two injections of either human recombinant IGF-I (0.1 mL Increlex©) or saline (control) into each patellar tendon were performed 24-h apart, respectively. Tendon collagen fractional synthesis rate (FSR) was measured by stable isotope technique in the hours after the second injection. Simultaneously, interstitial peritendinous (IGF-I) and (procollagen type I N-terminal propeptide, PINP), as a marker for type I collagen synthesis, were determined by microdialysis technique. Tendon collagen FSR and PINP were significantly higher in the IGF-I leg compared with the control leg. In conclusion, local IGF-I administration can directly enhance tendon collagen synthesis both within and around the human tendon tissue [13385].
IGF-1 in deer antler

Reported incidents of the use of nutritional supplements containing deer antler velvet by athletes has increased significantly in recent years. The supplements have been reported to contain insulin-like growth factor-1 (IGF-1), which is a banned substance included on the World Anti-Doping Agency (WADA) prohibited list. The presence of deer and human IGF-1 was tested in six commercially available supplements. IGF-1 was extracted from the six deer antler velvet supplements using chloroform and acetonitrile precipitation methods. Ultra-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) methods were developed to measure intact IGF-1 protein and IGF-1 trypsin peptides using a triple quadrupole mass spectrometer. Five deer-specific and five human-specific multiple-reaction monitoring (MRM) transitions for intact IGF-1 were measured as well as six deer-specific and seven human-specific MRM transitions for an IGF-1 trypsin peptide. The peak area from each MRM transition was used to calculate the product ion ratios relative to the most abundant transition. Product ion ratios measured in the supplements were matched to ratios measured in purified protein standards. A match to human IGF-1 was identified for all the MRM transitions measured in four of the supplements tested. The presence of a pharmaceutical protein, human IGF-1, was confirmed in four commercially available products sold as all natural, nutritional supplements. These methods can be used to screen additional products to further prevent the illegal sale of adulterated supplements [13387].

Markers for IGF-1

Insulin-like growth factor-I (IGF-I) is reportedly misused by elite athletes, either alone or with growth hormone (GH). The GH-2000 and GH-2004 research groups previously developed a method for detecting GH misuse based on the GH-sensitive markers IGF-I and procollagen type III amino-terminal propeptide (P-III-NP). Both markers increase in response to rhIGF-I/rhIGFBP-3 administration in recreational athletes. The aim of one pilot study was to assess the effect of rhIGF-I/rhIGFBP-3 administration on other serum markers of the GH-IGF axis and on other bone and collagen markers. Twenty-six female and 30 male recreational athletes were randomized to 28 days' treatment with placebo or rhIGF-I/rhIGFBP-3 complex, followed by 56 days' washout. GH-IGF axis markers (IGFBP-2, IGFBP-3, acid-labile subunit (ALS) and IGF-II) and bone and collagen markers (procollagen type I carboxy-terminal propeptide (PICP), type I collagen cross-linked carboxy-terminal telopeptide (ICTP) and osteocalcin) were measured using commercial immunoassays. In women in the high dose treatment group, mean IGF-II decreased by 53 percent on day 21. Mean IGFBP-2 increased by 119 percent and mean ALS decreased by 40 percent on day 21. There were no significant changes in IGFBP-3, osteocalcin, ICTP or PICP. In men in the high dose group, mean IGF-II decreased by 51 percent on day 21. Mean IGFBP-2 increased by 125 percent on day 21. There were no significant changes in IGFBP-3, ALS, osteocalcin, ICTP or PICP. Serum IGFBP-2 and IGF-II may be useful markers of rhIGF-I/rhIGFBP-3 administration in both women and men while ALS may also be a useful marker in women; these markers are now undergoing further evaluation [13386].

Laboratory techniques

Performance-enhancing substances are illicitly used in elite or amateur sports and may be obtained from the black market due to a cheaper and easier availability. Although various
studies have shown that black market products frequently do not contain the declared substances, enormous amounts of illegally produced and/or imported drugs are confiscated from athletes or at customs with alarming results concerning the outcome of the analyses of the ingredients. One case report describes the identification of His-tagged Long-R³-IGF-I, which is usually produced for biochemical studies, in an injection vial. The ingredients were isolated by immunoaffinity purification and identified by nano-UPLC, high-resolution/high accuracy mass spectrometry of the intact and trypsinated substance and by an enzyme-linked immunosorbent assay. Tandem mass spectra characterized the protein as Long-R³-IGF-I with a His₆-tag attached to the C-terminus by the linker amino acids Leu-Glu [10482].

Peptide analysis in doping controls by means of nano-UPLC coupled high resolution/high mass accuracy mass spectrometry provides the state-of-the-art technique in modern sports drug testing. The present study describes a recent application of this technique for the qualitative determination of different urinary insulin-like growth factor (IGF) related peptides. After simultaneous isolation by solid phase extraction and magnetic particle-based immunoaffinity purification, target analytes (IGF-1, IGF-2, Des1-3-IGF-1, R³-IGF-1 and longR³-IGF-1) were separated by nano-liquid chromatography prior to mass spectrometric detection. Endogenously produced IGF-1 and IGF-2, as well as the degradation product Des1-3-IGF-1, were frequently detected in urine samples from healthy volunteers in a concentration range of 20-400 pg/mL. The impact of IGF binding proteins (IGFBPs), being also present in urine, was potentially estimated by an additional ultrafiltration step in the sample preparation procedure. The synthetic analogue longR³-IGF-1, which is assumed to be subject to misuse by cheating athletes, was also analysed and detected in fortified urine samples. Besides the intact molecule, an N-terminally truncated degradation product Des1-10-longR³-IGF-1 was identified as the more stable target for doping controls using urine samples. The method was validated for qualitative purposes considering the parameters specificity, limit of detection (20-50 pg/mL), recovery (10-35 %), precision (<20 %), linearity, robustness and stability [11171].

Detection

Indirect biomarkers of recombinant human growth hormone (rhGH), insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II), insulin-like growth factor binding proteins (IGFBP-2 and IGFBP-3) and insulin (C-peptide) were measured together with urinary parameters of renal damage (beta₂-microglobulin and proteinuria) by immunoassays, in house validated for the purpose, in 61 subjects (36 elite athletes, 18 recreational athletes and 7 sedentary individuals) with different levels of physical fitness and endurance exercise. Validation parameters were good for the evaluated assays, excluding a high inter-assay imprecision and inaccuracy of 24 and 26 percent obtained for GH assay. The range of concentrations found in urine samples under investigation was generally covered by the calibration curves of the studied immunoassays. However, for the samples below or above the calibration curve, opportune dilution or concentration were performed. Particularly, C-peptide samples had to be diluted 1:5 and beta₂-microglobulin ones assayed using a triple sample volume, to fall within the calibration range. Urinary C-peptide was the only biomarker statistically higher in samples of elite athletes when compared to recreational athletes and sedentary individuals. Among elite athletes, tae-kwon-do athletes showed the highest IGF-II basal values while weightlifting athletes showed the lower IGF-I and IGFBP-3 basal values. The trend observed in weightlifters' basal samples was confirmed in their training samples: IGF-I, IGF-II, IGFBP-3 and beta₂-microglobulin were lower in with respect to those from synchronised swimming. Over the training season, within athlete variability was observed for IGFBP-3 for weightlifting athletes. In the studied subjects, no direct associations were found
between biomarkers of GH or insulin misuse and urinary parameters of renal damage, eventually due to high-workload endurance training. The variations observed in different biomarkers should be taken in consideration in the hypothesis of setting reference concentration ranges for doping detection [10483].

**Variation in measurements**

Insulin-like growth factor 1 (IGF-1) is a key mediator of growth hormone (GH) action and a well-characterized biomarker of GH abuse. Current immunoassays for IGF-1 suffer from poor concordance between platforms, which makes comparison of results between laboratories difficult. Although previous work has demonstrated good interlaboratory imprecision of LC-MS/MS methods when plasma is supplemented with purified proteins, the interlaboratory imprecision of an endogenous protein in the nanogram-per-milliliter concentration range has not been reported. It was deployed an LC-MS/MS method to quantify serum IGF-1 in 5 laboratories using 5 different instruments and analyzed 130 healthy human samples and 22 samples from patients with acromegaly. It was determined measurement imprecision (CV) for differences due to instrumentation, calibration curve construction, method of calibration, and reference material. Instrument-dependent variation, exclusive of digestion, across 5 different instrument platforms was determined to be 5.6 percent. Interlaboratory variation was strongly dependent on calibration. Calibration materials from a single laboratory resulted in less variation than materials made in individual laboratories (CV 5.2 % vs 12.8 %, respectively). The mean imprecision for 152 samples between the 5 laboratories was 16.0% when a calibration curve was made in each laboratory and 11.1% when a single-point calibration approach was used. The interlaboratory imprecision of serum IGF-1 concentrations is acceptable for use of the assay in antidoping laboratories and in standardizing results across clinical laboratories. The primary source of variability is not derived from the sample preparation but from the method of calibration [13388].

**In saliva**

To examine an immunoassay for measuring free IGF-I in a saliva specimen (free sIGF-I) and to study the levels in relation to the training conditions comparing young athletes and sedentary females an analysis was carried out by modifying a commercial kit for plasma matrix to measure the free sIGF-I. The plasma free and total IGF-I fractions, hGH and salivary total proteins were also measured. Saliva and blood specimens were collected from 15 well-trained young female volleyball athletes and from a control group of 14 young sedentary females. The calibration curve to assay free sIGF-I covered the range 0.05-5.00 microg/L. The detection limit was 0.07 microg/L. The within-run and between-run imprecision CVs were 10 percent and 13 percent respectively. The average recovery was 88 percent. Free sIGF-I, measured in 15 athletes and in 14 young sedentary females, was 0.10 ± 0.03 and 0.20 ± 0.05 microg/L respectively. There were decreased levels of free sIGF-I in well-trained athletes, compared with sedentary females. This decrease could be related to a greater tissue requirement by the active muscle subjected to intense exercise for several days [07158].
INSULIN

Men and women differ substantially in regard to degrees of insulin resistance, body composition, and energy balance. Adipose tissue distribution, in particular the presence of elevated visceral and hepatic adiposity, plays a central role in the development of insulin resistance and obesity-related complications. One review summarized published data on gender differences in insulin resistance, body composition, and energy balance, to provide insight into novel gender-specific avenues of research as well as gender-tailored treatments of insulin resistance, visceral adiposity, and obesity. English-language articles were identified from searches of the PubMed database through November 2008, and by reviewing the references cited in these reports. Searches included combinations of the following terms: gender, sex, insulin resistance, body composition, energy balance, and hepatic adipose tissue. For a given body mass index, men were reported to have more lean mass, women to have higher adiposity. Men were also found to have more visceral and hepatic adipose tissue, whereas women had more peripheral or subcutaneous adipose tissue. These differences, as well as differences in sex hormones and adipokines, may contribute to a more insulin-sensitive environment in women than in men. When normalized to kilograms of lean body mass, men and women had similar resting energy expenditure, but physical energy expenditure was more closely related to percent body fat in men than in women. Greater amounts of visceral and hepatic adipose tissue, in conjunction with the lack of a possible protective effect of estrogen, may be related to higher insulin resistance in men compared with women [09187].

An acute bout of endurance exercise enhances insulin sensitivity, but the effects of sprint interval exercise have not yet been described. It was compared insulin sensitivity at baseline and after an acute bout of exercise in healthy men (n=8) and women (n=5) (age 21 years; peak oxygen consumption (VO_{2} peak), 42.6 ± 1.7 mL/kg/min; <1.5 days/Week structured exercise; body fat, 21.1 ± 1.9 % ). Subjects underwent 3 oral glucose tolerance tests (OGTT) the day after each of the following 3 conditions: no exercise, baseline, sprint interval exercise at approximately 125 percent VO_{2} peak, and endurance exercise at approximately 75 percent VO_{2} peak). There were no differences by sex for any condition (men vs women). The findings demonstrated that an acute bout of endurance exercise, but not sprint interval exercise, increases insulin sensitivity relative to a no-exercise control condition in healthy males and females. While these findings underscore the use of regular endurance exercise as an effective intervention strategy against insulin resistance [09188].

Although physical exercise is an important part of metabolic control, endurance sports are considered hazardous for patients with type 1 diabetes because of the extreme physiological stress they represent. To further elucidate the metabolic challenge this form of exercise presented we investigated the performance of triathlon competitors with type 1 diabetes. Ten patients (32-61 years) with type 1 diabetes (disease duration 2-35 years) were followed for three years, during which each year they participated in one triathlon long-distance competitions (2.4 miles swimming, 26.2 miles running and 112 miles cycling; Ironman Germany 2005-2007). Glucose, cortisol, aldosterone, renin, thyroid hormones, testosterone, growth hormone and catecholamines were measured in blood and saliva. Five non-diabetic competitors served as controls. The performance equalled those of age-matched healthy athletes. Several participants experienced hyperglycemia early in the bike leg, whereas all of them developed low blood glucose levels during the marathon leg. Basal insulin supply was reduced up to 50 percent on race day. Hormone levels in athletes with type 1 DM and healthy controls were similar [09189].
Insulin and C-peptide have been proposed as possible biomarkers of human insulin hormone misuse in sport. An extended intra- and inter-laboratory validation of commercially available immunoassays was performed. Enzyme Amplified Sensitivity Immunoassay (EASIA) assays (Human Insulin-EASIA and C-peptide EASIA kits from BioSource) were evaluated for insulin and C-peptide in serum. The intra- and inter-laboratory precision and accuracy values were good for the evaluated assays with maximum imprecision and inaccuracy of 16 and 23 percent, respectively, obtained just for one day C-peptide assay evaluation. The range of concentrations found in serum samples under investigation was always covered by the calibration curves of the studied immunoassays. However, a 20 percent of the samples felt below the estimated insulin limit of quantification. High concordance between laboratory results was obtained for insulin assay, whereas that for C-peptide was lower. Evaluated immunoassays were used to measure serum concentrations of insulin and C-peptide in elite athletes of various sport disciplines at different moment of training season, in recreational athletes at baseline conditions and finally in sedentary individuals. Serum insulin was statistically lower both in recreational and elite athletes when compared to sedentary individuals. Among elite athletes, the specific sport affected serum insulin (e.g. weightlifting) and C-peptide (e.g. triathlon) concentrations. Over the training season, a within athletes variability was observed for taekwondo, swimming and weightlifting athletes. Variations due to those aspects should be taken in careful consideration in the hypothesis of setting reference concentration ranges for doping detection [09190].

Weight status and abnormal liver function are the two factors that influence whole-body insulin sensitivity. The main goal of the study was to compare insulin sensitivity in athletes (n=757) and physically active controls (n=670) in relation to the two factors. Homeostatic metabolic assessment for insulin resistance (HOMA-IR), weight status, and abnormal liver function (alanine aminotransferase and aspartate aminotransferase) were determined from 33 sports disciplines under morning fasted condition. The study was initiated in autumn 2006 and repeated in autumn 2007 (n=1508) to ensure consistency of all observations. In general, HOMA-IR and blood pressure levels in athletes were significantly greater than those in physically active controls but varied widely with sport disciplines. Rowing and short-distance track athletes had significantly lower HOMA-IR values and archery and field-throwing athletes had significantly higher values than the control group. Intriguingly, athletes from 22 sports disciplines displayed significantly greater body mass index values above control values. Multiple regression analysis showed that, for non-athlete controls, body mass index was the only factor that contributed to the variations in HOMA-IR. For athletes, body mass index and alanine aminotransferase independently contributed to the variation of HOMA-IR. Weight status and abnormal liver function levels appear to be the major contributors predicting insulin sensitivity for the physically active population [09191].

Insulin’s anabolic actions are believed to improve performance, by increasing protein synthesis and inhibiting protein catabolism and enhancing transport of selected amino acids in human skeletal muscle. Physiological hyperinsulinemia stimulates the activity of amino acid transport in human skeletal muscle, thereby stimulating protein synthesis [08261]. Insulin-treated diabetics are known to have increased lean body mass versus control [08262]. In addition to its role in regulating glucose metabolism, insulin increases amino acid transport into cells. Its stimulation of lipogenesis, and diminished lipolysis, is one of the reasons why bodybuilders and athletes will take rhGH in conjunction, to counteract this adverse effect, whilst optimizing protein synthesis [08042]. It is the inhibition of proteolysis that the athlete is interested in and the physiology of the diabetic patient has been extrapolated by the athlete to the sporting arena. Insulin administration is protein anabolic in the insulin-resistant state of chronic renal failure. It inhibits proteolysis and when administered with amino-acids, it increases net protein synthesis [08263]. The administration of exogenous insulin, establishes an in-vivo hyperinsulinemic clamp, increasing muscle glycogen before and in the recovery
stages of strenuous exercise. This may increase power, strength, and stamina and assist in recovery from strenuous exercise. Secondly, by inhibiting muscle protein breakdown and in conjunction with a high protein and high carbohydrate diet, insulin will have the action of increasing muscle bulk, potentially improving performance [08133].

In Gruber and Pope’s study [08132], 14 percent of surveyed subjects abused both insulin and anabolic steroids. Unless caught by urinalysis in the act, convicting a competitive sporting individual who has taken insulin is not possible [08133]. The few cases that have been published are case histories of individuals who have been admitted to hospital following accidental insulin overdose [08264-08273]. Dawson [08274] identified that 10 percent of 450 patients attending a needle exchange programme self-prescribed insulin for nontherapeutic purposes.

Simultaneously with insulin’s excitatory action in stimulating lipogenesis, insulin also exhibits an inhibitory action in preventing glycerol release. It is this inhibitory action on lipolysis (and also glycolysis, gluconeogenesis, ketogenesis, and proteolysis) that accounts for most of insulin’s physiological actions in man. The inhibitory actions are also responsible for insulin’s net anabolic actions [08133].

There is a correlation between substance misuse and inappropriate compliance with treatment in chronic medical conditions, which can induce anxiety and depressive disorders [08275]. Insulin dependent diabetics (type 1) have higher co-morbid substance misuse compared with the general population [08276] and their compliance with their medical management is reduced [08277]. Insulin dependent diabetics have an increased incidence of a psychiatric disorder, predominantly in the early course of their condition. Treating the psychiatric disorder improves diabetic control [08278]. Hypoglycemia is a regular occurrence in insulin-dependent diabetics and may be associated with cognitive and affective outcomes [08279]. Mood changes induced by fluctuations in glycemic control are characteristically individual, with depressive and anxiety affective states being most prevalent, however euphoria can also occur [08280]. The literature is interspersed with cases of deliberate misuse of insulin, associated with either suicide, parasuicide or factitious illness [08281, 08282]. Even intelligent individuals have abused their therapeutically prescribed insulin to induce feelings of “happiness, disorientation, drunkenness, altered perception of helplessness, and euphoria”. However, there always appears to be an underlying mood or personality disorder [08283]. There appears to be a class of individuals who are not diabetic and inject insulin to induce euphoria, to alter perception and for the “exquisite pleasure” associated with the risk of death [08284].

Accumulating data suggest that exercise may affect breast cancer risk and outcomes. Studies have demonstrated that high levels of insulin, often seen in sedentary individuals, are associated with increased risk of breast cancer recurrence and death. Now 101 sedentary, overweight breast cancer survivors were randomly assigned either to a 16-week cardiovascular and strength training exercise intervention or to a usual care control group. Fasting insulin and glucose levels, weight, body composition, and circumference at the waist and hip were collected at baseline and 16 weeks. Baseline and 16-week measurements were available for 82 patients. Fasting insulin concentrations decreased, significantly, by an average of 2.86 microU/mL in the exercise group, with no significant change in the control group (decrease of 0.27 microU/mL). The change in insulin levels in the exercise group seemed greater than the change in controls, but the comparison did not reach statistical significance. There was a trend toward improvement in insulin resistance in the exercise group but no change in fasting glucose levels. The exercise group also experienced a significant decrease in hip measurements, with no change in weight or body composition. This means that the relationship between physical activity and breast cancer prognosis may
be mediated, in part, through changes in insulin levels and/or changes in body fat or fat deposition [08285].

Insulin is a peptide hormone consisting of two peptide chains (A- and B-chain) that are cross-linked by two disulfide bonds. To obtain improved pharmacokinetic onset of action profiles of insulin treatment in diabetic patients, recombinant long-, intermediate-, and rapid-acting insulin analogs are produced, in which the C-terminal end of the B-chain plays an especially important role. A review of the veterinary literature reveals the low prevalence of equine type I diabetes mellitus, which indicates that the therapeutic use of insulin in racing horses is unlikely. Although there is no unequivocal evidence of an overall performance-enhancing effect of insulin, in human sports the misuse of insulin preparations is reported among elite athletes. The desired effects of insulin include the increase of muscular glycogen prior to sports event or during the recovery phase, in addition to a chalonic action, which increases the muscle size by inhibiting protein breakdown. In one study urinary insulin was detected in equine samples and differences between equine insulin, human insulin, as well as rapidly acting recombinant insulin variants were examined. Product ion scan experiments of intact proteins and B-chains enabled the differentiation between endogenously produced equine insulin, its DesB30 metabolite, human insulin and recombinant insulin analogs, and the assay allowed the assignment of individual product ions, especially those originating from modified C-termini of B-chains [08286].

Due to its versatile nature and its corresponding anabolic and anticatabolic properties, insulin has been prohibited in sports since 1999. Numerous studies concerning its impact on glycogen formation, protein biosynthesis, and inhibition of protein breakdown have illustrated its importance for healthy humans and diabetics as well as elite athletes. Various reports described the misuse of insulin to improve performance and muscle strength, and synthetic analogs were the subject of several studies describing the beneficial effects of biotechnologically modified insulins. Rapid- or long-acting insulins were developed to enhance the injection-to-onset profile as well as the controllability of administered insulin, where the slightest alterations in primary amino acid sequences allowed the inhibition of noncovalent aggregation of insulin monomers (rapid-acting analogs) or promoted microprecipitation of insulin variants upon subcutaneous application (long-acting analogs). Information on the metabolic fate and renal elimination of insulins has been rather limited, and detection assays for doping control purposes were primarily established using the intact compounds as target analytes in plasma and urine specimens. However, recent studies revealed the presence of urinary metabolites that have been implemented in confirmation methods of sports drug testing procedures. So far, no screening tool is available providing fast and reliable information on possible insulin misuse; only sophisticated procedures including immunoaffinity purification followed by liquid chromatography and tandem mass spectrometry have enabled the unambiguous detection of synthetic insulins in doping control blood or urine samples [10159].

The primary source of carbohydrate during exercise derives from muscle glycogen stores. The greater the amount of glycogen stored, the longer one should be able to exercise. Since insulin is a potent agent for the uptake of glucose and the subsequent glycogen storage in muscle, then the use of insulin ought to permit longer exercise time. In addition, insulin leads to the accumulation of amino acids in muscle and theoretically additional substrate for protein synthesis. Insulin may very well add to the anabolic properties of anabolic steroids and/or rhGH or rhIGF-I. The most likely scenario for a strength athlete would be to ingest a high carbohydrate diet, especially around the time of injecting rapidly acting insulin or its analogues [10001].

Insulin stimulates the uptake of glucose into muscle and fat by making available an increased
number of glucose transporters (Glut-4) at the cell membrane thus increasing the flux of glucose to the interior of the cell. However, its main effect is inhibitory to lipolysis, glycolysis, gluconeogenesis, ketogenesis and proteolysis. The theoretical efficacy to improve performance-muscle glycogen storage and the inhibition of proteolysis have not been shown in experimental protocols. That does not deter athletes from injecting insulin and its analogues, likely because insulin is but one of a "cocktail" or drugs along with training to enhance anabolic activity. In addition one must consider more rapid recovery from training and competition, but again there are no data to show this effect [10001].

The main adverse event is the most obvious, that of hypoglycemia. Most athletes who abuse insulin are likely adept at balancing the ingestion of carbohydrate when injecting rapidly-acting insulin analogues. Much of the use of insulin, which is readily available, is likely covert, kept even from friends and family. It is unlikely that one would tumble to the diagnosis of hypoglycemia in young healthy, non-diabetic individuals when they present to medical evaluation with confusion or coma. That can be a fatal mistake. There are no foolproof mechanisms for detecting insulin, unless one uses an analogue and that or one of its metabolites can be detected. One would think that anti-insulin antibody detection techniques would be definitive, but there is a low frequency of circulating anti-insulin antibodies in some normal (non-diabetic) individuals [10001].

One study evaluated the effects of various resistance exercise protocols on 24-hour postexercise insulin sensitivity. Seventeen participants with impaired fasting glucose (100-125 mg/dL) completed 4 separate bouts of resistance exercise under moderate intensity (65 % 1 repetition maximum, 1RM) or high intensity (85 % 1RM) conditions within the confines of single set and multiple set protocols. Intravenous fasting blood was taken at baseline and 24 hours postexercise for each exercise condition to measure fasting plasma glucose (G0) and fasting serum insulin (I0) to calculate insulin sensitivity. A minimum of 3 days washout was given between each exercise protocol. A 4 x 2 factorial analysis of variance was performed to compare insulin sensitivity and fasting glucose within subjects and between treatments. All of the exercise protocols significantly improved subsequent insulin sensitivity and G0. In comparison with single set, there was a significantly greater decrease in G0 24 hours after multiple set bouts. High intensity showed significant decreases in insulin sensitivity as compared with moderate intensity protocols. Effect size data suggest a dose response relationship between program variables of volume and intensity and 24-hour postexercise insulin sensitivity. High-intensity protocols resulted in greater effect sizes for insulin sensitivity (0.83 multiple set; 0.53 single set) as compared with moderate-intensity protocols. The high-intensity, multiple set bout yielded the greatest treatment effect in both fasting glucose (0.61) and insulin sensitivity (0.83). Overall, single set protocols were less effective than multiple set protocols in lowering fasting blood glucose. Findings suggest a dose-response relationship between volume and intensity on insulin sensitivity and fasting blood glucose. Results indicate that resistance exercise is an effective treatment for acutely enhancing insulin sensitivity and regulating blood glucose in individuals with impaired fasting glucose [10160].

To compare serum glucose and insulin responses to 3 preexercise snacks before, during, and after exercise in individuals with impaired fasting glucose (IFG) and healthy (H) men in an IFG population, the authors sought to determine whether a natural fruit snack (i.e., raisins) yields more desirable glucose and insulin concentrations than an energy bar or a glucose solution. The IFG (n=11, age 55 ± 1 years, fasting blood glucose 6.3 ± 0.1 mmol/L) and H groups (n=9, age 48 ± 3 years, fasting blood glucose 4.9 ± 0.1 mmol/L) cycled at 50 percent of VO2peak for 45 min on 4 occasions after consuming water or 50 g of carbohydrate from raisins (R), an energy bar (EB), or a glucose beverage (GLU). Metabolic markers were measured before, during, and after exercise. In all nutritional conditions, glucose concentrations of the IFG group were consistently higher than in the H group. Differences
between IFG and H groups in insulin concentrations were sporadic and isolated. In the IFG group, preexercise glucose concentration was lower in the R condition than in GLU. Ten and 20 min into exercise, glucose concentrations in the R and EB conditions were lower than in GLU. Insulin concentrations were lower in the R condition than in EB and GLU immediately before exercise and at minute 10 but at 20 min R remained lower than only GLU. Glucose concentrations were higher in the IFG group regardless of preexercise snack. Compared with the glucose solution, raisins lowered both the postprandial glycemic and insulinemic responses, whereas the energy bar reduced glycemia but not insulinemia [11172].

The purpose of one study was to gain insight into the current diabetes management practices of endurance athletes with type 1 diabetes and to compare these practices with the guidelines for athletes established by the American Diabetes Association (ADA). Participants included in this descriptive study were endurance athletes aged 18 years and older with type 1 diabetes. The survey questions were based on the current clinical recommendations for endurance athletes with type 1 diabetes, as established by the ADA. A link to the questionnaire was posted on the Web site of the Diabetes Exercise and Sports Association (DESA). A total of 38 questions were included in the survey, and 91 usable surveys were received. Analysis of variance was used for several comparisons using SPSS version 17. Half of the clinical guidelines were followed by the participants. Among these, about 50 percent followed many of the guidelines "most of the time" or "almost always" and 40 percent followed the guidelines "sometimes" or "most of the time." Results of ANOVA showed several trends (nonsignificant) in the occurrence of low blood glucose when the guidelines were not followed. It was concluded that endurance athletes with type 1 diabetes do not consistently follow the clinical guidelines for blood glucose management as recommended by the ADA. The results of this study reflect a need among athletes for diabetes management education programs that promote a better understanding of the potential negative side effects of suboptimal blood glucose control [11173].

The search for target analytes to uncover the misuse of long acting insulin analogues (Lantus, Insulin Glargine; Levemir, Insulin Detemir) in doping control samples led to the identification of several degradation products of insulin or its synthetic analogues. Specimens obtained from healthy volunteers or patients and athletes suffering from diabetes mellitus contained DesB30, DesB24-30, and DesB25-30 human insulin or DesB30-32, DesB31-32, and DesB24-32 Lantus, respectively. Analytes were purified from urine by immunoaffinity chromatography (IAC) with subsequent liquid chromatography-tandem mass spectrometry analysis. The employed analytical procedure was validated for qualitative determination considering the main metabolic products DesB30 human insulin and DesB30-32 Lantus. The occurrence of the identified Lantus degradation products in urine provided the direct and unambiguous evidence for an administration of this insulin analogue. For the determination of surreptitious Levemir or recombinant human insulin applications, an unequivocal argument was not detected, but promising approaches based on a modified insulin degradation profile with altered relative intensities of metabolites are presented [07161].

Human insulin has been considered relevant for sports drug testing since 1999 due to its assumed positive effects on muscle glycogen formation, antitabloc (so-called chalonic) actions on muscle protein, and improvements in protein biosynthesis. Besides conventional formulations consisting of recombinantly produced human insulin, numerous synthetic analogs, termed rapid- and long-acting insulins, have been introduced that possess improved injection-to-onset and pharmacokinetic profiles. In particular, the rapid-acting insulins such as Humalog, Novolog and Apidra have been targeted by new analytical methods, and top-down sequencing-based approaches have enabled their determination in human plasma and urine using immunoaffinity chromatography (IAC), solidphase extraction (SPE) and microbore LC-MS/MS procedures. While Humalog differs from human insulin only by the switched positions
of proline B28 and lysine B29, Novolog and Apidra comprise substituted amino acid residues, resulting in them having different molecular weights compared to that of human insulin (5807 Da). Product ion scan experiments yielded diagnostic ions that unequivocally identify the synthetic nature of these insulin analogs, and detection limits of 0.5 and 0.05 ng/mL, respectively, were obtained, enabling the determination of normal insulin levels in both matrices. Several athletes selected for doping control sample collection suffer from diabetes mellitus, and numerous authentic specimens from these tested "positive," providing the proof-of-principle of these new procedures [07050].

Given the fact that raising the plasma insulin concentration is key to stimulating muscle protein synthesis and limiting protein catabolism after exercise, it is not surprising that some athletes abuse insulin to increase skeletal muscle hypertrophy. Insulin injections reportedly can produce "rapid and noticeable (muscle) growth... almost immediately after starting insulin therapy". Most athletes choose to administer insulin immediately after a workout; they apparently realise that it is the most anabolic time to use this hormone. However, insulin abuse is extremely risky – one mistake in dose or diet can be fatal. Fortunately, recent studies have focused on safe insulinotropic nutritional mixtures containing protein hydrolysates, certain added amino acids (especially leucine), and high-glycaemic CHO; for example, dextrose and maltodextrine [06143].

Diabetes mellitus is the most common group of metabolic diseases and is characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Most patients with diabetes are type 2 (90 %); the remaining patients have type I disease. Athletes with diabetes range from the athlete participating in various youth sports to the competitive Olympic athlete and present a significant challenge to themselves and the medical staff who care for them on a daily basis. Each sport and the type of exercise have their own effects on diabetes management with numerous factors that significantly affect glucose levels, including stress, level of hydration, the rate of glycogenolysis and gluconeogenesis, and the secretion of counter-regulatory hormones. One article provided a general overview of diabetes mellitus, the effects of exercise on glucose levels, and a detailed review of the potential complications encountered in the management of diabetes in the athlete [12219].

Besides its particular importance as a widely used therapeutic agent, insulin (and its synthetic derivatives) has been suspected, purported, and proven to be a lethal weapon in numerous cases of attempted or successful homicide and suicide. In addition to blood and urine as common matrices for clinical diagnosis and post-mortem analysis, vitreous humour has gained considerable attention in autopsy and follow-up investigations due to its ability to provide valuable information on cause and time of death. However, post-mortem insulin analyses using such specimens have been rare due to the limited penetration of peptide hormones into the vitreous body, and immunoassays were exclusively employed in those studies. In the present communication, the determination of insulin(s) from vitreous humour by means of immunopurification combined with ultrahigh performance liquid chromatography-high resolution/high accuracy (tandem) mass spectrometry is reported. Exploiting the constantly increasing sensitivity and robustness of modern mass-spectrometry-based instruments, the option to identify insulin in post-mortem vitreous samples is demonstrated with a specimen collected from a non-diabetic victim who died from an insulin overdose. This communication represents the first successful mass-spectrometry-based analysis of post-mortem material related to an insulin poisoning case [12220].

Insulin, a polypeptide hormone secreted by pancreatic cells, is a key regulator in glucose homeostasis. Its deficiency leads to insulin-dependent (type I) diabetes whereas resistance to insulin is common in type II diabetes, obesity and a range of endocrine disorders. Its
determination is of considerable value, particularly in the clinical diagnosis of diabetes mellitus and the doping control of athletes. It has, additionally, been noted as a potential breast cancer marker (serum insulin levels being found to be raised in comparison to control patients). Electrochemical assays are potentially very cheap, highly sensitive, and very readily transposed to a point of care. Though there exist numerous examples of label free impedimetric or capacitative assaying of biomolecules, these are rarely demonstrated to be effective in complex biological mixtures or to be applicable to low molecular weight targets (since they operate through the interfacial displacement of water/ions and/or the steric blocking of a redox probe). It was reported an ultrasensitive electrochemical and label-free biosensor for insulin in blood serum with a clinically relevant linear range and detection limit of 1.2 pM. The transducing surfaces, based on readily prepared, antibody modified, polyethylene glycol monolayer modified polycrystalline gold surfaces, respond in a highly specific and re-useable manner to the target in up to 50 percent blood serum [13389].

To evaluate the effects of an overtraining (OT) protocol based on eccentric exercise (EE) sessions on the insulin and inflammatory signalling pathways in skeletal muscles of Swiss mice the rodents were divided into control (C; sedentary mice), trained (TR; performed the aerobic training protocol) and overtrained (OTR; performed the OT protocol). The incremental load test (ILT) and exhaustive test (ET) were used to measure performances before and after exercise protocols. Twenty-four hours after the exhaustive test performed at the end of week 8, the extensor digitorum longus (EDL) and soleus muscles were removed for subsequent protein analysis by immunoblotting. The phosphorylation of insulin receptor beta (pIRbeta; Tyr1146) diminished for EDL and soleus in OTR compared to C. The phosphorylation of insulin receptor substrate 1 (pIRS-1; Ser307) increased for EDL and soleus in OTR compared to C and TR. The phosphorylation of protein kinase B (pAkt; Ser473) diminished for EDL and soleus in OTR compared to C and TR. The phosphorylation of protein kinase B (pAkt; Ser473) diminished for EDL and soleus in OTR compared to C and TR. The phosphorylation of IκB kinase alpha and beta (pIKKalpha/beta; Ser176/180), stress-activated protein kinases/Jun amino-terminal kinases (pSAPK/JNK; Thr183/Tyr185) and the protein levels of suppressor of cytokine signaling 3 (SOCS3) increased for EDL and soleus in OTR compared to C and TR. In summary, the current used OT protocol based on EE sessions impaired the insulin signalling pathway with concomitant increases of IκK, SAPK/JNK and SOCS 3 protein levels [13390].

Insulin analogues represent a major and growing class of biotherapeutics, and their quantitation is an important focus of commercial and public effort across a number of different fields. As LC-MS has developed, it has become an increasingly practicable and desirable alternative to ligand-binding-based approaches for quantitation of this class of compounds. The sensitivity challenge of measuring trace levels of this large peptide molecule in a protein-containing matrix is considerable; however, different approaches to detection, extraction and separation are described to overcome this challenge, including immunoaffinity capture, SPE and low-flow HPLC. Considerations such as bioanalytical assay acceptance criteria and antidrug antibody effects during drug development are included, alongside descriptions of recent sports doping and clinical applications. Factors affecting the correlation and agreement of MS with biological ligand-binding methods are discussed, with ways to anticipate and appreciate differences between the values derived from each technique. A high degree of scientific creativity, combined with science-defined regulatory approaches that define suitable validation criteria, will be needed to meet the demanding requirements for high-throughput analysis of insulin by LC-MS [13391].

In weightlifting
Use of performance-enhancing drugs (PEDs) is common among strength-trained individuals, and a growing concern is the misuse of insulin. A 99-item Internet-based survey was posted on discussion boards of various fitness, bodybuilding, weightlifting, and anabolic steroid Web sites between February and June 2009. A case series of 41 nondiabetic insulin users is described. The typical insulin user was 31 ± 9 years old, male (98 %), and Caucasian/white (87 %) who classified himself as a "recreational exerciser" (48 %). The average insulin user also used anabolic steroids (95 %) and practiced polypharmacy by incorporating 16 ± 6 PEDs in his or her yearly routine. Hypoglycemia was reported by most of the subjects (57 %), and one individual reported unconsciousness. Insulin was obtained most commonly from local sources (e.g. friends, training partners, gym member/dealer; 41 %) and community pharmacies (38 %), with most (81 %) finding it "easy" to acquire their insulin. Strategies aimed to prevent insulin misuse are needed [12221].

**Amino acid-stimulated insulin secretion**

The importance of amino acid availability for the stimulatory effects of insulin to be evident was reported. Insulin, given with sufficient amino acids, can stimulate leg and whole body protein balance by mechanisms including stimulation of protein synthesis and inhibition of protein breakdown. This is in line with the recent data showing that protein balance over the muscle remains negative after resistance exercise when only carbohydrate (CHO) is ingested. In sharp contrast, amino acid ingestion alone significantly increases muscle protein anabolism after resistance exercise. However, consumption of both amino acids and CHO results in much greater effects on muscle protein anabolism, suggesting an interactive effect between insulin, amino acid availability, and resistance exercise. Also, it is well established that the stimulatory effect of amino acids on muscle protein synthesis is greater after exercise than at rest. Thus, nutrient timing is also an important consideration. Appropriate resistance exercise leads to significant skeletal muscle hypertrophy, which can occur through an increase in protein synthesis, a decrease in protein degradation, or both. Although stimulation – that is, resistance exercise – is important for muscle hypertrophy, nutrient availability appears to be a critical factor regulating the degree of hypertrophy. Obviously, the hormonal milieu of the muscle also has a major impact on protein synthesis. It is now apparent that both increased insulin and increased availability of amino acids are important for maximizing muscle protein anabolism. If hyperinsulinaemia is not supported by an exogenous amino acid supply, plasma as well as muscle free amino acid concentrations fall because of reduced splanchnic release. On the other hand, if amino acid concentrations are maintained at normal or higher concentrations, net protein deposition in muscle will occur because of stimulation of synthesis and possibly because of a simultaneous decrease in breakdown. Dietary supplements and other ergogenic aids are popular among athletes. Recent studies have shown that nutritional mixtures containing protein hydrolysates, added leucine, and high-glycaemic carbohydrates greatly augment insulin secretion compared with high-glycaemic carbohydrates only. When post-exercise hyperinsulinaemia is supported by hyperaminoacidaemia induced by protein hydrolysate and leucine ingestion, net protein deposition in muscle should occur. Thus, consumption of post-exercise recovery drinks containing these nutrients in conjunction with appropriate resistance training may lead to increased skeletal muscle hypertrophy and strength. However, the long-term effects on body composition and exercise performance remain to be determined [06143].

Formerly, it was believed that insulin secretion was controlled almost entirely by blood glucose concentration. However, it later become apparent that amino acids also play a very important role in controlling insulin secretion. Certain amino acids cause insulin release in humans even under conditions where the blood sugar changes little from its basal level. However, changes in blood sugar concentrations markedly influence the responsiveness of
beta cells to individual amino acids. Studies on isolated perfused rat pancreas and islets have shown that physiological amino acid mixtures and even pharmacological concentrations of individual amino acids require the presence of permissive concentrations of glucose (2.5-5.0 mM) to be effective beta cell stimulants. However, leucine is an exception. Contrary to popular belief, oral arginine is not an effective insulin secretagogue [06143].

**Effects of leucine on post-exercise muscle protein synthesis**

The key branched-chain amino acid leucine acts as a nutrient signal to stimulate muscle protein anabolism. Leucine affects muscle protein metabolism by decreasing the rate of protein degradation, probably by increasing circulating insulin. In addition, leucine affects phosphorylation of key proteins involved in the regulation of protein synthesis, which has been shown to occur even in the absence of an increase in circulating insulin. After exercise, recovery of muscle protein synthesis requires dietary protein or branched-chain amino acids to increase tissue concentrations of leucine. The important bottom line is that insulin and leucine allow skeletal muscle to coordinate protein synthesis with physiological state and dietary intake [06143].

**Protein hydrolysates**

Protein hydrolysates are produced from purified protein sources by heating with acid or, preferably, addition of proteolytic enzymes, followed by purification procedures. Extreme bitterness is a negative attribute of most protein hydrolysates. Fortunately, specific “debittering” strategies have focused on the application of proline-specific exopeptidases and endopeptidases given the contribution of proline residues to hydrolysate bitterness. The hydrolytic process mimics our own digestive actions; thus some feel it is an ideal way to process dietary protein. Extensively hydrolysed proteins containing mostly dipeptides and tripeptides are absorbed more rapidly than free-form amino acids and much more rapidly than intact (non-hydrolysed) proteins. The considerably greater absorption rate of amino acids from dipeptides and tripeptides than from an amino acid mixture appears to be the result of uptake by a system that has a greater transport capacity than the amino acid carrier system, thus minimising competition between its substrates. Each protein hydrolysate is a complex mixture of peptides of different chain length together with free amino acids, which can be defined by a global value known as degree of hydrolysis, which is the fraction of peptide bonds that have been cleaved in the starter protein. However, two protein hydrolysates made by different methods – for example, oligopeptides/significant free amino acids versus mainly dipeptides and tripeptides – may have a similar degree of hydrolysis even though their absorption kinetics are probably quite different. Consequently, it has been suggested that it is better to use the term “peptide chain length profile”. It seems that only dipeptides and tripeptides, which remain after luminal and brush-border peptidase digestion, are absorbed intact. Tetrapeptides and higher peptides appear to require prior brush-border hydrolysis before their hydrolysis products can be absorbed. Although the starter protein and method of hydrolysis affect absorptive characteristics, the peptide chain length is the most important variable. Protein hydrolysates produced from various sources showed increased amino acid absorption in humans when the proportion of dipeptides and tripeptides was increased. Thus, to maximise absorption rate, the ideal protein hydrolysate should contain mainly dipeptides and tripeptides. Such a protein hydrolysate seems to produce the most immediate hyperaminoacidaemia. In general, it is the kinetics of the absorption (rather than the net absorption of amino acids) that determines the greater nutritional value of the protein hydrolysates. The use of a protein hydrolysate in post-exercise drinks is preferred because it results in a faster increase in plasma amino acid concentrations during a 2 h period than does intact protein, and in turn the concentrations of essential amino acids in the blood.
regulate muscle protein synthesis. A practical advantage is that one can ingest a protein hydrolysate-containing supplement immediately after exercise without becoming bloated and not excessively suppressing appetite, so one can eat another meal sooner, possibly optimising the post-exercise “anabolic window”. In addition, protein hydrolysate ingestion has a strong insulinotropic effect [06143].

Whey

Clearly, hydrolysed whey protein is the most popular protein hydrolysate among athletes. Whey protein has been singled out as the ultimate source of protein on the basis of an excellent amino acid profile. Whey may offer other benefits too. Casein hydrolysate is also used in some commercial protein mixtures. It should be realised that the biological value of hydrolysed collagen (also known as gelatin) is zero; thus, collagen supplementation as a protein source is not recommended. However, it has been suggested that hydrolysed collagen may be useful in counteracting degenerative joint diseases. Finally, some commercial products are enriched with wheat gluten hydrolysate; that is, “glutamine peptides”. Wheat gluten has a unique amino acid profile: glutamine residues account for about 40 protein of the amino acids. Glutamine is an important fuel for some cells of the immune system and may have specific immunostimulatory effects. It is worth noting that the “classical” model of protein metabolism, which views nitrogen intake in terms of the flux of free amino acids from dietary protein and their exchange between plasma and intracellular compartments and between free and protein-bound amino acids, is misleading because it ignores the flux of amino acids through intermediate pools of small peptides [06143].

It has been shown that consumption of whey protein induced a rapid aminoacidemia with a greater amplitude than did casein protein. Whey protein consumption resulted in a rapid stimulation of whole-body protein synthesis and also oxidation, whereas casein resulted in a suppression of whole-body proteolysis. The result was that casein ingestion induced a more positive whole-body leucine balance than did whey. Recent findings showed that milk proteins appear superior to, or at least equivalent to, either isolated whey or casein alone in terms of supporting postprandial dietary nitrogen utilization [07309].

Timing of nutrient ingestion has been demonstrated to influence the anabolic response of muscle following exercise. Previously, it was demonstrated that net amino acid uptake was greater when free essential amino acids plus carbohydrates were ingested before resistance exercise rather than following exercise. However, it is unclear if ingestion of whole proteins before exercise would stimulate a superior response compared with following exercise. One study was designed to examine the response of muscle protein balance to ingestion of whey proteins both before and following resistance exercise. Healthy volunteers were randomly assigned to one of two groups. A solution of whey proteins was consumed either immediately before exercise (PRE; n=8) or immediately following exercise (POST; n=9). Each subject performed 10 sets of 8 repetitions of leg extension exercise. Phenylalanine concentrations were measured in femoral arteriovenous samples to determine balance across the leg. Arterial amino acid concentrations were elevated by approximately 50 percent, and net amino acid balance switched from negative to positive following ingestion of proteins at either time. Amino acid uptake was not significantly different between PRE and POST when calculated from the beginning of exercise or from the ingestion of each drink. Thus the response of net muscle protein balance to timing of intact protein ingestion does not respond as does that of the combination of free amino acids and carbohydrate [07359].

Myostatin decreases muscle mass and this is accomplished, in part, by inhibiting muscle satellite cell proliferation and differentiation by regulating the expression of cell cycle-related proteins (e.g. p21 and cdk2) and myogenic regulatory factors (e.g. myogenin and MyoD).
The purpose of one investigation was to determine whether protein ingestion before and after a resistance exercise (RE) bout affects myostatin and cell cycle-related gene expression. Strength-trained middle-aged to older men were divided into a protein group (61 years, n=9) or a placebo group (62 years, n=9). Muscle biopsies from the vastus lateralis muscle were taken at rest and 1 and 48 h after a 5 x 10 repetition leg press RE bout. Protein (15 g whey) or non-caloric placebo was taken immediately before and after the RE bout. mRNA expression levels of myostatin and related genes (AcvrIIb, FLRG, p21, p27, cdk2, myogenin and MyoD) were determined by Taqman probe-based real-time RT-PCR and normalized to GAPDH mRNA. Myostatin mRNA decreased after a RE bout, but only in the placebo group. Conversely, myostatin binding protein FLRG and cell cycle kinase cdk2 mRNA increased only in the protein group. p21 mRNA was increased at 1 h post-RE in placebo and tended to be increased in the protein group. Myostatin, its binding protein and cell cycle-related gene expressions are affected by single RE bout and these responses are further modified by whey protein intake. Therefore, controlling nutrition intake is important when studying gene expression responses to exercise.

Whey protein is a supplemental protein source often used by athletes, particularly those aiming to gain muscle mass; however, direct evidence for its efficacy in stimulating muscle protein synthesis (MPS) is lacking. We aimed to determine the impact of consuming whey protein on skeletal muscle protein turnover in the post-exercise period. Eight healthy resistance-trained young men (age 21) participated in a double-blind randomized crossover trial in which they performed a unilateral leg resistance exercise workout (EX: 4 sets of knee extensions and 4 sets of leg press; 8-10 repetitions/set; 80 % of maximal), such that one leg was not exercised and acted as a rested (RE) comparator. After exercise, subjects consumed either an isocaloric whey protein plus carbohydrate beverage (WHEY: 10 g protein and 21 g fructose) or a carbohydrate-only beverage (CHO: 21 g fructose and 10 g maltodextran). Subjects received pulse-tracer injections of L-[ring-2H5]phenylalanine and L-[15N]phenylalanine to measure MPS. Exercise stimulated a rise in MPS in the WHEY-EX and CHO-EX legs, which were greater than MPS in the WHEY-RE leg and the CHO-RE leg, respectively. The rate of MPS in the WHEY-EX leg was greater than in the CHO-EX leg. It was concluded that a small dose (10 g) of whey protein with carbohydrate (21 g) can stimulate a rise in MPS after resistance exercise in trained young men that would be supportive of a positive net protein balance, which, over time, would lead to hypertrophy.

Ingestion of whey or casein yields divergent patterns of aminoacidemia that influence whole-body and skeletal muscle myofibrillar protein synthesis (MPS) after exercise. Direct comparisons of the effects of contrasting absorption rates exhibited by these proteins are confounded by their differing amino acid contents. The objective was to determine the effect of divergent aminoacidemia by manipulating ingestion patterns of whey protein alone on MPS and anabolic signaling after resistance exercise. In separate trials, 8 healthy men consumed whey protein either as a single bolus (BOLUS; 25-g dose) or as repeated, small, "pulsed" drinks (PULSE; ten 2.5-g drinks every 20 min) to mimic a more slowly digested protein. MPS and phosphorylation of signaling proteins involved in protein synthesis were measured at rest and after resistance exercise. BOLUS increased blood essential amino acid (EAA) concentrations above those of PULSE (162 % compared with 53 %, 60 min after exercise, whereas PULSE resulted in a smaller but sustained increase in aminoacidemia that remained elevated above BOLUS amounts later (180-220 min after exercise). Despite an identical net area under the EAA curve, MPS was elevated to a greater extent after BOLUS than after PULSE early (1-3 h: 95 % compared with 42 %) and later (3-5 h: 193 % compared with 121 %). There were greater changes in the phosphorylation of the Akt-mammalian target of rapamycin pathway after BOLUS than after PULSE. Thus, rapid aminoacidemia in the postexercise period enhances MPS and anabolic signaling to a greater extent than an
identical amount of protein fed in small pulses that mimic a more slowly digested protein. A pronounced peak aminoacidemia after exercise enhances protein synthesis [11394].

It was recently shown that a week-long, high-fat diet reduced whole body exercise efficiency in sedentary men by >10 percent. To test if a similar dietary regime would blunt whole body efficiency in endurance-trained men and, as a consequence, hinder aerobic exercise performance, 16 endurance-trained men were given a short-term, high-fat (70 % kcal from fat) and a moderate carbohydrate (50 % kcal from carbohydrate) diet, in random order. Efficiency was assessed during a standardized exercise task on a cycle ergometer, with aerobic performance assessed during a 1-h time trial and mitochondrial function later measured using $^{31}$P-magnetic resonance spectroscopy. The subjects then underwent a 2-wk wash-out period, before the study was repeated with the diets crossed over. Muscle biopsies, for mitochondrial protein analysis, were taken at the start of the study and on the 5th day of each diet. Plasma fatty acids were 60 percent higher on the high-fat diet compared with moderate carbohydrate diet. However, there was no change in whole body efficiency and no change in mitochondrial function. Endurance exercise performance was significantly reduced, most probably due to glycogen depletion. Neither diet led to changes in citrate synthase, ATP synthase, or mitochondrial uncoupling protein 3. It was concluded that prior exercise training blunts the deleterious effect of short-term, high-fat feeding on whole body efficiency [11397].

With a growing number of dietary interventions that claim to improve lipid profile, it is important to ensure that these claims are evidence based. The objective of one study was to make recommendations for dietary regimens by analyzing their effectiveness and the level of evidence. It was searched MEDLINE as well as the Cochrane Database of Systematic Reviews for nutritional studies. Meta-analyses and randomized controlled trials published in English and including data on the effect on blood lipid levels were used. Randomized controlled trials were included if they were at least 4 weeks in duration and had a minimum of 50 participants. It was identified 22 different dietary interventions and reviewed 136 studies published between January 1990 and December 2009 that met our inclusion criteria. The literature review showed that to improve lipid profile, the following regimens can be recommended fully: Mediterranean and Portfolio diets; low-fat diet; diet high in soy protein, fibre, or phytosterols; whole grain foods, and omega-3 fatty acid supplementation. The consumption of nuts, a diet high in carbohydrates and protein, green tea, and red wine, as well as the supplementation with policosanol and red yeast rice extract, can be considered for improvement of the lipid profile, while the supplements of guggulipid, garlic, chromium, vitamin C, magnesium-pyridoxal-phosphate-glutamate, tocotrienols, and absorbitol cannot be recommended [11398].

**Effects of insulinotropic nutritional mixtures on insulin secretion**

A study was implemented to determine the effects of different protein-containing solutions on insulin response and amino acid availability in healthy humans. Four different 600 mL solutions were used. The glucose solution (control) contained only glucose (25 g/L), and the three additional solutions contained the same quantity of glucose plus protein (0.25 g/kg body mass) but proteins were derived from different sources: whey hydrolysate, pea hydrolysate, and a complete cow's milk solution. This study indicated that:

- Ingestion of glucose and protein hydrolysate results in a synergistic and fast increase in plasma insulin. In fact, protein hydrolysates stimulated an increase in plasma insulin that was two and four times greater than that produced by the intact milk protein solution and glucose solution, respectively
Protein hydrolysates are absorbed at a faster rate from the small intestine than are intact milk proteins, as reflected by the rapid increase in the plasma concentration of branched-chain amino acids in peripheral blood. Whey protein hydrolysate elicited the greatest availability of amino acids during the 3 h postprandial period. The authors attributed this difference to the rapid increase in plasma amino acids evoked during the first 40 min of the digestive period, during which the increase was about 37 percent greater after the ingestion of whey protein hydrolysate solution than that after ingestion of the intact milk protein solution.

It is likely that the high concentrations of plasma amino acids and increased insulin explains the superiority of protein hydrolysates over intact proteins in promoting nitrogen utilisation. The co-ingestion of carbohydrate appears to affect the absorption kinetics, as one study showed that whey and casein proteins and their respective hydrolysates administered alone produce similar rates of intestinal absorption of amino acids. Alternatively, it is possible that this study used protein hydrolysates containing mainly oligopeptides [06143].

It was also determined the extent to which the combined ingestion of high-glycaemic CHO and a casein protein hydrolysate with or without additional free leucine can increase insulin concentrations during post-exercise recovery. Fourteen male athletes were participated in three randomised crossover trials in which they performed 2 h of exercise. Thereafter, the subjects were studied for 3.5 h during which they ingested CHO only (0.8 g/kg/h), CHO + protein hydrolysate (0.8 and 0.4 g/kg/h, respectively), or CHO + protein hydrolysate + free leucine (0.8, 0.4, and 0.1 g/kg/h, respectively) in a double-blind fashion. The results revealed that plasma insulin responses were 108 percent and 190 percent greater in the CHO + protein hydrolysate and CHO + protein hydrolysate + leucine trial, respectively, compared with the CHO only trial. The study also indicated that addition of free phenylalanine, as applied in earlier studies, is not necessary to obtain such high post-exercise insulin responses. Similarly, it was examined plasma insulin responses after co-ingestion of casein protein hydrolysate with and without additional free leucine with a single bolus of high-glycaemic CHO. Again, the subjects participated in three trials in which blood insulin responses were determined after the ingestion of beverages of different composition: CHO only (0.7 g/kg), CHO + protein hydrolysate (0.7 and 0.3 g/kg, respectively) or CHO + protein hydrolysate + free leucine (0.7, 0.3 and 0.1 g/kg, respectively). The result indicated that plasma insulin responses were 66 and 221 percent greater in the healthy subjects in the CHO + protein hydrolysate and CHO + protein hydrolysate + free leucine trials, respectively, compared with those in the CHO only trial. In other words, this study also showed that co-ingestion of a protein hydrolysate with additional leucine strongly augments insulin secretion after the consumption of a single bolus of CHO [06143].

The notion that the protein hydrolysates have strong insulinotropic properties is also supported by the studies examining the effects of intact protein-containing post-exercise drinks. Ivy et al[46] compared the effects of CHO + intact protein (80 g CHO, 28 g protein, 6 g fat), low-CHO (80 g CHO, 6 g fat), or high-CHO (108 g CHO, 6 g fat) and reported that plasma insulin concentrations did not differ at any time among treatments. However, it has also been observed that plasma insulin concentrations for the CHO + intact protein treatment (112 and 40.7 g, respectively) were somewhat higher than those for the CHO treatment (112 g CHO). A post-exercise drink containing a mixture of free amino acids also has a potent effect on insulin secretion. However, a large dose of amino acids can cause gastrointestinal discomfort. This may have something to do with the drink's osmolarity. A protein hydrolysate containing dipeptides and tripeptides reduces osmolarity because equal solution weights of dipeptides and tripeptides have the same and one-third the osmolarity of free amino acids, respectively [06143].
A sophisticated study investigated post-exercise muscle protein synthesis and whole body protein balance after the combined ingestion of high-glycaemic CHO with or without whey protein hydrolysate and/or leucine. Their nutritional protocol was rather rigorous; the subjects received a beverage volume of 3 ml/kg every 30 min to ensure a given dose of 0.3 g high-glycaemic CHO/kg and 0.2 g of a protein hydrolysate/kg every hour, with or without the addition of 0.1 g/kg/h free leucine. Repeated boluses were taken every 30 min until 330 min after exercise. The results revealed that the rates of whole body protein synthesis were highest in the CHO + protein hydrolysate + leucine trial. Similarly, the fractional synthetic rate in the vastus lateralis muscle was significantly higher in the CHO + protein hydrolysate + leucine trial than the CHO trial, with intermediate values observed in the CHO + protein hydrolysate trial. Furthermore, the investigators found plasma insulin responses to correlate negatively with whole body protein degradation, whereas whole body protein synthesis correlated positively with plasma insulin response. However, the fractional synthetic rate did not correlate with the plasma insulin response, whereas the mixed muscle protein fractional synthetic rate did correlate with the amount of leucine that was ingested. It is difficult to interpret these results given the massive supplementation. Nevertheless, the authors concluded that, “the additional ingestion of free leucine in combination with protein and carbohydrate likely represents an effective strategy to increase muscle anabolism following resistance exercise.” Other recent studies have shown that relatively small doses of leucine can improve exercise performance and enhance the acquisition of strength [06143].

**Protein shake**

Contrary to popular belief, higher protein intake has no adverse effects on healthy kidneys, fluid status, or bone. In fact, proteins appear to have positive effects on bone health, as they increase circulating insulin-like growth factor I (IGF-I), which plays an important role in bone formation. It was reported that a protein supplement during a strength and conditioning programme led to an increase in plasma concentrations of IGF-I in those subjects compared with the concentrations in a group who also trained but consumed an isocaloric CHO supplement. Also, serum bone alkaline phosphatase concentrations increased over time and tended to be higher in the protein group than in the CHO group, indicating increased bone formation. In addition, IGF-I plays a critical role in development, growth, repair, and maintenance of skeletal muscle. Thus, it may partially explain why many strength/power athletes (especially bodybuilders) feel that a very high protein intake is beneficial for skeletal muscle hypertrophy. Indeed, studies indicate increased positive nitrogen balance when protein intake is increased. Traditionally, the term “protein requirement” has meant the amount of dietary protein that must be consumed to provide the amino acids needed for the synthesis of those proteins irreversibly catabolised in the course of the body's metabolism. It should be noted, however, that strength/power athletes are not concerned with the minimum amount of protein necessary to sustain normal body functions, but, rather, their absolute gains in muscle mass and strength. Other potential benefits of higher protein intake should be considered too. Interestingly, a placebo-controlled study reported that post-exercise protein/CHO supplementation [06143]:

- reduces bacterial/viral infections
- decreases the number of medical visits for muscle or joint problems
- diminishes episodes of heat exhaustion
- reduces muscle soreness
- improves rifle scores in US marine recruits during basic training.
Effects of post-exercise hyperinsulinaemia on fat oxidation and de novo lipogenesis

The chief lipid-related functions of insulin are inhibition of lipolysis and lipid oxidation (at 13 and 44 microU/ml, respectively). When insulin concentration falls below 13 microU/ml, lipolysis is powerfully and exponentially stimulated. It was reported that a very-low-CHO diet significantly decreased serum insulin (~34 %) and that about 70 percent of the variability in fat loss was accounted for by the decrease in serum insulin concentrations. Further, exogenous insulin promotes body fat accumulation, so one could speculate that insulinotropic supplements have similar effects. However, I feel this is hardly a concern for healthy athletes when these supplements are ingested immediately after rigorous exercise when the muscle cells are highly receptive to insulin and “screaming” for new fuel. The physiological state of a sedentary person and that of a well-trained athlete after exercise are poles apart. AMP-activated protein kinase acts as a “metabolic switch” in multiple tissues after exercise; the net effect of its activation is to increase fatty acid oxidation and diminish glycerolipid synthesis [06143].

To investigate the hormonal and metabolic adaptations occurring when high-glycaemic CHOs are ingested after exercise, it was compared the fate of a 100 g oral glucose load in healthy volunteers after an overnight fast at rest either without previous exercise or after 3 h of exercise performed on a treadmill at about 50 percent of the individual VO2max. Indirect calorimetry indicated that glucose ingestion in post-exercise recovery was associated with decreased CHO oxidation and increased lipid oxidation when compared with control conditions. It has also been reported that de novo lipogenesis was totally suppressed after exercise, even when a very large CHO load was ingested, and that fat oxidation remained high in subjects who had exercised after both the small and large CHO meal. Certainly, this does not mean that insulinotropic supplements should be used while watching TV. It is possible that the hyperinsulinaemic condition prevalent in obese, insulin-resistant individuals is responsible for the repartitioning of fatty acids away from oxidation and toward storage [06143].

Anti-inflammatory effects of insulin

It has been proposed that insulin is the ideal anti-inflammatory agent for critically ill patients, because it normalises plasma glucose concentrations (glucose is proinflammatory) while exerting its anti-inflammatory effect. In addition, insulin suppresses generation of reactive oxygen species and the expression of p47phox, a key component of NADPH oxidase, the enzyme that generates the superoxide radical. Thus, insulin has antioxidant effects too. As strenuous exercise produces muscle inflammation as well as increases generation of reactive oxygen species, it is possible that post-exercise hyperinsulinaemia offers additional benefits beyond muscle protein synthesis [06143].

Laboratory techniques

Insulin, a polypeptide hormone secreted by pancreatic cells, is a key regulator in glucose homeostasis. Its deficiency leads to insulin-dependent (type I) diabetes whereas resistance to insulin is common in type II diabetes, obesity and a range of endocrine disorders. Its determination is of considerable value, particularly in the clinical diagnosis of diabetes mellitus and the doping control of athletes. It has, additionally, been noted as a potential breast cancer marker (serum insulin levels being found to be raised in comparison to control patients). Electrochemical assays are potentially very cheap, highly sensitive, and very
readily transposed to a point of care. Though there exist numerous examples of label free impedimetric or capacitative assaying of biomolecules, these are rarely demonstrated to be effective in complex biological mixtures or to be applicable to low molecular weight targets (since they operate through the interfacial displacement of water/ions and/or the steric blocking of a redox probe). It was reported an ultrasensitive electrochemical and label-free biosensor for insulin in blood serum with a clinically relevant linear range and detection limit of 1.2 pM. The transducing surfaces, based on readily prepared, antibody modified, polyethylene glycol monolayer modified polycrystalline gold surfaces, respond in a highly specific and re-useable manner to the target in up to 50 percent blood serum [12222].

Insulin and related synthetic therapeutics have been prohibited by the World Anti-Doping Agency for athletes demonstrably not suffering from diabetes mellitus. The primary specimen for doping controls has been urine, but the renal excretion of intact human insulin as well as synthetic analogues such as the rapid-acting products Humalog LisPro, Novolog Aspart, and Apidra Glulisine has been reported negligible owing to metabolic degradation. Nevertheless, employing solid-phase extraction in combination with immunoaffinity purification followed by a top-down sequencing-based mass spectrometric approach, an assay was established allowing the identification of three intact rapid-acting synthetic insulins in doping control urine samples. A volume of 25 mL of urine was concentrated, insulin analogues were isolated from the concentrate by immunoaffinity chromatography, and the eluate was analyzed using microbore liquid chromatography/tandem mass spectrometry. Characteristic product ion spectra obtained from 5-fold protonated intact analytes as well as isolated insulin B-chains allowed the unambiguous identification of target analytes with detection limits of 0.05 ng/mL (9 fmol/mL). Moreover, assay validation demonstrated recoveries between 72 and 80 percent for Humalog LisPro, Novolog Aspart, and Apidra Glulisine, and assay precisions ranged from 9 to 16 percent. A reliable tool is provided that allows the qualitative determination of rapid-acting insulins in urine specimens collected for sports drug testing [06144].
OTHER DEFINED PEPTIDE HORMONES

Gonadotropins

The administration of gonadotropins is prohibited in sport but the effect in men of recently available recombinant hCG and LH on serum and urine concentrations of gonadotropins and androgens has not been systematically evaluated in the anti-doping context. To determine the time-course of recombinant LH (LHrh) and hCG (rhCG) on blood and urine hormone profiles in men to develop effective tests to detect rhLH and rhCG doping. Two randomized controlled studies with a 2 x 2 factorial design used healthy male volunteers aged 18-45 years. In the rhLH study, men were randomized into (a) either of two single doses of rhLH (75 IU or 225 IU) and (b) suppression of endogenous LH and testosterone by nandrolone or no suppression. In the rhCG study men were randomized into (a) either of two single doses of rhCG (250 mug or 750 mug) and (b) suppression of endogenous LH and testosterone by nandrolone decanoate or no suppression. Nandrolone suppression comprised a single dose of 200 mg nandrolone decanoate 3 days prior to, and in the rhCG study an additional dose 1 day after, gonadotropin injection. Neither rhLH dose produced a significant increase in serum or urine LH or testosterone or in the T/E or T/LH ratios regardless of nandrolone-induced suppression of endogenous LH and testosterone. Nor did an even higher dose (750 IU) in 3 healthy men with unsuppressed gonadal axis. These findings were confirmed with two different commercial LH immunoassays together with adjustment for any influence of urine sediment and dilution. Both rhCG doses produced a steep, dose-proportional increase in serum and urine hCG with increases in serum and urine testosterone and suppression of serum and urine LH, regardless of hCG dose. Serum but not urine testosterone was lowered by nadrolone suppression. The testosterone/LH ratio showed a progressive increase unrelated to rhCG dose or nandrolone suppression whereas both rhCG and nandrolone suppression minimally increased T/E ratio. This means that both rhCG doses produce a striking increase in serum hCG and testosterone with suppression of serum LH but, at single doses up to 750 IU, rhLH has no influence on serum or urine LH or testosterone. Effective rhLH doping, which relies on a sustained increases in endogenous testosterone, would require much higher and more frequent daily rhLH doses. Use of LH immunoassays optimised for serum to detect rhLH doping by urine LH measurement requires more standardisation and validation and, at present, is unreliable. The testosterone/LH ratio is, however, a useful screening test for hCG doping although its utility requires further evaluation [09186].

Parenteral administration of human chorionic gonadotropin (hCG) or luteinizing hormone (LH) stimulates the production of testosterone in males and these gonadotropins can therefore be used by athletes to enhance muscle strength. However, they are more expensive and less efficient than testosterone and anabolic steroids. Therefore their main use is probably to stimulate gonadal testosterone production during and after self-administration of testosterone or anabolic steroids. A positive effect of hCG on muscle strength has not been demonstrated in women and elevated concentrations of hCG in females are often caused by pregnancy. The use of gonadotropins is therefore prohibited only in males but not in females. HCG occurs at low but measurable concentrations in plasma and urine of healthy males and can be measured by sensitive methods. However, the characteristics of the method to be used for doping control have not been defined. Virtually all commercially available hCG assays have been designed for determination of hCG in serum rather than urine, which is used for doping control. Methods based on mass spectrometric detection of fragments derived from hCG extracted from urine by immunoadsorption have been developed but their suitability for doping control remains to be
determined. The concentrations of LH in serum and urine are variable and more then 10-fold higher than those hCG. It is therefore difficult to detect illicit use of LH. The characteristics and reference values for hCG and LH assays used in doping control and the cutoff values need to be defined [08287].

Doping with (glyco)protein hormones represent an extremely challenging, analytical problem as nearly all are constitutively present at low concentrations that fluctuate according to circadian or alternative periodical, or external stimuli. Thus the mere concentration in a biological sample is only resolutive when this surpasses extreme values. As the vast majority of these molecules are produced by recombinant DNA technology it is believed that the exogenous molecules could bear the signature of the host cell. In particular, these could comprise structural differences originated from co or post-translational differences. In one study it was employed both proteomics and glycomics strategies to compare recombinant and urinary human chorionic gonadotrophin in order to evaluate this hypothesis. As anticipated the recombinant hormone could be shown to contain N-glycolyl neuraminic acid, a sialic acid that cannot be produced by humans. Furthermore, differences were observed in the overall glycosylation, in particular the presence of abundant hybrid-type glycans that were much less pronounced in the recombinant species. These differences were determined to occur predominantly in the alpha-subunit for which antidoping strategies focussed on these elements could be used for both chorionic gonadotrophin and lutrophin as they share the same alpha-subunit [08288].

Concentrations of intratesticular (IT) testosterone (T) are known to be 100-200 times those of serum T; however, the IT concentrations of T’s precursors, their testicular to serum gradients, gonadotropin dependence, and response to stimulation with human chorionic gonadotropin (hCG) have not been studied in detail. It was hypothesized that serum and IT androstenedione (ADD) and IT dehydroepiandrosterone (DHEA) would be significantly suppressed by the administration of a GnRH antagonist and increased when stimulated by hCG, without a similar suppression of serum DHEA. It was suppressed gonadotropins in 23 normal men with the GnRH antagonist acyline and randomly assigned them to one of four doses of hCG, 0, 15, 60, or 125 IU sc every other day for 10 d. Blood and IT fluid for the measurement of serum and IT hormones were obtained at baseline and after 10 d of treatment. Baseline IT ADD was 629 nmol/liter, and IT DHEA was 564 nmol/liter, which were 175 and 27 times higher than their respective serum concentrations. IT ADD and IT DHEA were suppressed by 98 and 82 percent, respectively, by acyline and significantly increased with hCG administration. Likewise, serum ADD was suppressed by 50 percent, but serum DHEA was unchanged. It was thus found that ADD and DHEA are highly concentrated within the human testes compared with serum. Serum and IT ADD and IT DHEA are markedly suppressed with GnRH administration and stimulated by hCG, but serum DHEA is not, suggesting that most circulating DHEA is not of testicular origin [11174].

The synthesis and secretion of the gonadotropic hormones involves coordination of signal transduction, gene expression, protein translation, post-translational folding and modification and finally secretion. The production of biologically active gonadotropin thus requires appropriately folded and glycosylated subunits that assemble to form the heterodimeric hormone. It was overviewed recent literature on regulation of gonadotropin subunit gene expression and current understanding of the assembly and secretion of biologically active gonadotropic hormones. Finally, it was discussed the therapeutic potential of understanding glycosylation function towards designing new forms of gonadotropins based on observations of physiologically relevant parameters such as age related glycosylation changes. The glycoprotein hormone family includes lutinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH) and chorionic gonadotropin (CG). Production of these hormones occurs in either the pituitary (LH, FSH, TSH) or by the chorion of a
developing fetus (CG). These hormones are heterodimeric, consisting of a common alpha subunit and a unique beta subunit, and they are also glycosylated. Therefore, their production requires coordination of transcriptional regulation of the subunit genes [11476].

**Human chorionic gonadotropin**

Human chorionic gonadotrophin (hCG) is routinely analyzed in doping control samples of males athletes due to its ability to stimulate the testicular production of testosterone. Today, two immunoassays are recommended by WADA to be used as initial testing and confirmation tools. The implementation of such immunoassays into sports drug testing routine protocols demonstrated the fitness-for-purpose of both the screening (Immulite 2000 XPi) and the confirmatory (Delfia Xpress) test for human urine. While the initial testing assay recognizes several different hCG variants including the intact heterodimeric hCG and the beta-core fragment of hCG, the Delfia Xpress confirmation test detects intact hCG and nicked hCG only. Eventually, only urinary concentrations of intact hCG exceeding 5 IU/L should be considered as indication for an anti-doping rule violation [13009].

Determination of human chorionic gonadotropin (hCG) is used for diagnosis and monitoring of pregnancy, pregnancy related disorders, for trophoblastic and some nontrophoblastic tumors. In addition, hCG is determined for doping control in males. Assay of hCG is complicated by the occurrence of different molecular forms, which are detected to various degrees by different assays. The main form of hCG in circulation and in patients with trophoblastic tumors is intact heterodimeric hCG. The free beta subunit (hCG-beta) is a minor form in plasma in both conditions, but it may be the major form in aggressive trophoblastic cancer. Therefore, assays measuring hCG and hCGβ together are mainly used for diagnosis of pregnancy and trophoblastic diseases. When excreted into urine, most of hCG (and hCG-beta) is broken down to the core fragment of hCGβ (hCG-beta-cf), which is the main immunoreactive form of hCG in urine during pregnancy. Specific determination of hCG-beta is of value in screening for Down's syndrome and diagnosis of nontrophoblastic cancer. hCG-beta-cf is of limited utility but it is important because it may disturb assay of hCG in pregnancy [13392].

Human chorionic gonadotrophin (hCG) as well as LH are prohibited in sports (for males only) due to their ability to stimulate testosterone production and release. Both substances are commonly analyzed by ELISA, which was recently shown to be critical particularly concerning hCG if urine samples collected for doping controls are stored at -20°C. In a study a significant loss (up to 100 %) of immunoreactive hCG was observed in urine specimens stored at -20°C, attributed to a negative impact of urea. Noteworthy, at +4°C and -80°C the same samples (with identical urea concentrations) did not decrease in hCG immunoreactivity, which might be relevant to consider in case of doping control sample transportation and storage. In a complementary and indirect manner, the option to determine hCG abuse in sport by steroid profiling as well as LH measurements in blood and urine was evaluated. While commonly used urinary steroid profiles as such did not exhibit the required sensitivity to detect hCG abuse, accurate quantification of the testosterone concentration, T/LH ratio as well as the direct analysis of hCG were found to reliably contribute to an efficient detection of hCG abuse [13012].

Human chorionic gonadotrophin (hCG) is dimeric glycoprotein produced by placenta in pregnancy and also in low levels by pituitary gland. The main clinical use for exogenous hCG-administration is typically linked to infertility. The desired effect of hCG misuse in sport is due to the enhancement of testicular production of testosterone. Therefore, hCG is listed by the World Anti-Doping Agency (WADA) as a prohibited substance in male athletes and
according to the recently published WADA guideline urinary concentrations of hCG > 5 IU/L may be an indicator of doping. In this study two independent immunoassays were used to implement the new WADA guideline. The assay for initial testing (Siemens Immulite 2000 XPi hCG assay) recognizes various hCG variants (e.g. hCG and β-core fragment of hCG) whereas the confirmatory assay (PerkinElmer DELFIA Xpress hCG) is sensitive to intact and nicked hCG only. Both assays showed adequate sensitivity and were proven fit-for-purpose in routine doping control. Population-based distribution of the assays was in good agreement with results of earlier studies and supported well the current threshold of 5 IU/L [13393].

The performance of a method for MS determination of human chorionic gonadotropin (hCG) was compared with a reference method currently used in World Anti-Doping Agency accredited doping laboratories - the DELFIA® immunoassay. A strong correlation was demonstrated for the serum samples. However, for the urine samples, DELFIA reported significantly lower quantitative hCG measurements than the MS method. This was explained by the relatively unstable content of intact hCG-heterodimer in urine during storage compared with in serum. Discrepancies observed for the urine analyses might be related to the molecular dissociation of intact hCG-heterodimer into free subunits during storage, and the direct effect this has on the intact hCG measurements provided by DELFIA. The MS method quantified both intact hCG and free hCG β-subunit simultaneously, and was thus less susceptible to this problem. However, both methods detected illicit levels of serum hCG an equally long time after administration. The presented work advocated the implementation of this MS method as a confirmatory method for hCG determination in doping laboratories [13394].

Human chorionic gonadotropin (hCG) is dimeric glycoprotein produced by placenta in pregnancy and also in low levels by pituitary gland. The main clinical use for exogenous hCG-administration is typically linked to infertility. The desired effect of hCG misuse in sport is due to the enhancement of testicular production of testosterone. Therefore, hCG is listed by the World Anti-Doping Agency (WADA) as a prohibited substance in male athletes and according to the recently published WADA guideline urinary concentrations of hCG > 5 IU/L may be an indicator of doping. In this study two independent immunoassays were used to implement the new WADA guideline. The assay for initial testing (Siemens Immulite 2000 XPi hCG assay) recognizes various hCG variants (e.g. hCG and beta-core fragment of hCG) whereas the confirmatory assay (PerkinElmer DELFIA Xpress hCG) is sensitive to intact and nicked hCG only. Both assays showed adequate sensitivity and were proven fit-for-purpose in routine doping control. Population-based distribution of the assays was in good agreement with results of earlier studies and supported well the current threshold of 5 IU/L [13395].

Regulation of chorionic gonadotropin expression is less well understood in part because no obvious placental signal has been identified. Chorionic gonadotropins have been found in only two groups of mammals, primates and equids. When only human and horse chorionic gonadotropins were well characterized, chorionic gonadotropin expression was apparently exclusively placental in humans, as a separate pituitary LH existed, whereas in the horse, pituitary LH and placental CG were found to be comprised of identical polypeptides. In the human, the CGβ gene appeared to be derived from an ancestral LHβ gene, which was located on the same chromosome and in the same gene cluster. In the horse it was not known if a similar gene duplication was followed by loss of one gene, as donkeys and zebras also appear to possess only a LH/CGβ-like gene. Recent studies of new world monkey CG may provide an example of a path from separate LH and CG β-subunit genes to a single LH/CGβ gene. The common marmoset (Callithrix jacchus) was found to possess both LH and CGβ-subunit genes, however, only the CGβ gene was expressed in the pituitary. As the structural gene appeared normal, the absence of expression probably reflects changes in regulatory elements. Two other new world monkeys, the squirrel monkey (Saimiri
Sciureus) and the owl monkey (Aotus trivirgatus) were also reported to express only the CGbeta gene in the pituitary. The potential for pituitary expression of CGbeta exists even in humans, as the presence of pituitary-derived hCG was confirmed by its possession of pituitary-specific sulfated glycans [11476].

The objective of the study was to review the rationale underlying the banning of human chorionic gonadotropin (hCG) and estrogen blockers (antiestrogens, specific estrogen receptor modulators, aromatase inhibitors) in sports for male and female athletes in the light of gender differences in regulation of reproductive physiology. It was reviewed well-controlled clinical studies of exogenous testosterone effects on human muscle size and strength in men and all available evidence relevant to the effects of hCG and estrogen blockers on blood testosterone in men and women. Well-designed placebo-controlled clinical studies in men with suppressed pituitary-testicular axis establish a strong case that, across a wide range from sub- to supraphysiological doses, muscle growth and strength is proportional to exogenous testosterone dose and resulting blood testosterone concentrations. In men, there is unequivocal evidence that hCG and estrogen blockers cause consistent and sustained rise in blood testosterone concentrations. In women, although there has been no direct testing of ergogenic or myotrophic properties of exogenous testosterone in healthy women, either hCG or estrogen blockers do not produce any consistent or biologically significant increase blood testosterone concentrations. It was concluded that in men undergoing potential stimulation of endogenous blood testosterone concentrations, blood testosterone concentration is a reasonable surrogate measure for muscle growth and increased strength in men. Because hCG and estrogen blockers produce marked increase in blood testosterone concentration in men, this provides strong evidence to support the banning of hCG and estrogen blockers in men. In women, however, the negligible effect on blood testosterone suggests that drug-induced performance enhancement by hCG or estrogen blockers is highly unlikely. Furthermore, routine urinary hCG testing in young women risks invasion of privacy by detecting unrecognized pregnancy. These considerations suggest that prohibition of hCG and estrogen blockers should be restricted to men in which they are well justified [06127].

Although there is increasing concern about the possible abuse of erythropoietin, GH, and glucocorticoids, the major form of sports doping remains androgen abuse. Although androgen physiology differs fundamentally in men and women, at suitable doses exogenous androgens enhance muscle mass and strength in all athletes. As a result, since the early 1970s exogenous androgens have been banned for men and women in sports. This ban has been enforced by urine testing using mass spectrometry-based methods to detect illicit administration of natural and synthetic androgens. The notorious toleration of androgen abuse in U.S. baseball and football, undermining both the integrity of sport and its respectability as the pinnacle of healthfulness, exemplifies the likely fate of other sports if they failed to go beyond outmoded and laissez faire self-regulatory policing of drug cheating. In recent years, human chorionic gonadotropin (hCG) and estrogen blockers were added to the WADA Prohibited List for women as well as men. Whereas banning these agents appears well justified for men, there are doubts about the validity as well as adverse privacy implications for hCG testing when applied to female athletes. Similarly, the justification for prohibiting estrogen blockers in women is also dubious based on the absence of any plausible mechanism for potential estrogen-mediated ergogenic effects. One review focused on the reproductive endocrinology of these agents in men and women and the validity of their prohibition [06127].

The presence of proteolytic enzymes in urine samples, coming from exogenous or endogenous sources, enhances the cleavage of human chorionic gonadotropin (hCG). Moreover, elevated temperatures occurring occasionally during the delayed transportation of sport urine samples, favor the nicking of the hCG molecule. The aim of one study, funded by
the World Anti-Doping Agency (WADA), was the application of a stabilization mixture in athletes' urine samples to chemically inactivate proteolytic enzymes coming from exogenous or endogenous sources so as to prevent the degradation of hCG. The stabilization mixture applied, already tested for the stabilization of endogenous steroids and recombinant erythropoietin (rEPO), was a combination of antibiotics, antimycotic substances, and protease inhibitors. Incubation experiments were conducted in the presence or absence of the stabilization mixture in urine aliquots spiked with six proteases (first series of experiments) and one microorganism associated with urinary tract infections (UTI) (second series of experiments). Intact hCG levels were evaluated by using the EIAgene Total hCG kit. In the first series of experiments, hCG levels were reduced in the untreated aliquots following incubation at 37 degrees C. The addition of the chemical stabilization mixture prevented degradation of hCG induced by four of the proteases applied. In the second series of experiments, no significant difference was found in urine inoculated with E. coli, between aliquots treated with chemical mixture and the untreated aliquots. The addition of the proposed chemical stabilization mixture improves the quality of athletes' urine samples against possible deterioration due to high temperatures or attempts of proteolytic manipulation [10367].

The presence of proteolytic enzymes in urine samples, coming from exogenous or endogenous sources, enhances the cleavage of human chorionic gonadotropin (hCG). Moreover, elevated temperatures occurring occasionally during the delayed transportation of sport urine samples, favor the nicking of the hCG molecule. The aim of one study, funded by the World Anti-Doping Agency (WADA), was the application of a stabilization mixture in athletes' urine samples to chemically inactivate proteolytic enzymes coming from exogenous or endogenous sources so as to prevent the degradation of hCG. The stabilization mixture applied, already tested for the stabilization of endogenous steroids and recombinant erythropoietin (rEPO), was a combination of antibiotics, antimycotic substances, and protease inhibitors. Incubation experiments were conducted in the presence or absence of the stabilization mixture in urine aliquots spiked with six proteases (first series of experiments) and one microorganism associated with urinary tract infections (UTI) (second series of experiments). Intact hCG levels were evaluated by using the EIAgene Total hCG kit. In the first series of experiments, hCG levels were reduced in the untreated aliquots following incubation at 37 degrees C. The addition of the chemical stabilization mixture prevented degradation of hCG induced by four of the proteases applied. In the second series of experiments, no significant difference was found in urine inoculated with E. coli, between aliquots treated with chemical mixture and the untreated aliquots. The addition of the proposed chemical stabilization mixture improves the quality of athletes' urine samples against possible deterioration due to high temperatures or attempts of proteolytic manipulation [10367].

Human chorionic gonadotropin (hCG) is a 37-kDa glycoprotein predominantly produced by the placenta during pregnancy, and its use has been prohibited for male athletes since 1987. Its ability to induce the secretion of endogenously produced testosterone from testes has necessitated its detection in doping controls, which have primarily been accomplished using immunological methods. A new approach was presented, based on immunoaffinity purification followed by trypsin digestion and subsequent LC-MS/MS analysis of marker peptide beta-T5. Thus, qualitative and quantitative evidence for hCG was obtained, allowing detection limits of 5 mIU/mL of urine to be obtained. This approach provided the discriminative power required, which common immunological test methods lack due to possible cross-reactivity [07050].

The principal objective of one study was to compare the analytical performance of the Elecsys2010 (Roche Diagnostics) system with the IMx (Abbott laboratories) system for beta-
hCG assay in order to assess its possible utility as a confirmation test for the quantitative measurement of beta-hCG in urine for doping control purposes. Urine samples with spiked standard known concentrations of beta-hCG and different urine samples from athletes were used in order to determine the calibration curve stability and linearity, detection limit, total, within-run and between-run precision, and method comparison for the IMx and Elecsys2010 systems for beta-hCG assay, along with the stability of samples, at room temperature and at 4 degrees C. The IMx assay was linear up to 500 IU/L, whereas the Elecsys2010 assay was linear up to 1000 IU/L. The detection limit for the IMx and Elecsys2010 systems were 0.75 IU/L and 0.25 IU/L, respectively. The total precision of the IMx and Elecsys2010 systems were \( \leq 5 \) percent for beta-hCG concentrations ranging from 20 to 900 IU/L and from 7 to 55 IU/L, respectively. The within-run precision tests for the IMx and Elecsys2010 systems yielded CV results of 2-3 percent and 3-4 percent respectively, while the between-run precision for the Elecsys2010 was less than 4 percent, whereas that of IMx was 9-16 percent. Sample stability studies evidenced a nonsignificant effect at 4 degrees C and room temperature (25 degrees C) for up to 1 month. It was concluded that for doping control, the IMx and Elecsys2010 may be used to screen and confirm the presence of beta-hCG in urine of athletes, respectively. Additional studies are needed to verify this finding. This study also underscores the need for beta-hCG assays standardization for doping control [07159].

Several factors influencing the carbon isotope ratios (CIR) of endogenous urinary steroids have been identified in recent years. One of these should be the metabolism of steroids inside the body involving numerous different enzymes. A detailed look at this metabolism taking into account differences found between steroids excreted as glucuronides or as sulphates and hydrogen isotope ratios of different steroids pointed out possibility of unequal CIR at the main production sites inside the male body – the testes and the adrenal glands. By administration of beta-HCG it is possible to strongly stimulate the steroid production within the testes without influencing the production at the adrenal glands. Therefore, this treatment should result in changed CIR of urinary androgens in contrast to the undisturbed pre-treatment values. Four male volunteers received three injections of beta-HCG over a time course of 5 days and collected their urine samples at defined intervals after the last administration. Those samples showing the largest response in contrast to the pre-administration urines were identified by steroid profile measurements and subsequent analysed by GC/C/IRMS. CIR of androsterone, etiocholanolone, testosterone, 5alpha- and 5beta-androstanediol and pregnanediol were compared. While pregnanediol was not influenced, most of the investigated androgens showed depleted values after treatment. The majority of differences were found to be statistically significant and nearly all showed the expected trend towards more depleted delta\(^{13}\)C-values. These results support the hypothesis of different CIR at different production sites inside the human body. The impact of these findings on doping control analysis will be discussed [12202].

Human chorionic gonadotrophin (hCG) is measured on a routine basis in all doping control urine samples collected from male athletes, most commonly by means of immunological methods. Stability issues have been observed particularly with hCG in the past. In the course of establishing a generic urine sample preservation protocol, the impact of chemical protease inhibitors on the stability of hCG in doping control urine samples at different storage temperatures was tested. Degradation of hCG was reduced by four of the tested proteases and temperature rather than bacterial contamination was found to be a relevant aspect in degradation tests [12016].

The applicability of a mass spectrometry (MS)-based method for determination of various forms of human chorionic gonadotropin (hCG) in doping analysis was demonstrated. A clinical study involving the hCG-containing pharmaceuticals Pregnyl and Ovitrelle was carried out, comprising a single injection of one pharmaceutical per participant to a total of 24
healthy male voluntaries. Hereafter, serum and urine samples were collected over a period of 14 days. The analysis of the samples using immuno-MS demonstrated elimination profiles of intact hCG for both pharmaceuticals, with last day of detection following administration at day 7 in serum, and at day 10 in urine, at limit of detections as defined by the World Anti-Doping Agency. Furthermore, the method allowed detection and differentiation of the various forms of hCG known to be present in serum and urine as a function of metabolism. For both pharmaceuticals, only the intact hCG was detected in serum, whereas in urine the injection of Pregnyl as hCG source (containing urinary hCG, i.e., most hCG variants) was shown to generate a more complex hCG variant pattern compared to Ovitrerelle (contains only intact hCG). By detecting hCG using this MS-based approach in doping analysis, strong analytical evidence is provided minimizing the risk of false-positive and false-negative results [12203].

Physiology
hCG is a dimeric glycoprotein consisting of an alpha- and beta-subunit normally produced by the human placenta. The alpha-subunit is the product of a single copy gene identical with the alpha-subunit of the other three pituitary glycoprotein hormones LH, FSH, and TSH. The β-beta-subunit of hCG is derived from a multicopy gene arising by duplication of the homologous single copy LH beta-subunit gene. Crucially, hCG beta-subunit includes a read-through C-terminal extension of 29 amino acids, containing four O-linked sialic acid capped glycosylation sites that markedly prolong the circulating half-life and biopotency of hCG, making it a naturally occurring long-acting analog of LH. Endogenous hCG is produced by the normal placenta in pregnancy or placental trophoblastic (hydatidiform mole, choriocarcinoma), gonadal (ovarian, testicular or extragonadal teratoma), or ectopic and nontrophoblastic tumors. In clinical practice, the identification of hCG immunoreactivity in blood or urine is used for early pregnancy diagnosis as well as a tumor marker. Biologically active heterodimeric hCG is manufactured pharmaceutically as a biological product either purified from human pregnancy urine or as a recombinant glycoprotein purified from genetically engineered mammalian cells [06127].

Effects in men
For men, the prohibition on hCG and estrogen blockers is unequivocally justified. Both produce sustained and significant increases in endogenous testosterone production and blood testosterone concentrations. Whereas there are no direct studies of the ergogenic effects of hCG or of estrogen blockers, the case for prohibition is well established using blood testosterone concentrations as a reliable surrogate variable for increases in muscle mass and strength. One study of 40 healthy older men (>60 yr of age) showed increased lean (muscle) mass but no increase in shoulder or knee strength measured by dynamometry during 3 months treatment with recombinant hCG, compared with placebo. It is likely, however, that the modest, replacement dosage and older age of the participants may underestimate the ergogenic potential of hCG for elite male athlete who would seek to abuse this drug [06127].

Clinically, hCG is used as a naturally occurring long-acting and potent LH analog. The only legitimate clinical indication for hCG is to restore endogenous testosterone production and normalize blood testosterone concentrations in gonadotrophin-deficient men including delayed male puberty. There are virtually no proven off-label uses for hCG in routine clinical endocrinology practice. Among androgen abusers, however, hCG is apparently misused by male athletes in two settings according to the underground androgen abuse folklore. In one scenario men who have developed sustained inhibition of their hypothalamo-pituitary testicular axis from prolonged high-dose androgen abuse seek to rectify this by increasing testicular testosterone production using hCG. In reality, this continues hypothalamic-pituitary suppression, which causes the reduced testis size and testosterone production and defers the problem of hypothalamo-pituitary recovery, which is usually slow but complete, so that
hCG treatment, although feasible is rarely justified clinically. The other setting is that of androgen abusers seeking to avoid detection of synthetic androgens or exogenous testosterone by stimulating endogenous testosterone production. Preliminary information suggests that the testosterone-to-epitestosterone (T/E) ratio is unaffected by hCG treatment consistent with its stimulation of endogenous testosterone production by Leydig cells, producing a characteristic testosterone to estrogen ratio for that individual, which is no different from natural endogenous testosterone production [06127].

In normal men, hCG produces a sustained and dose-dependent increase in blood testosterone concentrations through stimulation of Leydig cell testosterone secretion. This is well established for purified urinary and recombinant hCG. Typically, the basal blood testosterone concentrations (about 20 nmol/liter) are increased to concentrations of 30-40 nmol/L, peaking between 2 and 4 d after a single injection at typical clinical doses. Both the peak blood testosterone responses and the time of peak are log-dose dependent for purified urinary and recombinant hCG. These markedly increased blood testosterone concentrations, with increments of 10-30 nmol/L, are within the range defined experimentally as having a log-linear relationship with increased muscle mass and strength in men. They are therefore highly likely to increase muscle mass and strength. Hence, prohibition of hCG for men is well justified. An unintended consequence of testing for male athletes urine for hCG is the incidental diagnosis of hCG-secreting germ cell tumors. Whereas minute amounts of hCG are detectable in highly concentrated urine from healthy young men, readily detectable quantities usually signify the diagnosis of a germ cell tumor of testicular or, rarely, of extratesticular origin or an ectopic hCG-secreting tumor. Such incidental diagnoses are made through sports doping tests [06127].

Effects in women
The prohibition of hCG in women is not clearly justified in terms of performance enhancement or athlete safety. In addition, routine urine hCG measurement results in significant invasion of female athlete privacy as a result of the unintended screening for pregnancy. The available evidence suggests that hCG has negligible, if any, stimulation of blood testosterone concentrations in healthy young women. Together with the prevailing low blood testosterone levels in women, this makes it highly unlikely that any myotrophic or ergogenic effects are produced by administration of hCG to women [06127].

A major consideration setting the framework for considering the effects of hCG in women is the relatively high population prevalence of women with PCOS and its variants. These conditions span a wide spectrum of ovarian disorders all featuring mild increased blood testosterone concentrations in the female range, although still an order of magnitude lower than in men. Key features of PCOS are multiple ovarian cysts associated with ovulatory and menstrual dysfunction, hyperandrogenism (acne, hirsutism), and insulin resistance (obesity, metabolic syndrome). Definitions of the disorder vary from higher prevalence rates using a European definition with a focus on ultrasound criteria, compared with the American definition, which focus on clinical criteria requiring ovarian dysfunction and hyperandrogenism. Because asymptomatic polycystic ovaries are a relatively common finding among unselected women with pooled prevalence estimates approximately 20 percent, population estimates depend heavily on the criteria used. Using the narrower American definition for PCOS, the population prevalence has been estimated at 4 percent, whereas the broader European definition identifies 8-10 percent of unselected women as having PCOS. An even larger proportion of women have isolated features such as acne, hirsutism, and obesity insufficient to make the formal diagnosis of PCOS. Mild increase in blood testosterone concentration is a very common, near universal feature of women with PCOS and, to a lesser extent, women with incomplete forms of PCOS. Hence, blood testosterone concentrations of up to 3-4 nmol/L in untreated women with severe PCOS are
typical, compared with women with normal ovarian function (upper limit is 2–2.5 nmol/L). It is tacitly accepted that women with even severe PCOS are not barred from sports. Indeed, it is common experience that they appear to be overrepresented in power sports. This indicates that mild hyperandrogenism is already an accepted feature in female sports and thereby sets an existing upper limit for what is considered acceptable. This yardstick has relevance to considering the acceptability of the minimal, if any, increases in blood testosterone produced by hCG in women [06127].

The best available evidence indicates that administration of 250 microg recombinant hCG to healthy young women produces a rise of approximately 0.25 nmol/liter in blood testosterone concentrations. This increase is less than (about half) the diurnal rhythm in blood testosterone concentrations in women. Based on the findings increases of such small magnitude are highly unlikely to have any measurable effect on muscle mass or strength. Corroborative findings are available showing minimal or no increase in blood testosterone concentrations in women treated with urinary-purified hCG at chronic low dose or conventional high dose. Administration of urinary hCG (5,000 to 10,000 IU, about 330-660 microg) to young women with normal ovarian function produced either no or minimal (about 1 nmol/L) increase in blood testosterone concentrations. The minority of women with PCOS with higher baseline blood testosterone concentrations have slightly higher increases (2-3 nmol/L), but these remain of small magnitude in quantitative terms for myotrophic effects. An important caveat on blood testosterone measurements in some of these studies is the unreliability of conventional commercial immunoassays for blood testosterone, compared with well validated in-house immunoassays. In the low range of blood testosterone concentrations, such as in samples from women, children, or castrate men, the validity of commercial testosterone immunoassays has been described as comparable with random number generation. Because most studies of hCG effects in women have used unreliable testosterone assays, their findings remain questionable and will require further critical evaluation using mass spectrometry based methods. Beyond evaluating blood testosterone concentrations, measurements of muscle mass in women with PCOS suggest blood testosterone concentrations may be significantly correlated with their muscle mass; however, the significance of these correlations at such low blood testosterone concentrations remains speculative as far as performance capabilities go. As usual, the directionality of such correlations cannot be reliably determined from observational data [06127].

Effects of trauma to the brain
Traumatic brain injury (TBI) is a common cause of death and disability in young adults with consequences ranging from physical disabilities to long-term cognitive, behavioral, psychological and social defects. Recent data suggest that pituitary hormone deficiency is not infrequent among TBI survivors; the prevalence of reported hypopituitarism following TBI varies widely among published studies. The most common cause of TBI is motor vehicle accidents, including pedestrian-car and bicycle car encounters, falls, child abuse, violence and sports injuries. Prevalence of hypopituitarism, from total to isolated pituitary deficiency, ranges from 5 to 90 %. The time interval between TBI and pituitary function evaluation is one of the major factors responsible for variations in the prevalence of hypopituitarism reported. Endocrine dysfunction after TBI in children and adolescents is common. Adolescence is a time of growth, freedom and adjustment, consequently TBI is also common in this group. Sports-related TBI is an important public health concern, but many cases are unrecognized and unreported. Sports that are associated with an increased risk of TBI include those involving contact and/or collisions such as boxing, football, soccer, ice hockey, rugby, and the martial arts, as well as high velocity sports such as cycling, motor racing, equestrian sports, skiing and roller skating. The aim of this paper is to summarize the best evidence of TBI as a cause of pituitary deficiency in children and adults [13397].
Laboratory techniques

The applicability of a mass spectrometry (MS)-based method for determination of various forms of human chorionic gonadotropin (hCG) in doping analysis was demonstrated. A clinical study involving the hCG-containing pharmaceuticals Pregnyl and Ovitrelle was carried out, comprising a single injection of one pharmaceutical per participant to a total of 24 healthy male voluntaries. Hereafter, serum and urine samples were collected over a period of 14 days. The analysis of the samples using immuno-MS demonstrated elimination profiles of intact hCG for both pharmaceuticals, with last day of detection following administration at day 7 in serum, and at day 10 in urine, at limit of detections as defined by the World Anti-Doping Agency. Furthermore, the method allowed detection and differentiation of the various forms of hCG known to be present in serum and urine as a function of metabolism. For both pharmaceuticals, only the intact hCG was detected in serum, whereas in urine the injection of Pregnyl as hCG source (containing urinary hCG, i.e. most hCG variants) was shown to generate a more complex hCG variant pattern compared to Ovitrelle (contains only intact hCG). By detecting hCG using this MS-based approach in doping analysis, strong analytical evidence is provided minimizing the risk of false-positive and false-negative results [12204].

Immuno-massspectrometry. The applicability of a mass spectrometry (MS)-based method for determination of various forms of human chorionic gonadotropin (hCG) in doping analysis was demonstrated. A clinical study involving the hCG-containing pharmaceuticals Pregnyl and Ovitrelle was carried out, comprising a single injection of one pharmaceutical per participant to a total of 24 healthy male voluntaries. Hereafter, serum and urine samples were collected over a period of 14 days. The analysis of the samples using immuno-MS demonstrated elimination profiles of intact hCG for both pharmaceuticals, with last day of detection following administration at day 7 in serum, and at day 10 in urine, at limit of detections as defined by the World Anti-Doping Agency. Furthermore, the method allowed detection and differentiation of the various forms of hCG known to be present in serum and urine as a function of metabolism. For both pharmaceuticals, only the intact hCG was detected in serum, whereas in urine the injection of Pregnyl as hCG source (containing urinary hCG, i.e., most hCG variants) was shown to generate a more complex hCG variant pattern compared to Ovitrelle (contains only intact hCG). By detecting hCG using this MS-based approach in doping analysis, strong analytical evidence is provided minimizing the risk of false-positive and false-negative results [13396].

Chorionic gonadotrophin (CG) and luteinizing hormone (LH)

Human chorionic gonadotrophin (hCG) as well as LH are prohibited in sports (for males only) due to their ability to stimulate testosterone production and release. Both substances are commonly analyzed by ELISA, which was recently shown to be critical particularly concerning hCG if urine samples collected for doping controls are stored at -20 °C. In a complementary and indirect manner, the option to determine hCG abuse in sport by steroid profiling as well as LH measurements in blood and urine was evaluated. While commonly used urinary steroid profiles as such did not exhibit the required sensitivity to detect hCG abuse, accurate quantification of the testosterone concentration, T/LH ratio as well as the direct analysis of hCG were found to reliably contribute to an efficient detection of hCG abuse [12017].

Clomiphene

Many AAS users take additional drugs or supplements to counteract the adverse effects of AASs. Among 500 users of AASs, more than 50 percent reported taking clomiphene, antiaromatases (e.g. anastrozole), or the antiestrogen tamoxifen; 40 percent admitted to
using human chorionic gonadotropin. Human chorionic gonadotropin and clomiphene are taken at the end of or after an AAS cycle to reduce hypogonadotropic hypogonadism and reverse testicular atrophy and infertility. Some studies have shown maintenance of spermatogenesis with the concurrent use of human chorionic gonadotropin, but there are still significantly more abnormal and hypokinetic spermatozoa. The maintenance of spermatogenesis by human chorionic gonadotropin occurs without an increase in FSH. The low FSH concentration explains why sperm quality remains abnormal. The effect on offspring is unknown. The side effects of human chorionic gonadotropin include hyperglycemia, insulin resistance, decreased thyroid function, adrenal insufficiency, carpal tunnel syndrome, arthralgia, myopathy, pancreatitis, hepatotoxicity, and an increased risk for certain malignancies. Clomiphene stimulates the release of gonadotropins and is used in women with infertility; however, clomiphene may not increase serum gonadotropins when taken by power athletes during an AAS cycle. Antiaromatase or antiestrogen drugs, such as anastrozole and tamoxifen, are taken to counteract the effects of aromatization of the AAS to estrogens (e.g. gynecomastia). There is no data to support their effectiveness, and they also have side effects.

Clomiphene is a selective estrogen receptor modulator (SERM) that increases production of gonadotropins by inhibiting negative feedback on the hypothalamus. Clomifene inhibits estrogen receptors in hypothalamus, inhibiting negative feedback of estrogen on gonadotropin release, leading to up-regulation of the hypothalamic–pituitary–adrenal axis. Clomiphene is prohibited by World Anti Doping Agency (WADA) out-of-competition and in-competition. As it is extensively metabolized, further investigation of clomiphene metabolic profile will be essential to routine anti-doping analysis. The metabolic pathway and the different metabolites of clomiphene in human urine collected from three healthy volunteers during 1 week were studied by liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QTOFMS) based on accurate mass measurement. Seven unreported metabolites were identified and characterized, and all of the newly found urinary metabolites belonged to a new metabolic pathway (hydrogenation). An approach for the metabolism study of clomiphene and its analogs by LC-QTOFMS was presented. Two metabolites, 3,4-dihydroxy-dihydro-clomiphene (m/z 440.1991) and 3,4-dihydroxy-dihydro-deethyl-clomiphene (m/z 412.1674), are the potential biomarkers for monitoring oral administration of clomiphene in doping control.

Clomiphene, a representative of “other anti-estrogenic substances” (S4.3), was also studied concerning its metabolism in humans. As a major difference to earlier studies, the renal elimination of dihydrogenated and subsequently hydroxylated and/or methoxylated compounds was suggested, based on LC-MS/MS data with high resolution/high accuracy mass spectrometry. Since also here the evidence by derivatization, H/D-exchange, or chemical synthesis is missing, the postulated structures can only be considered as tentative and serve for screening rather than confirmatory purposes.

**Release of GnRH**

The canonical regulator of gonadotropin secretion is the hypothalamic decapeptide, GnRH, which can induce stimulus-secretion coupling as well as gene expression. However, it is clear now that many paths coalesce to achieve the complexity and disparate regulation of LH and FSH expression, a necessary first step to production and secretion. GnRH mediated gonadotropin secretion can be enhanced by the transcription factor Isl-1, which is elevated in response to GnRH as well as by activin and leptin stimulation, although the responses to activin and leptin were higher and more prolonged than that induced by GnRH. However, subsequent experiments indicated Isl-1 mainly is responsible for leptin-mediated FSHbeta.
and LHβ production, not GnRH- or activin-mediated secretion, as knocking down Isl-1 expression had no significant effect on gonadotropin regulation by either GnRH or activin. The secretion of LH appears to be induced, whereas the secretion of FSH is constitutive, as will be discussed below. The release of GnRH from hypothalamic neurons is pulsatile. Given the effect of GnRH as a releasing hormone, one might expect that all secretagogues including GnRH would engender gene expression that would co-vary with pulse. Although a mechanism for differential gonadotropin gene expression, which can posit transcriptional factor dampening of transcription is compelling, it also suggests that adenylate cyclase is not the only downstream effector of GnRH. In this regard, it has been shown quite elegantly that the prostaglandin biosynthetic pathway, and in particular PGF2α and PGI, can regulate not only LHβ and FSHβ gene transcription, but also the levels of GnRH receptor. Since 1976 evidence has been provided, which showed the presence of the α subunit protein in non-pituitary and non-placental tissues. It is reasonable to suggest that the expression of the α subunit is not cell type dependent and the promoter likely embodies cyclic AMP response element (CRE) binding sites for CRE binding protein transcription factors. In addition, the control of this promoter can also be anticipated to respond to transcription factors that are regulated by intracellular levels of calcium. Of course, every reproductive physiologist knows that castration gives rise to elevated gonadotropins in blood. Indeed, as ovarian function wanes in mid-life, and gonadal steroid production is decreased, FSH rises. Therefore, the long loop feedback of gonadal steroids on gonadotropin production was a harbinger of the potential for regulation of gonadotropin gene expression by gonadal steroids. It is also important to mention the hypothalamic factor, Gonadotropin Inhibitory Hormone (GnIH or RFRP-1,-2,-3 peptides), which inhibits release of gonadotropins mediated by GnRH. During the 1970s a search for non-steroidal factors that regulate gonadotropin release led to the identification of inhibin, which suppresses FSH secretion, and then serendipitously activin, which increases FSH secretion. Both inhibin and activin are members of the TGFβ superfamily which appear to preferentially control the secretion of FSH. Activin acts as an autocrine/paracrine factor in the anterior pituitary, and stimulates FSHβ gene transcription, whereas inhibin, acting as an ovarian hormone, is inhibitory [11476].

**Luteinizing hormone**

Urine luteinizing hormone (LH) concentration is routinely measured in all anti-doping laboratories to exclude recombinant LH abuse and to test any potential alteration of the hypophyseal-gonadal axis. Before establishing proper reference values among professional top level athletes, an extended validation of two commercial immunoassays for LH measurements was performed. Elecsys 1010 and Access are two automated immunoanalyzers for central laboratories. The limits of detection, the limits of quantification, intra-laboratory, inter-technique correlation, precision, accuracy were determined. Furthermore, reference urinary LH distribution values for male and female top level athletes were determined. Stability studies of LH in urine following freezing and thawing cycles (n=3) as well as storage conditions at room temperature, 4 degrees C and -20 degrees C were performed. Male and female subjects showed important urinary corrected (specific gravity correction) LH distribution differences. Intra-assay precision for the Access analyzer was less than 8.0 percent whereas inter-assay was close to 11 percent. Intra and inter-assay precision for the Elecsys 1010 analyzer was slightly better. No urinary LH loss was observed after two freezing and thawing cycles. On the other hand, time and bad storage conditions such as elevated temperature can deteriorate rapidly urinary LH. In conclusion, both analyzers showed acceptable performances and are suitable for screening anti-doping analyses. Each anti-doping laboratory has to settle its own reference distribution values and then determine when to launch a confirmation procedure. This takes place then depending on the positivity criteria the anti-doping laboratory has established and validated. This study also clearly showed that the time delay between the urine collection and the analysis should be reduced.
as much as possible and urine samples should be transported in optimal conditions (low
temperature and quickly) to decrease urinary LH deterioration [07160].

Urine provides a convenient non-invasive alternative to blood sampling for measurement of
certain hormones. Urinary luteinizing hormone (LH) measurements have been used for
endocrinology research and anti-doping testing. However, the commercially available LH
immunoassays are developed and validated for human blood samples but not urine so that
LH assays intended for use with urine samples need thorough validation. Therefore, the
present study evaluated the measurement of urinary LH immunoreactivity using previously
validated immunofluorometric (IF) and immunochemiluminometric (ICL) LH assays after
prolonged frozen storage. LH was measured in serial urine samples following administration
of a single injection of one of two doses of recombinant human chorionic hormone (rhCG)
with assays run at the end of study (2008) and again after four years of frozen (-20 °C)
storage where samples were stored without adding preservatives. The ICL assay showed
quantitatively reproducible LH measurements after prolonged -20 °C storage. However, the
IF immunoassay gave consistently lower LH levels relative to ICL (2008) with a further
proportionate reduction after four years of sample storage (2012). Yet, both the assays
displayed similar patterns of the time-course of urine LH measurement both before and after
four years of frozen storage. In conclusion, we found that both immunoassays are suitable
for urinary LH measurements with ICL assay being more robust for quantitative urinary LH
measurement such as for anti-doping purposes, whereas the IF could be applicable for
research studies where urine LH levels are compared within-study but not in absolute terms
[13398].

In boxing
Luteinizing hormone (LH) is physiologically produced by the anterior pituitary gland. Male
athletes may use pharmaceutical LH for doping since it increases the production of
testosterone by testes. The World Anti-Doping Agency (WADA) has put luteinizing hormone
(LH) on its list of prohibited substances for male athletes. Indeed, this gonadotropin, which is
one of the numerous hormones produced by the anterior pituitary gland, stimulates the
conversion of cholesterol to testosterone in the Leydig cells of testes. Pharmaceutical LH is
obtained either by extraction from the urine of postmenopausal women or by genetic
engineering to produce recombinant LH. No method is currently able to differentiate between
endogenous and pharmaceutical LH. Anti-doping control is based on the assay of this
gonadotropin in urine in order to detect abnormally elevated levels. This approach requires a
reliable threshold value for the limit of physiological levels. Since there is no consensus
value, each anti-doping laboratory has to estimate a reference interval (normal range) that
depends on the assay method. In the course of conducting this analysis, one laboratory
observed disturbing results related to boxing. It was observed a highly significant prevalence
of abnormal results in samples taken after a boxing match. Comparison of the descriptive
statistics for 426 LH values observed in boxing and other sports showed significant
differences. An experimental study comparing urinary LH levels in 17 boxers before and after
a match demonstrated a clear increase after the match. The same observation was made for
urinary follicle stimulating hormone (FSH) in all of the eight boxers tested for this other
pituitary gonadotropin. These observations have consequences for anti-doping controls, as
the reference range for urinary LH levels must take into account the specificities of boxers.
They also suggest consequences for the health of boxers. Although to our knowledge such
observations have never been described, other pituitary disorders have been reported. Our
results deserve further investigation from a medical point of view [13399].

Laboratory techniques
Urine luteinizing hormone (LH) concentration is routinely measured in all anti-doping
laboratories to exclude recombinant LH abuse and to test any potential alteration of the
hypophyseal-gonadal axis. Before establishing proper reference values among professional top level athletes, an extended validation of two commercial immunoassays for LH measurements was performed. Elecsys 1010 and Access are two automated immunoanalyzers for central laboratories. The limit of detection, the limit of quantification, intra-laboratory, inter-technique correlation, precision, accuracy were determined. Furthermore, reference urinary LH distribution values for male and female top level athletes were determined. Stability studies of LH in urine following freezing and thawing cycles (n=3) as well as storage conditions at room temperature, 4 degrees C and -20 degrees C were performed. Male and female subjects showed important urinary corrected (specific gravity correction) LH distribution differences. Intra-assay precision for the Access analyzer was less than 8.0% whereas inter-assay was close to 11%. Intra and inter-assay precision for the Elecsys 1010 analyzer was slightly better. A good inter-technique correlation was obtained. No urinary LH loss was observed after two freezing and thawing cycles. On the other hand, time and bad storage conditions such as elevated temperature can deteriorate rapidly urinary LH. In conclusion, both analyzers showed acceptable performances and are suitable for screening anti-doping analyses. Each anti-doping laboratory has to settle its own reference distribution values and then determine when to launch a confirmation procedure. This takes place then depending on the positivity criteria the anti-doping laboratory has established and validated. The study also clearly showed that the time delay between the urine collection and the analysis should be reduced as much as possible and urine samples should be transported in optimal conditions (low temperature and quickly) to decrease urinary LH deterioration [06142].

Conceptually, a mechanism to maintain normal testosterone concentrations could be an enhanced pulsatile release of LH. Decreased testosterone concentrations relieve the inhibition of the negative feedback loop at the hypothalamus/pituitary gland, resulting in an enhanced release of gonadotropin-releasing hormone and subsequently enhancing LH secretory dynamics. For example, compared with younger men, older men tend to possess greater LH secretion, which is hypothesized to represent a biological attempt to maintain normal testosterone concentrations. Endurance-trained athletes have also shown decreased testosterone and increased LH concentrations. The literature with regard to acute exercise stress has been equivocal. It has also been reported decreased concentrations of testosterone and increased LH concentrations for up to 2 days after a marathon and suggested that elevated catecholamines may be partially responsible for the decreased testicular sensitivity. LH pulsatility within hours in a number of different species. Thus, although LH pulsatility is slowly restored in women, there is evidence to suggest that gonadotropin-releasing hormone neurons may rapidly respond to caloric intake [06141].

Natural agonists

The gonadotropins, luteinizing hormone, human chorionic gonadotropin and follicle-stimulating hormone, are key regulators of reproduction. As a result of this function, they have been the focus of research for many years. Isolated or recombinant proteins have been successfully used therapeutically for the treatment of infertility; and, in the case of compounds that block gonadotropin activity, for their potential utility in contraception. Until recently, selective small molecules modulating gonadotropin receptor activity have proven difficult to identify. The gonadotropins are glycoproteins that are released into the plasma as differently glycosylated isoforms and bind to specific G protein-coupled receptors. The degree of glycosylation on the gonadotropins has been shown to be important for the biological activities of these hormones and is differentially regulated depending on the steroidal status. Recent data from the study of glycosylated variants of LH, hCG and FSH have revealed that these isoforms have distinct signaling properties that allow for gonadotropin pleiotropic signals to be transduced effectively at the level of the receptor.
Thus, glycosylated variants of the gonadotropins behave as biased agonists. Recently, newly developed, small molecule, synthetic allosteric compounds have been identified that are capable of mimicking this biased signaling. This opens the door to development of orally available, drug-like therapies for reproductive disorders that offer similar pleiotropic richness as that offered by the complex, endogenous hormones [11477].

**Secretagogues: gonadotrophin-releasing hormone**

GHRP-2 (pralmorelin, D-Ala-D-(beta-naphthyl)-Ala-Ala-Trp-D-Phe-Lys-NH(2)), which belongs to a class of growth hormone secretagogue (GHS), is intravenously used to diagnose growth hormone (GH) deficiency. Because it may be misused in expectation of a growth-promoting effect by athletes, the illicit use of GHS by athletes has been prohibited by the World Anti-Doping Agency (WADA). Therefore, the mass spectrometric identification of urinary GHRP-2 and its metabolite D-Ala-D-(beta-naphthyl)-Ala-Ala-OH (AA-3) was studied using liquid chromatography/electrospray ionization tandem mass spectrometry for doping control purposes. The method consists of solid-phase extraction using stable-isotope-labeled GHRP-2 as an internal standard and subsequent ultra-performance liquid chromatography/tandem mass spectrometry, and the two target peptides were determined at urinary concentrations of 0.5-10 ng/mL. The recoveries ranged from 84 to 101%, and the assay precisions were calculated as 1.6-3.8 percent (intra-day) and 1.9-4.3 percent (inter-day). Intravenous administration of GHRP-2 in ten male volunteers was studied to demonstrate the applicability of the method. In all ten cases, unchanged GHRP-2 and its specific metabolite AA-3 were detected in urine [10363].

The decapeptide gonadotrophin-releasing hormone (GnRH) is endogenously produced in the hypothalamus and secreted into the microcirculation between hypothalamus and pituitary gland. Here, the bioactive hormone is responsible for the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the systemic circulation. Because an intermittent application of exogenous GnRH in young males increases the testosterone plasma level by stimulation of the Leydig cells, the potential misuse of the administered substance offers a reasonable relevancy for doping controls and is prohibited in accordance to the list of banned substances of the World Anti-Doping Agency. One presented method provides a mass spectrometric approach to determine the nondegraded hormone in regular doping control samples by utilizing a sample preparation procedure with solid phase extraction, immunoaffinity purification and a subsequent separation by liquid chromatography with ESI-MS/MS detection. For liquid chromatography/mass spectrometry two alternative instrumental equipments were tested. In urine specimens provided from healthy volunteers GnRH was not detected in accordance to the recent literature, but in postadministration samples urinary concentrations between 20 to 100 pg/ml of the intact peptide were determined. The method offered good validation results considering the parameter specificity, linearity (5-300 pg/ml), limit of detection (LOD, approx. 5 pg/ml), precision (inter/intraday, < 20 %) and accuracy (105 %) using Des-pGlu(1)-GnRH as internal standard to control each sample preparation step [08248].

**Myostatin**

A decade has passed since myostatin was first identified as a negative regulator of muscle growth. Since then, studies in both humans and animals have demonstrated that decreasing the levels of this growth factor or inhibiting its function can dramatically increase muscle size, and a number of therapeutic applications of myostatin inhibition to the treatment of
myopathies and muscle atrophy have been proposed. As such treatments would be likely to also stimulate muscle growth in healthy individuals, there is a growing concern among anti-doping authorities that myostatin inhibitors may be among the next generation of ergogenic pharmaceuticals or even in the vanguard of “gene doping” technology. While the ability to stimulate muscle growth through myostatin inhibition is well documented, a growing body of evidence suggests such increases may not translate into an improvement in athletic performance [08289].

Myostatin is a member of the transforming growth factor-beta/bone morphogenetic protein (TGF-beta/BMP) superfamily of secreted factors that functions as a potent inhibitor of skeletal muscle growth. Moreover, considerable evidence has accumulated that myostatin also regulates metabolism and that its inhibition can significantly attenuate the progression of obesity and diabetes. Although at least part of these effects on metabolism can be attributable to myostatin’s influence over skeletal muscle growth and therefore on the total volume of metabolically active lean body mass, there is mounting evidence that myostatin affects the growth and metabolic state of other tissues, including the adipose and the liver. In addition, recent work has explored the role of myostatin in substrate mobilization, uptake, and/or utilization of muscle independent of its effects on body composition. Finally, the effects of both endurance and resistance exercise on myostatin expression, as well as the potential role of myostatin in the beneficial metabolic adaptations occurring in response to exercise, have also begun to be delineated in greater detail. The purpose of this review was to summarize the work to date on the expression and function of myostatin in obesity, diabetes, and exercise adaptation [11478].

Myostatin is a potent negative regulator of skeletal muscle mass, but its role in human skeletal muscle hypertrophy and atrophy is sparsely described. Muscle biopsies were obtained from young male subjects before and after 30 and 90 days of resistance training as well as after 3, 10, 30, 60 and 90 days of subsequent detraining. Myostatin mRNA increased significantly with detraining. It was observed a 28 kDa myostatin immunoreactive protein, which, however, was also present in myostatin knock out mice skeletal muscle. As a novel finding it was consistently detected a 10 kDa band, which may represent a mature myostatin monomer under reducing conditions or a novel, unknown myostatin form. Further, it was observed a significant increase in this 10 kDa band after 3 days of detraining preceding the rapid type II fiber atrophy, in which almost half of the acquired fiber area was lost after only 10 days of detraining. Accordingly, an increase in the level of the 10 kDa protein is associated with rapid type II fiber atrophy, suggesting myostatin-mediated specific type II fiber atrophy, which in combination with the mRNA data support a role for myostatin in the negative regulation of adult human skeletal muscle mass [09216].

Inhibition of myostatin signalling or its biological activity has recently emerged as a potential remedial approach against muscle wasting and degenerative diseases such as muscular dystrophies. In one study it was systemically administered a recombinant AAV8 vector expressing a mutated myostatin propeptide (AAV8ProMyo) to healthy mice in order to assess its impact on the histological, cellular and physiological properties of the skeletal muscle, exploiting the fact that myostatin is naturally inhibited by its own propeptide. It was reported that a single intravenous administration of AAV8ProMyo leads to increases in muscle mass of tibialis anterior, extensor digitorum longus and gastrocnemius muscles 8 weeks post-injection and tibialis anterior, gastrocnemius and rectus femoris muscles 17 weeks post-injection. Moreover, treatment resulted in muscle fibre hypertrophy but not hyperplasia, with IIB myofibres responding to the greatest extent following propeptide-induced myostatin inhibition. Additionally, myofibre nuclear:cytoplasmic ratio was decreased in the AAV8ProMyo treated animals. Importantly, the hypertrophic EDL muscle 8 weeks after AAV8ProMyo treatment did not show the dramatic decrease in specific force displayed by
The objective of one study was to examine the effects of short-term exercise training, myostatin inhibition (PF-354), and exercise + PF-354, all relative to a vehicle control, on performance and metabolic measures in 24-month-old mice. At study termination, PF-354-treated mice exhibited significantly greater muscle weights. Performance measures revealed that exercise + PF-354 increased treadmill running time and distance to exhaustion (more than twofold) and increased habitual activity. Measures of strength were not different; however, all treatment groups demonstrated more than 30 percent reductions in muscle fatigue. Metabolic measures showed that basal metabolic rates were higher in PF-354- and exercise + PF-354-treated mice, and exercise and exercise + PF-354 groups exhibited significantly greater insulin sensitivity. PF-354 was associated with decreased Smad3 phosphorylation and increased peroxisome proliferator-activated receptor gamma coactivator-1alpha expression and, similar to exercise, decreased MuRF-1. The data suggest that the combination of exercise training and myostatin blockade may significantly improve physical function and whole-body metabolism in older individuals.

Methodological problems, including binding of myostatin to plasma proteins and cross-reactivity of assay reagents with other proteins, have confounded myostatin measurements. Here it was described development of an accurate assay for measuring myostatin concentrations in humans. Monoclonal antibodies that bind to distinct regions of myostatin served as capture and detector antibodies in a sandwich ELISA that used acid treatment to dissociate myostatin from binding proteins. Serum from myostatin-deficient Belgian Blue cattle was used as matrix and recombinant human myostatin as standard. The quantitative range was 0.15-37.50 ng/mL. Myostatin protein was undetectable in sera of Belgian Blue cattle and myostatin knockout mice. Recovery in spiked sera approximated 100 percent. ActRIIB-Fc or anti-myostatin antibody MYO-029 had no effect on myostatin measurements when assayed at pH 2.5. Myostatin levels were significantly higher in young than older men 8.0 ± 0.3 ng/mL versus 7.0 ± 0.4 ng/mL. In men treated with graded doses of testosterone, myostatin levels were significantly higher on day 56 than baseline in both young and older men; changes in myostatin levels were significantly correlated with changes in total and free testosterone in young men. Myostatin levels were not significantly associated with lean body mass in either young or older men.

It was examined associations among myostatin 2379 A > G and 163 G > A and follistatin (FST) -5003 A > T and -833 G > T single nucleotide polymorphisms (SNP) on the muscle size and the strength response to resistance training (RT). There were 645 subjects (age = 24 ± 0.2 yr, body mass index = 24.2 ± 0.2 kg) who self-disclosed themselves as Caucasian (79 %), African American (4 %), Asian (8 %), Hispanic (5 %), or other (4 %). They were genotyped for myostatin 2379 A > G (n = 645), myostatin 163 G > A (n = 639), FST -5003 A > T (n = 580), and FST -833 G > T (n = 603). It was assessed dynamic (one repetition maximum [1RM]) and isometric (maximum voluntary contraction [MVC]) muscle strength and size (cross-sectional area [CSA]) of the elbow flexors before and after 12 weeks of unilateral upper-arm RT. Baseline MVC was greater among African Americans who were carriers of the myostatin G(2379) allele (AG/GG, n=15) than the A2379A homozygotes. African Americans who were carriers of the FST T(-5003) allele (n=12) had greater baseline 1RM and CSA than African Americans with the A-5003A genotype (n = 14; P < 0.05). No myostatin or FST genotype and muscle phenotype associations were found among the other ethnic groups.

Dexamethasone, alone or in association with estrogens, is often illegally administered per os at very low dosage as a growth promoter in beef cattle, with effects that are opposite to the muscle wasting and atrophy induced by repeated administration at therapeutic dosages. In
vitro and in vivo studies have investigated the catabolic effects of dexamethasone at therapeutic doses on skeletal muscle, demonstrating an increase in the expression of GDF8 (myostatin) gene, a well-known negative regulator of skeletal muscle mass, in a dose-dependent way. This suggested a direct role of myostatin in dexamethasone-induced muscle wasting. In one study, an oligonucleotide microarray platform was used to compare expression profiles of beef cattle muscle in animals treated with either dexamethasone or dexamethasone plus 17-beta estradiol administered at subtherapeutic dosage, against untreated controls. Data analysis demonstrates that the expression profiles were strongly affected by dexamethasone treatment with hundreds of genes upregulated with relevant fold-change, whereas seven genes were downregulated including the myostatin gene. On the contrary, the number of differentially regulated genes was lower in response to the addition of estradiol to the dexamethasone treatment. Differentially regulated genes were analyzed to describe the effects of these treatments on muscle physiology, highlighting the importance of specific pathways (e.g. Wnt or cytokine signaling) and cellular processes (e.g. cell shape and motility). Finally, the observed differences in the expression profile will allow the development of indirect bio-markers to detect illegal dexamethasone treatments in beef cattle using quantitative RT-PCR [09221].

Myostatin is a dominant inhibitor of skeletal muscle development and growth. As transgenic over-expression of myostatin propeptide dramatically enhanced muscle mass, we hypothesized that administration of myostatin propeptide will increase muscle growth. In this study, the wild-type form of porcine myostatin propeptide and its mutated form at the cleavage site of metalloproteinases of BMP-1/TLD family were produced from insect cells. In vitro A204 cells reporter assays showed that both wild-type and the mutated propeptides depressed myostatin activity. The recombinant propeptides at four-fold myostatin concentration can effectively block myostatin function during co-incubation with A204 cells. In particular, the mutated propeptide appeared much more effective than wild-type propeptide over a long period during the in vitro co-incubation. Administration of the mutated propeptide to neonatal mice at the age of 11 and 18 days was tested and showed significant increase in growth performance by 11-15 percent from the age of 25 to 57 days. The major skeletal muscles of mice that were injected with mutated propeptide were 14-25 percent heavier than the control group – a significant difference – as a result of muscle fiber hypertrophy. In conclusion, administration of the mutated myostatin propeptide during the neonatal period is an effective way for promoting muscle growth [09222].

Myostatin, a negative regulator of muscle growth, has recently been found to be expressed in tendons. Myostatin-deficient mice have weak and brittle tendons, which suggest that myostatin could be important for tendon maintenance. Follistatin expression in the callus tissue after tendon transection is influenced by loading. It was found that follistatin antagonises myostatin, but not GDF-5 or OP-1 in vitro. To study if myostatin might play a physiological role in soft tissue, it was transected 64 rat Achilles tendons and studied the gene expression for myostatin and its receptors at four different time-points during healing. Intact tendons were also studied. All samples were studied with or without mechanical loading. Unloading was achieved with botulinum toxin injections in the calf muscles. The expression of the myostatin gene was more than 40 times higher in intact tendons than in the callus tissue during tendon healing. The expression of myostatin was also influenced by loading status in both intact and healing tendons. Thereafter, it was measured the mechanical properties of healing tendons after local myostatin administration. This treatment increased the volume and the contraction of the callus after 8 days, but did not improve its strength. The results indicate that myostatin plays a positive role in tendon maintenance and that exogenous protein administration stimulates proliferation and growth of early repair tissue. However, no effect on further development towards connective tissue formation was found [09223].
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therapeutic doses on skeletal muscle, demonstrating an increase in the expression of GDF8 (myostatin) gene, a well-known negative regulator of skeletal muscle mass, in a dose-dependent way. This suggested a direct role of myostatin in dexamethasone-induced muscle wasting. In one study, an oligonucleotide microarray platform was used to compare expression profiles of beef cattle muscle in animals treated with either dexamethasone or dexamethasone plus 17-beta estradiol administered at subtherapeutic dosage, against untreated controls. Data analysis demonstrates that the expression profiles were strongly affected by dexamethasone treatment with hundreds of genes upregulated with relevant fold-change, whereas seven genes were downregulated including the myostatin gene. On the contrary, the number of differentially regulated genes was lower in response to the addition of estradiol to the dexamethasone treatment. Differentially regulated genes were analyzed to describe the effects of these treatments on muscle physiology, highlighting the importance of specific pathways (e.g. Wnt or cytokine signaling) and cellular processes (e.g. cell shape and motility). Finally, the observed differences in the expression profile will allow the development of indirect bio-markers to detect illegal dexamethasone treatments in beef cattle using quantitative RT-PCR [09227].

Myostatin propeptide (MYOPRO) and follistatin (FOLLI) are potent myostatin inhibitors. In one study it was analysed effects of training and androgens on MYOPRO and FOLLI concentrations in blood and skeletal muscle using Immuno PCR. Young healthy males performed either a 3-month endurance training or a strength training. Blood and biopsy samples were analysed. Training did not significantly affect MYOPRO and FOLLI concentrations in serum and muscle. To investigate whether total skeletal muscle mass may affect circulating MYOPRO and FOLLI levels, blood samples of tetraplegic patients, untrained volunteers and bodybuilders were analysed. MYOPRO was significantly increased exclusively in the bodybuilder group. In orchiectomised rats MYOPRO increased in blood and muscle after treatment with testosterone. In summary the data demonstrate that moderate training does not affect the concentrations of MYOPRO to FOLLI. In contrast androgen treatment results in a significant increase of MYOPRO in skeletal muscle and serum [10368].

There is mounting evidence that skeletal muscle produces and secretes biologically active proteins or "myokines" that facilitate metabolic cross talk between organ systems. The increased expression of myostatin, a secreted anabolic inhibitor of muscle growth and development, has been associated with obesity and insulin resistance. Despite these intriguing findings, there have been few studies linking myostatin and insulin resistance. To explore this relationship in more detail, we quantified myostatin protein in muscle and plasma from 10 insulin-resistant, middle-aged (53 ± 6 year) men before and after 6 months of moderate aerobic exercise training . To establish a cause-effect relationship, we also injected C57/Bl6 male mice with high physiological levels of recombinant myostatin protein. Myostatin protein levels were shown to decrease in muscle and matching plasma samples with aerobic exercise. Furthermore, the strong correlation between plasma myostatin levels and insulin sensitivity suggested a cause-effect relationship that was subsequently confirmed by inducing insulin resistance in myostatin-injected mice. A modest increase (44 %) in plasma myostatin levels was also associated with significant reductions in the insulin-stimulated phosphorylation of Akt (Thr308) in both muscle and liver of myostatin-treated animals. These findings indicate that both muscle and plasma myostatin protein levels are regulated by aerobic exercise and, furthermore, that myostatin is in the causal pathway of acquired insulin resistance with physical inactivity [10485].

Both 19-norandrostenedione (estr-4-ene-3,17-dione, NOR) and desoxymethyltestosterone (17alpha-methyl-5alpha-androst-2-en-17beta-ol, DMT or "madol") are “designer steroids” misused for doping purposes in the bodybuilding scene. It has previously been characterized
the pharmacological profile of madol and identified potential adverse side effects. The aim of one study was to investigate the anabolic potency of NOR, madol and the reference substance testosterone propionate (TP). Besides wet weight of the M. levator ani (LA), it was examined the effects on muscle fiber type composition and myosin heavy chain (MHC) expression in the M gastrocnemius (Gas) muscle as additional markers for anabolic potency. A Hershberger assay was performed, where orchiectomized (orchi) male Wistar rats were treated subcutaneously with NOR, madol, TP or vehicle control (all 1 mg/kg BW/day) for 12 days. Wet weights of the Gas, LA, prostate and seminal vesicle were examined to determine anabolic and androgenic effects. Fiber type composition of the Gas muscle was analyzed using ATPase staining, and MHC protein profiles were determined by silver stain and Western blot analysis. NOR and madol exhibited strong anabolic and weak androgenic potency by stimulating growth of the LA but not the prostate and seminal vesicle. Skeletal muscle fiber type composition characterized by ATPase staining was not significantly altered between the treatment groups, although there was a tendency toward lower levels of type IIB and increased type IIA fibers in all treatment groups relative to orchi. MHC protein expression determined by Western blot and silver stain analysis revealed that MHC IId/x was significantly up-regulated, while MHC IIb was significantly down-regulated in NOR, madol and TP groups relative to orchi. There were no significant differences for MHC Ila and MHC I expression between groups. Results suggest that the observed MHC expression shift could serve as a molecular marker to determine anabolic activity of anabolic steroids at least in skeletal muscle of orchi rats. The molecular mechanisms as well as the androgen-dependent regulation of MHC expression in intact skeletal muscle remain to be further investigated [11180].

It was hypothesized that suppression of endogenous testosterone blunts mRNA expression post strength training (ST). Twenty-two young men were randomized for treatment with the GnRH analogue goserelin (3.6 mg every 4 weeks) or placebo for a period of 12 weeks. The ST period of 8 weeks started at week 4. Strength test, blood sampling, muscle biopsies, and whole-body dual-energy X-ray absorptiometry (DXA) scan were performed at weeks 4 and 12. Muscle biopsies were taken during the final ST session (pre, post 4 h, and post 24 h). Resting serum testosterone decreased significantly in the goserelin group, whereas it remained unchanged in the placebo group. An acute increase of serum testosterone was observed during the final ST session in the placebo group, whereas a decreased response was observed in the goserelin group. mRNA expression of IGF-IE(bc) and myogenin increased, while expression of myostatin decreased; however, no differences were observed between the groups. Muscle strength and muscle mass showed a tendency to increase more in the placebo group than in the goserelin group. In conclusion, despite blocked acute responses of testosterone and 10- to 20-fold lower resting levels in the goserelin group, ST resulted in a similar mRNA expression of myoD, myogenin, IGF-IE(abc), myostatin and androgen receptor as observed in the placebo group. Therefore, in the present study, the molecular events were the same, despite divergent muscle hypertrophy and strength gains [07162].

Myostatin, a member of the transforming growth factor-beta (TGF-beta) superfamily, is a critical autocrine/paracrine inhibitor of skeletal muscle growth. Since the first observed double-muscling phenotype was reported in myostatin-null animals, a functional role of myostatin has been demonstrated in the control of skeletal muscle development. The deletion of myostatin in mice induces a dramatic and widespread increase in skeletal muscle mass due to both muscle hypertrophy and hyperplasia. The double-muscling phenotype of some cattle and sheep breeds is caused by mutations in their myostatin genes. Myostatin appears to trigger signaling by directly binding to its serine/threonine kinase receptor. Downstream of the receptors, Smads are first demonstrated to be mediators of signals for myostatin. Beyond the canonical Smad-mediated signal pathway, Non-Smad signal
pathways have recently been reported to participate in myostatin signaling. Myostatin expression is increased in glucocorticoid-induced muscle atrophy as well as skeletal muscle degeneration-related diseases, such as HIV infection and chronic illnesses. The increased myostatin expression is also associated with metabolic disorders, such as obesity and diabetes. Therefore, the most important myostatin function is the regulation of skeletal muscle growth. However, the biological function of myostatin is not only restricted to muscle growth inhibition but also might have other redundant functions. However, beyond the confines of its traditional role in muscle growth inhibition, myostatin has recently been shown to play an important role in metabolism. During the past several years, it has been well established that Smads are canonical mediators of signals for myostatin from the receptors to the nucleus. However, growing evidence supports the notion that Non-Smad signal pathways also participate in myostatin signaling. Myostatin expression is increased in muscle atrophy and metabolic disorders, suggesting that changes in endogenous expression of myostatin may provide therapeutic benefit for these diseases. MicroRNAs (miRNAs) are a class of non-coding RNAs that negatively regulate gene expression and recent evidence has accumulated supporting a role for miRNAs in the regulation of myostatin expression. The functional role of myostatin in the control of skeletal muscle development is well established. However, the biological function of myostatin is not only restricted to suppressing skeletal muscle growth. Muscle mass is tightly regulated by the balance between muscle cell replication and protein synthesis and muscle proteolysis and cell death. There is evidence that myostatin may reduce muscle mass by decreasing protein synthesis. Studies in vitro reported that myostatin inhibit protein synthesis in both myoblasts and particularly in myotubes. In vivo studies examined the rates of myofibrillar protein synthesis in normal mice and in mice with constitutive myostatin deficiency. They found that mice with constitutive myostatin deficiency have increased the rate of myofibrillar protein synthesis. Similar results were observed in mice with anti-myostatin antibody. In addition, it was reported that follistatin, an inhibitor of myostatin, stimulates protein synthesis in skeletal muscle of neonatal rats. In these in vivo studies, myostatin does not influence muscle proteolysis, which suggests that increased protein synthesis is probably the major mechanism for the muscle fiber hypertrophy arising from absence or blockade of myostatin. Recently, numerous studies have provided evidence that myostatin acts as a negative regulator of the Akt/mTOR signaling pathway, consistent with its inhibitory effect on protein synthesis. Taken together, these findings uncover a novel role for myostatin in protein metabolism and, more specifically, in the regulation of protein synthesis. Myostatin thus negatively regulates skeletal muscle growth, an effect attributed to inhibition of both myoblast proliferation and differentiation. Myostatin appears to inhibit myoblast proliferation by arresting cell cycle at the G1-phase. A number of studies have provided evidence that myostatin-induced myoblast inhibition is associated with the up-regulation of the cyclin-dependent kinase (Cdk) inhibitor p21, the down-regulation of Cdk2, the decrease in Cdk2 activity, and the decrease phosphorylation of retinoblastoma (Rb). In addition, myostatin was reported to augment cyclin D1 protein degradation to cause G1 cell cycle arrest through PI3K/Akt/GSK-3β signaling pathway. Myostatin appears to inhibit myoblast differentiation by downregulating expression of differentiation related genes, such as MyoD, myogenin, and Myf5, as well as the activity of their downstream target creatine kinase. Satellite cells are quiescent muscle stem cells that promote postnatal muscle growth and repair. Myostatin has also been shown to maintain the satellite cells in a quiescent state. Cell cycle analysis confirms that myostatin up-regulates the levels of p21 protein and decreases the levels and activity of Cdk2 protein, suggesting that myostatin inhibits the G1 to S phase transition and thus maintains the quiescent status of satellite cells. The approaches to blocking myostatin activity show promise for clinical application. Several strategies to depress myostatin activity have been developed over the years since it was cloned and identified as a novel TGF-beta superfamily member in 1997. Myostatin propeptide, an endogenous myostatin specific inhibitor, has been developed to suppress myostatin activity. Myostatin propeptide is capable of inhibiting
myostatin activity by binding to it directly and maintaining it in a latent state. In addition, a single intravenous administration of a recombinant AAV8 vector expressing a mutated myostatin propeptide leads to increases in mice muscle mass of tibialis anterior, extensor digitorum longus and gastrocnemius muscles 8 weeks post-injection and tibialis anterior, gastrocnemius and rectus femoris muscles 17 weeks post-injection. In addition to myostatin inhibitors, neutralizing antibody has been developed to inhibit myostatin activity. Blockade of endogenous myostatin by using intraperitoneal injections of anti-myostatin monoclonal antibody increased skeletal muscle mass in mice. More recently, it was reported that antibody-directed myostatin blockade enhances the mass and function of skeletal muscles of 21-mo-old aging mice. There are evidence that a neutralizing antibody to myostatin not only promotes gains in muscle mass, but improves glucose tolerance and lipid profiles in obese insulin resistant mice. These findings highlight the therapeutic potential of antibody-directed myostatin inhibition not only for muscular dystrophy but also for metabolic diseases, such as obesity and type II diabetes. In addition to myostatin inhibitors, neutralizing antibody has been developed to inhibit myostatin activity. Blockade of endogenous myostatin by using intraperitoneal injections of anti-myostatin monoclonal antibody increased skeletal muscle mass in mice. Inhibition of myostatin activity has also potential benefits for livestock producers, as it can increase growth performance of livestock and lean meat percentage of carcass. At present, only a few reports are available about suppression of myostatin activity in livestock species [13341]

The purpose of one pilot study was to investigate the impact of training, anabolic steroids and endogenous hormones on myostatin-interacting proteins in order to identify manipulations of myostatin signalling. To identify whether analysis of the myostatin interacting proteins follistatin and myostatin propeptide is suitable to detect the abuse of anabolic steroids, their serum concentrations were monitored in untrained males, bodybuilders using anabolic steroids and natural bodybuilders. In addition, it was analysed follistatin and myostatin propeptide serum proteins in females during menstrual cycle. The results showed increased follistatin concentrations in response to anabolic steroids. Furthermore, variations of sex steroid levels during the menstrual cycle had no impact on the expression of follistatin and myostatin propeptide. In addition, we identified gender differences in the basal expression of the investigated proteins. In general, follistatin and myostatin propeptide concentrations were relatively stable within the same individual both in males and females. In conclusion, the current findings provide an insight into gender differences in myostatin-interacting proteins and their regulation in response to anabolic steroids and endogenous hormones. Therefore the data provide new aspects for the development of doping prevention strategies [13400].

**Effect of psychological stress**

Psychological stress is known to attenuate body size and lean body mass. We tested the effects of 1, 3, or 7 days of two different models of psychological stress, 1 h of daily restraint stress (RS) or daily cage-switching stress (CS), on skeletal muscle size and atrophy-associated gene expression in mice. Thymus weights decreased in both RS and CS mice compared with unstressed controls, suggesting that both models activated the hypothalamic-pituitary-adrenal axis. Body mass was significantly decreased at all time points for both models of stress but was greater for RS than CS. Mass of the tibialis anterior (TA) and soleus (SOL) muscles was significantly decreased after 3 and 7 days of RS, but CS only significantly decreased SOL mass after 7 days. TA mRNA levels of the atrophy-associated genes myostatin (MSTN), atrogin-1, and the phosphatidylinositol 3-kinase inhibitory subunit p85alpha were all significantly increased relative to unstressed mice after 1 and 3 days of RS, and expression of MSTN and p85alpha mRNA remained elevated after 7 days of RS.
Expression of muscle ring finger 1 was increased after 1 day of RS but returned to baseline at 3 and 7 days of RS. MSTN, atrogin-1, and p85alpha mRNA levels also significantly increased after 1 and 3 days of CS but atrogin-1 mRNA levels had resolved back to normal levels by 3 days and p85alpha with 7 days of CS. p21CIP mRNA levels were significantly decreased by 3 days of CS or RS. Finally, body mass was minimally affected, and muscle mass was completely unaffected by 3 days of RS in mice null for the MSTN gene, and MSTN inactivation attenuated the increase in atrogin-1 mRNA levels with 4 days of RS compared with wild-type mice. Together these data suggest that acute daily psychological stress induces atrophic gene expression and loss of muscle mass that appears to be MSTN dependent [10369].

**Experimental**

The growth factor myostatin (Mstn) is a negative regulator of skeletal muscle mass. Mstn(-/-) muscles are hypertrophied, stronger, and more glycolytic than Mstn(+/+) muscles, suggesting that they might not perform endurance exercise as well as Mstn(+/+) mice. Indeed, it has previously been shown that treadmill exercise training reduces triceps weight in Mstn(-/-) mice. To analyze the response of Mstn(-/-) muscle to endurance exercise in detail, it was carried out endurance training over 4 weeks to examine muscle mass, histology, and oxidative enzyme activity. We found that muscle mass was reduced with training in several muscles from both genotypes, with no evidence of muscle damage. Citrate synthase activity was increased with training in control and mutant mice. Non-trained Mstn(-/-) mice did, however, have lower maximal exercise capacity compared with Mstn(+/+) mice. These results show that Mstn(-/-) muscle retains the metabolic plasticity necessary to adapt normally to endurance training [10370].

Myostatin is a TGF-β family member that normally acts to limit skeletal muscle mass. Follistatin is a myostatin-binding protein that can inhibit myostatin activity in vitro and promote muscle growth in vivo. Mice homozygous for a mutation in the Fst gene have been shown to die immediately after birth but have a reduced amount of muscle tissue, consistent with a role for follistatin in regulating myogenesis. Here, it was shown that Fst mutant mice exhibit haploinsufficiency, with muscles of Fst heterozygotes having significantly reduced size, a shift toward more oxidative fiber types, an impairment of muscle remodeling in response to cardiotoxin-induced injury, and a reduction in tetanic force production yet a maintenance of specific force. It was shown that the effect of haploinsufficient loss of Fst is at least partially retained in a Mstn-null background, implying that follistatin normally acts to inhibit other TGF-β family members in addition to myostatin to regulate muscle size. Finally, it was presented genetic evidence suggesting that activin A may be one of the ligands that is regulated by follistatin and that functions with myostatin to limit muscle mass. These findings potentially have important implications with respect to the development of therapeutics targeting this signaling pathway to preserve muscle mass and prevent muscle atrophy in a variety of inherited and acquired forms of muscle degeneration [10371].

**Mechanical growth factors**

Recently much interest has been shown in developing a treatment of muscle wasting associated with a range of diseases as well as in ageing, which are major medical and socioeconomic problems. Emerging molecular techniques have made it possible to gain a better understanding of the growth factor genes involved and how they are activated by physical activity including the IGF-1 gene that can be spliced to give rise to different isoforms, one of which is called Mechanical Growth Factor, MGF, that activates muscle progenitor cells
that provide the extra nuclei required for muscle hypertrophy, repair and maintenance. This fact that MGF 'kick starts' the hypertrophy process clearly has potential for abuse and has already attracted the attention of body builders [08290].

Growth factors (GFs) act as signalling agents for cells and become a more and more popular mean to influence the human body and its tissues. This review gives an overview of the current possibilities to use such agents in the field of sports related injuries and thus providing the athlete with a whole new potential to minimize recovery time. GFs and its application have been studied intensively for a long time starting with animal studies. For some of these GFs this research has been brought onto the next level to clinical phase trials. Agents such as insulin like growth factor 1 (IGF-1), mechano growth factor (MGF), basic fibroblast growth factor (B-FGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-beta), bone morphogenetic protein (BMP) and leukemia inhibitory factor (LIF) are being discussed in this review. These GFs not only have the potential to be used to cure injuries but also are being in the centre of interest for doping abusers and are a powerful yet not fully understood technique to gain performance [09215].

Although not approved for clinical use, illicitly distributed peptidic drug candidates such as the fibroblast growth factor (FGF) and mechano growth factor (MGF) were obtained from custom seizures and analyzed/characterized. FGF was obtained in an unlabelled vial and identified by means of 1D- and 2D-PAGE followed by bottom-up LC-MS/MS analysis, providing evidence for the presence of approximately 2 µg of FGF bearing a modified (or truncated) N-terminus. The presence of C-terminally amidated MGF (primary structure: YQPSTNKTSSQRRGSTFEERK) in injection vials was demonstrated by LC-HRMS and −MS/MS, further supporting the growing evidence that peptidic drugs are readily available via internet-based suppliers [13012].

Stretching

Stretching of skeletal muscle induces expression of the genes which encode myogenic transcription factors or muscle contractile proteins and results in muscle growth. Anabolic steroids are reported to strengthen muscles. It was previously studied the effects of muscle stretching on gene expression. The mechanism of mechanotransduction has been elucidated. Skeletal muscle stretch/overload increases the mRNA expression of IGF-I, particularly the specific autocrine IGF-I splicing variant mechano-growth factor (MGF). Mechanical environment and changes in muscle structure and physiology suggest that there may be pathways within muscle cells through which mechanical signals can be converted into chemical signals that in turn generate numerous, specific downstream events that determine the muscle's form and function. Several studies have reported that muscle stretching induces muscle growth and hypertrophy. Such mechanical stimulation is thought to be helpful for patients with muscle weakness and also applicable to clinically unconscious patients and those with paralysis. Administration of anabolic steroids also produces muscle hypertrophy. However, the effects of anabolic steroids on the results of mechanical stimulation to promote muscle strengthening are not well known. Here, it was studied the effect of a combination of passive stretching and the administration of an anabolic steroid on mRNA expression of a muscle growth factor, insulin-like growth factor-I autocrine variant, or mechano-growth factor (MGF). Twelve 8-week-old male Wistar rats were used. Metenolone was administered and passive repetitive dorsiflexion and plantar flexion of the ankle joint performed under deep anesthesia. After 24 h, the gastrocnemius muscles were removed and the mRNA expression of insulin-like growth factor-I autocrine variant was measured using quantitative real-time polymerase chain reaction. Repetitive stretching in combination with metenolone, but not stretching alone, significantly increased MGF mRNA expression. It was
concluded that anabolic steroids enhance the effect of passive stretching on MGF expression in skeletal muscle [13401].

Antibodies against human MGF E-peptide

Since 2005, as one of prohibited substances on the Prohibited List of the World Anti-Doping Agency (WADA), the occurrence of mechano growth factor (MGF) abuse in sport has likely increased. However, there is still no WADA-validated and -approved method for its detection. Four polyclonal antibodies (Ab-K01, Ab-B01, Ab-B02 and Ab-K02) against MGF C-terminal peptides were generated, purified and evaluated by western blot, ELISA and reverse-phase protein microarray, respectively. It was found that all the antibodies could identify their corresponding antigen in mouse serum by reverse-phase protein microarray, in particular, Ab-K01 showed the highest affinity among them and might be a potential tool for the detection of antibody affinity. Furthermore, Ab-B01 and Ab-K01 were successfully used for the determination of MGF-40 by reverse competitive ELISA. The quantitative measurement of MGF-40 has laid the foundation for doping detection of MGF and further biological research on MGF [13402].

Fibroblast growth factor (FGF) and mechano growth factor (MGF)

Although not approved for clinical use, illicitly distributed peptidic drug candidates such as the fibroblast growth factor (FGF) and mechano growth factor (MGF) were obtained from custom seizures and analyzed/characterized. FGF was obtained in an unlabelled vial and identified by means of 1D- and 2D-PAGE followed by bottom-up LC-MS/MS analysis, providing evidence for the presence of approximately 2 mg of FGF bearing a modified (or truncated) N-terminus [12017].

AGRP

The ability of acute exercise to stimulate appetite and food intake depends on intensity, duration, and agouti-related protein (AGRP) levels. Fasting, as well as any negative energy balance, has been reported to increase AGRP expression in the arcuate nucleus (ARC) of the hypothalamus and other extra-hypothalamic tissues in human and rats. The purpose of the present study was to investigate the response of plasma AGRP, GH and insulin to a single circuit-resistance exercise. Twenty volunteer male college students completed a single bout of circuit-resistance training (10 exercises at 35 % of 1RM). Blood samples were collected before, immediately and 30 min following the exercise protocol. Plasma AGRP and GH levels showed a significant increase immediately after exercise and returned to pre exercise values during the recovery period. The data indicate that exercise protocol was able to increase plasma AGRP and GH levels. A higher plasma AGRP level might result in an acute exercise-induced hyperphagia and help to fuel post-exercise restoration processes [07163].
CAFFEINE

Overviews

Caffeine, nicotine, ethanol and tetrahydrocannabinol (THC) are among the most prevalent and culturally accepted drugs in western society. For example, in Europe and North America up to 90 percent of the adult population drinks coffee daily and, although less prevalent, the other drugs are also used extensively by the population. Smoked tobacco, excessive alcohol consumption and marijuana (cannabis) smoking are addictive and exhibit adverse health effects. These drugs are not only common in the general population, but have also made their way into elite sports because of their purported performance-altering potential. Only one of the drugs (i.e. caffeine) has enough scientific evidence indicating an ergogenic effect.

Apart from water, tea and coffee are among the most popular beverages worldwide. The main pharmacologically active substance in both is the purine alkaloid of the xanthines class, 1,3,7-,trimethylxanthine or caffeine. According to European and North American statistics, about 90 percent of the adult population consider themselves as daily coffee users with an average daily caffeine consumption of about 200 mg or 2.4 mg/kg/day (about 2 cups of coffee). It is therefore considered the world’s most widely consumed pharmacologically active substance. Caffeine is both water and fat soluble and is quickly distributed in the body after absorption mainly by the small intestine and the stomach with peaking plasma levels after 15-120 min and a half-life of about 5-6 hours with individual variation. Due to its lipophilic nature, caffeine also crosses the blood-brain barrier, and is metabolized by the liver into paraxanthine, theophylline, and theobromine [130008].

The effect of caffeine to promote improvements in mood, cognition, and exercise performance has been well established in young and athletic adults. However, little is known about whether such nutritional ergogenic aids are effective in enhancing psychological well-being, physiological or cognitive performance in older adults. One study assessed the ergogenic effect of caffeine on mood, perceptual-motor coupling, and muscular strength in an older human population. Following a familiarization session, 12 apparently healthy volunteers (nine females and three males; 69 ± 6 years) completed two laboratory visits. "Pre ingestion" trials of mood state Brunel Mood State Inventory (BRUMS) and coincidence anticipation performance (Bassin anticipation timer) at slow (3 mph) and fast (8 mph) stimulus speeds were completed on both visits. Using a randomized, double-blind, cross-over design, participants consumed either caffeine (3 mg/kg body mass) or a placebo. Sixty minutes post-ingestion participants repeated the trials before completing a set of 10 consecutive repetitions of maximal knee extension using isokinetic dynamometry. Rating of perceived exertion (RPE) was assessed following the fifth and final repetition. Caffeine ingestion significantly improved mood state scores for vigor by 17 percent and reduced absolute error by 35 percent during coincidence anticipation assessment at 8 mph compared to placebo. There were no other significant effects. Caffeine ingestion failed to augment maximal voluntary contraction of the knee extensors and RPE did not prove to be significantly different to from placebo. Acute caffeine ingestion may not be an effective ergogenic aid for improving muscular strength in older adults but could possibly be used as a nutrition supplement for enhancing mood and improving cognitive performance in daily living tasks where interceptive timing skills are required [13435].

Numerous studies to date have shown that caffeine ingested prior to and during prolonged sub-maximal and high intensity exercise can improve performance. It is often cited that caffeine induces its ergogenic effects by an increase in fat oxidation through the sympathetic nervous system, and a sequential sparing of muscle glycogen. However, there is very little
support for an increase in fat oxidation or an enhancement to the sympathetic nervous system being the principal mechanism of caffeine's ergogenic effect. Since, recent investigations have elucidated that the principal mechanism of caffeine's ergogenic effects is through its ability to act as an adenosine receptor antagonist to induce effects on both central and peripheral nervous system to reduce pain and exertion perception, improve motor recruitment and excitation-contraction coupling. In the literature to date, the ergogenic effects are well documented with the time to exhaustion test at a fixed power output being the predominant performance measure used. It was questioned whether assessing endurance capacity in this way would have sufficient ecological validity to translate results to real life events. However since then, a number of studies have confirmed the ergogenic effects of caffeine using time trial protocols, which involves completing an energy based target or set distance in as fast as time possible, thus simulating variable intensities that are likely to occur during competitive events. In most of these studies pure (anhydrous) caffeine was ingested through capsules or dissolved in water. Based on this research it is often assumed that ingesting caffeine in a variety of dietary sources, such as coffee, will result in the same ergogenic effect. Very few studies, however, have shown a positive effect of coffee on exercise performance. Coffee improved performance in some, but not all studies. This may seem surprising as reports have shown that coffee is the most concentrated dietary source of caffeine as well as being one of the largest sources of caffeine used by athletes prior to competition. Amongst the current studies, only two investigations have actually used coffee rather than decaffeinated coffee plus anhydrous caffeine, with only one of these studies showing an ergogenic effect of the coffee. This further identifies the equivocal evidence surrounding the performance effects of coffee. It has been suggested that other components in coffee known as chlorogenic acids, may have antagonised the physiological responses of caffeine. However, in this study chlorogenic acids in the coffee or in the plasma were not measured. Chlorogenic acids are a group of phenolic compounds that possess a quinic acid ester of hydroxycinnamic acid. The consumption of chlorogenic acids varies significantly in coffee ranging from 20-675 mg per serving. It has previously been shown in vitro that chlorogenic acids antagonize adenosine receptor binding of caffeine and cause blunting to heart rate, blood pressure and cause a dose-dependent relaxation of smooth muscle. For this reason, it is unclear what role chlorogenic acids, found in coffee, will have on the physiological and metabolic effects of coffee and caffeine during exercise in humans. Therefore, due to the large variation of chlorogenic acids between coffee beverages and the unclear performance effects of coffee to date, it is yet to be determined if coffee causes differences in the performance and metabolic effects during exercise when compared to caffeine alone. Therefore the primary aim of the present study was to investigate whether acute intake of coffee (5 mg CAF/kg BW) and anhydrous caffeine (5 mg CAF/kg BW) are ergogenic to cycling performance compared to decaffeinated coffee or placebo beverages when using a validated 45-minute time trial performance test. In addition, completing a steady state exercise bout prior to the time trial performance test is a routine protocol used in one laboratory.. For this reason it provided any opportunity to also investigate the effect of acute anhydrous caffeine or coffee intake on substrate oxidation and plasma metabolite responses during 30-min steady state exercise (55 % VO2max). However, whether caffeine ingested through coffee has the same effects is still subject to debate. The primary aim of the study was to investigate the performance enhancing effects of caffeine and coffee using a time trial performance test, while also investigating the metabolic effects of caffeine and coffee. In a single-blind, crossover, randomised counter-balanced study design, eight trained male cyclists/triathletes (age 41 ± 7 years) 30 min of steady-state (SS) cycling at approximately 55 percent VO2max followed by a 45 min energy based target time trial (TT). One hour prior to exercise each athlete consumed drinks consisting of caffeine (5 mg CAF/kg BW), instant coffee (5 mg CAF/kg BW), instant decaffeinated coffee or placebo. The set workloads produced similar relative exercise intensities during the SS for all drinks, with no observed difference in carbohydrate or fat oxidation. Performance times during the TT were
significantly faster (5%) for both caffeine and coffee when compared to placebo and decaf. The significantly faster performance times were similar for both caffeine and coffee. Average power for caffeine and coffee during the TT was significantly greater when compared to placebo and decaf. No significant differences were observed between placebo and decaf during the TT. The present study illustrates that both caffeine (5 mg/kg/BW) and coffee (5 mg/kg/BW) consumed 1 h prior to exercise can improve endurance exercise performance [13436].

One study thus examined the effects of acute intake of coffee (5 mg CAF/kg BW) and caffeine (5 mg CAF/kg BW) on time trial cycling performance, as well as substrate utilisation during SS exercise. Numerous studies to date have shown the efficacy of acute caffeine ingestion for improving prolonged endurance exercise performance. The effects of caffeine on time trial endurance performance (>5 min) have recently been reviewed in a well conducted meta-analysis. The authors concluded that of the 12 studies that investigated caffeine intake (1-6 mg CAF/kg BW), performance was improved by about 3 percent. Fewer studies have investigated the ergogenic effects of coffee, with results being mixed thus far. In agreement with the literature, the current study found an improvement in performance following caffeine intake of 4.9 and 4.5 percent when compared to decaf coffee and placebo, respectively. Interestingly, the current study also showed that coffee improved performance to the same extent as caffeine when compared to decaf coffee and placebo, 4.7 and 4.3 percent respectively. Thus, this is a study that demonstrate that coffee consumed 1 h prior to exercise, at a high caffeine dose (5 mg CAF/kg BW), is equally as effective as caffeine at improving endurance exercise performance. The findings are in line with a number of studies that have shown improvements to performance following coffee intake. For example, decaf coffee plus caffeine (330 mg), improved exercise time to exhaustion (80 % VO_{2max}) compared with decaffeinated coffee (about 18%). More recently, it was shown that coffee was able to improve 1500 m treadmill running performance when compared to decaffeinated coffee (about 3%). Despite conflicting evidence from other studies, the current study clearly demonstrates that coffee is as effective as caffeine at improving endurance exercise performance. The composition and preparation of coffee in each of the studies may also explain the discrepancies in the ergogenic effects of coffee. Coffee is about 2 percent caffeine, with the remainder composed of chlorogenic acids, ferulic acid, caffeic acid, nicotinic acid as well as other unidentifiable compounds. It is evident that the source of coffee beans, roasting, storage and preparation (brewing and filtering) dramatically alters the caffeine and chlorogenic acid content of the coffee. In accordance, recent evidence has shown that the chlorogenic acid content of commercially available espresso coffees range from 24-422 mg/serving. Further, in vitro studies suggest that chlorogenic acids antagonize adenosine receptor binding of caffeine and cause blunting to heart rate and blood pressure in rats. Yet, in vivo there is no evidence to suggest that chlorogenic acids, especially at the low nanomolar concentration typically observed, impact on the mechanisms of action of caffeine that lead to the ergogenic effects. In support of this notion, regular coffee (1.1 mg/kg/BW) consumed prior to the ingestion of different doses of caffeine (3-7 mg/kg/BW) has been shown not to affect the ergogenic effects of caffeine. Interestingly, despite coffee producing similar ergogenic effects as caffeine, the metabolite responses were not identical. It was observed that the significant increase in plasma glucose, FA and glycerol with caffeine was paralleled with an attenuated response for coffee, and a significantly blunted response with decaf coffee when compared to placebo. This is likely due to the compounds in coffee inducing subtle effects on antagonism of adenosine receptors in a variety of exercising and non exercising tissues. It was previously shown that coffee resulted in a blunted adrenaline response when compared to caffeine at rest in humans, which was attributed to chlorogenic acids antagonizing adenosine receptor binding of caffeine. In addition nicotinic acid, a fatty acid ester found in coffee known to inhibit lipolysis, has been shown to lower FA concentrations in patients suffering from hyperlipidemia. Chlorogenic acids are also believed
to improve glucose uptake at the skeletal muscle when compared to caffeine, also by altering the antagonism of adenosine receptors. More recently, caffeic acid has been found to stimulate skeletal muscle glucose transport, independent of insulin, when accompanied with an elevation in AMPK in vitro. Despite the aforementioned evidence, it remains unclear why compounds in coffee appear to modulate the metabolite response but not the ergogenic effects of coffee [13436].

To determine the oral dose of caffeine needed to increase muscle force and power output during all-out single multi-joint movements 13 resistance-trained men, underwent a battery of muscle strength and power tests in a randomized, double-blind, cross over design, under four different conditions: a) placebo ingestion (PLAC) or with caffeine ingestion at doses of; b) 3 mg/kg body weight (CAFF3mg); c) 6 mg/kg (CAFF6mg) and; d) 9 mg/kg (CAFF9mg). The muscle strength and power tests consisted in the measurement of bar displacement velocity and muscle power output during free-weight full-squat (SQ) and bench press (BP) exercises against 4 incremental loads (25 %, 50 %, 75 % and 90 % 1RM). Cycling peak power output (PPO) was measured using a 4s inertial load test. Caffeine side-effects were evaluated at the end of each trial and 24 h later. Mean propulsive velocity at light loads (25 %-50 % 1RM) increased significantly above PLAC for all caffeine doses (5.4-8.5 %). At the medium load (75 % 1RM), CAFF3mg did not improve SQ or BP muscle power or BP velocity. CAFF9mg was needed to enhance BP velocity and SQ power at the heaviest load (9 0% 1RM) and cycling PPO (6.8-11.7 %). The CAFF9mg trial drastically increased the frequency of the adverse side-effects (15-62 %). It was concluded that the ergogenic dose of caffeine required to enhance neuromuscular performance during a single all-out contraction depends on the magnitude of load used. A dose of 3 mg/kg is enough to improve high velocity muscle actions against low loads, whereas a higher caffeine dose (9 mg/kg) is necessary against high loads, despite the appearance of adverse side-effects [13436].

Coffee is the leading worldwide beverage after water and its trade exceeds USD10 billion worldwide. Controversies regarding its benefits and risks still exist as reliable evidence is becoming available supporting its health promoting potential; however, some researchers have argued about the association of coffee consumption with cardiovascular complications and cancer insurgence. The health-promoting properties of coffee are often attributed to its rich phytochemistry, including caffeine, chlorogenic acid, caffeic acid, hydroxyhydroquinone (HHQ), etc. Many research investigations, epidemiological studies, and meta-analyses regarding coffee consumption revealed its inverse correlation with that of diabetes mellitus, various cancer lines, Parkinsonism, and Alzheimer's disease. Moreover, it ameliorates oxidative stress because of its ability to induce mRNA and protein expression, and mediates Nrf2-ARE pathway stimulation. Furthermore, caffeine and its metabolites help in proper cognitive functionality. Coffee lipid fraction containing cafestol and kahweol act as a safeguard against some malignant cells by modulating the detoxifying enzymes. On the other hand, their higher levels raise serum cholesterol, posing a possible threat to coronary health, for example, myocardial and cerebral infarction, insomnia, and cardiovascular complications. Caffeine also affects adenosine receptors and its withdrawal is accompanied with muscle fatigue and allied problems in those addicted to coffee. An array of evidence showed that pregnant women or those with postmenopausal problems should avoid excessive consumption of coffee because of its interference with oral contraceptives or postmenopausal hormones. One review article was an attempt to disseminate general information, health claims, and obviously the risk factors associated with coffee consumption to scientists, allied stakeholders, and certainly readers [11198].

A large body of scientific evidence describes the beneficial effects of human caffeine consumption on a number of physiologic systems. The consumption of moderate amounts of caffeine [10498]:

1183
- increases energy availability
- increases daily energy expenditure
- decreases fatigue
- decreases the sense of effort associated with physical activity
- enhances physical performance
- enhances motor performance
- enhances cognitive performance
- increases alertness, wakefulness, and feelings of "energy"
- decreases mental fatigue
- quickens reactions
- increases the accuracy of reactions
- increases the ability to concentrate and focus attention
- enhances short-term memory
- increases the ability to solve problems requiring reasoning
- increases the ability to make correct decisions
- enhances cognitive functioning capabilities and neuromuscular coordination

Caffeine is a unique compound. It is a drug with no nutritive value so entrenched in our food supply that it enjoys social acceptance and widespread use by the majority of adults around the world. Its well-known effects of reducing fatigue and increasing alertness are prized by populations who need to prolong their capacity for occupational activities, for example, students studying for exams, shiftworkers, long-haul truck drivers, members of the military forces and athletes. In fact, new products such as non-prescription medications, “energy drinks”, confectionery and sports supplements containing caffeine or guarana are now being specifically manufactured to allow caffeine to be consumed as an ergogenic (work-enhancing) aid. Caffeine (C₈H₁₀N₄O₂) has been used as an aid to sports performance for more than a century and has been widely studied by exercise scientists for the past 40 years. From 1980 to 2003, it was included on the list of substances banned by the International Olympic Committee, with limits on urinary caffeine levels above which caffeine use would be deemed to be a doping offence. These levels were intended to discriminate the intake of large amounts of caffeine – typically, above 6–9 mg/kg of an athlete’s body mass (kg BM). In 2004, however, caffeine was removed from the list of prohibited substances and methods of the World Anti-Doping Agency, meaning that athletes who compete under this code can consume caffeine either in their background diets or for the specific purposes of performance enhancement without fear of sanctions. Several aspects of the relationship between caffeine and exercise are intriguing, and differ from the situation with other ergogenic aids. First, caffeine appears to exert positive effects on exercise capacity (prolonging the duration for which exercise of a given intensity can be maintained) over a diverse range of protocols including prolonged submaximal exercise (>90 min), sustained high-intensity work (20-60 min) and short duration supra-maximal exercise (1–5 min). Of course, athletes are more interested in the effects of caffeine in trained individuals on measurements of sports performance. A much smaller number of studies in laboratory and field conditions show that caffeine supplementation is likely to be beneficial across a range of sports including endurance events, “stop and go” events (e.g. team and racquet sports) and sports involving sustained high-intensity activity lasting from 1-60 min (e.g. swimming, rowing, middle and distance running races). The direct effects on single events involving strength and power such as lifts, throws and sprints are unclear. The benefits of caffeine appear to be achieved by a number of different protocols of use, with variables including the timing and amount of the caffeine dose. Although the traditional supplementation regimen involves a single intake of about 6 mg/kg BM, 1 h pre-exercise, one studies show that ergogenic effects from caffeine intake may occur at very modest levels of intake (1-3 mg/kg BM or 70–200 mg caffeine). In fact, several studies suggest there is no dose-response relationship between caffeine intake
and benefits to endurance exercise or, if it exists, there is a plateau at about 3 mg/kg or about 200 mg. The intake of caffeine from the traditionally available sources (coffee, tea and cola drinks) is typically around 50–150 mg of caffeine per serving. However, it is possible to find products that provide 300–500 mg of caffeine per serving. In terms of variations to the timing of intake of caffeine doses, it appears, at least in endurance sports, that caffeine can be consumed pre-event or as single or multiple doses spread throughout an exercise bout or just prior to the onset of fatigue. The effects of caffeine can be long lasting, with one study showing that people who ingest caffeine to enhance a morning exercise task may still receive benefits during a session undertaken later in the day [10174].

The effect caffeine elicits on endurance performance is well founded. However, comparatively less research has been conducted on the ergogenic potential of anaerobic performance. Some studies showing no effect of caffeine on performance used untrained subjects and designs often not conducive to observing an ergogenic effect. Recent studies incorporating trained subjects and paradigms specific to intermittent sports activity support the notion that caffeine is ergogenic to an extent with anaerobic exercise. Caffeine seems highly ergogenic for speed endurance exercise ranging in duration from 60 to 180 seconds. However, other traditional models examining power output (i.e. 30-second Wingate test) have shown minimal effect of caffeine on performance. Conversely, studies employing sport-specific methodologies (i.e. hockey, rugby, soccer) with shorter duration (i.e. 4-6 seconds) show caffeine to be ergogenic during high-intensity intermittent exercise. Recent studies show caffeine affects isometric maximal force and offers introductory evidence for enhanced muscle endurance for lower body musculature. However, isokinetic peak torque, one-repetition maximum and muscular endurance for upper body musculature are less clear. Since relatively few studies exist with resistance training, a definite conclusion cannot be reached on the extent caffeine affects performance. It was previously thought that caffeine mechanisms were associated with adrenaline (epinephrine)-induced enhanced free-fatty acid oxidation and consequent glycogen sparing, which is the leading hypothesis for the ergogenic effect. It would seem unlikely that the proposed theory would result in improved anaerobic performance, since exercise is dominated by oxygen-independent metabolic pathways. Other mechanisms for caffeine have been suggested, such as enhanced calcium mobilization and phosphodiesterase inhibition. However, a normal physiological dose of caffeine in vivo does not indicate this mechanism plays a large role. Additionally, enhanced Na+/K+ pump activity has been proposed to potentially enhance excitation contraction coupling with caffeine. A more favourable hypothesis seems to be that caffeine stimulates the CNS. Caffeine acts antagonistically on adenosine receptors, thereby inhibiting the negative effects adenosine induces on neurotransmission, arousal and pain perception. The hypoalgesic effects of caffeine have resulted in dampened pain perception and blunted perceived exertion during exercise. This could potentially have favourable effects on negating decreased firing rates of motor units and possibly produce a more sustainable and forceful muscle contraction. The exact mechanisms behind caffeine's action remain to be elucidated [09251].

Caffeine is the pharmacologically active substance found in tea, coffee, and cola. The amount of caffeine present varies according to the type of drink and the way it has been prepared. Caffeine may also be a constituent of some common medicines such as cold preparations and pain relief treatments, usually in quantities of less than 100 mg per dose. Caffeine produces mild CNS stimulation, similar to that of amphetamines, reducing fatigue and increasing concentration and alertness. Physiological effects include increased heart rate and output, metabolic rate, and urine production. High doses can cause anxiety, insomnia, and nervousness. In 2004 caffeine was removed from the list of prohibited substances and is now part of the monitoring programme [06171].
Caffeine is a naturally occurring plant alkaloid. It is classified as a methylxanthine. Other examples of methylxanthines include theophylline and theobromine. It is found in over 60 different plant species including Caffea arabica (coffee), Thea sinenis (tea), and Cola acuminata (cola). Caffeine is consumed in a variety of forms, including coffee, soft drinks, and chocolate. It is also found in a variety of over the counter stimulants, appetite suppressants, analgesics, and cold and sinus preparations. In fact, caffeine is the world’s most commonly used and widely consumed pharmacologic substance. Approximately 75 percent of caffeine is consumed in the form of coffee. In terms of international commerce, coffee is second only to oil in dollar amount traded. The US imports approximately 30% of the world’s coffee and Americans consume about 45 million pounds of coffee annually. Consumption of caffeine is actually higher in the United Kingdom and Scandinavian countries (400 mg/person/d compared with 238 mg/person/d in the United States) [06183].

History of coffee drinking

Caffeine has been consumed in the form of coffee since around 850 AD when its use was popularized in Egypt. Caffeine has long held interest as a potential ergogenic aid. In fact, the fatigue-masking effects of caffeine have been known since the early 1900s. Research in the late 1970s indicating improved exercise performance with caffeine popularized its use as a potential ergogenic aid [06183].

Presence of caffeine in society

Caffeine (1,3,7-trimethylxanthine) is one of the most consumed drugs in sports. A recent study has shown that 3 out of 4 elite athletes consume caffeine prior to competing, based on the post-exercise urinary caffeine concentrations of 20,686 urine samples obtained for doping analysis. However, the manner in which athletes consume caffeine is diverse. Caffeine is present in coffee and chocolate beans, tea leaves and cola nuts and so can be consumed from natural sources (coffee, tea, chocolate, etc). In addition, caffeine can be artificially synthesized and included in food and drinks, like the recently created energy drinks. These beverages contain moderate amounts of caffeine (32 mg/100 mL) in addition to carbohydrates, taurine, glucoronolactone and B- group vitamins. Due to their low cost, accessibility, and the relatively low frequency of deleterious side-effects derived from their consumption, caffeine-containing energy drinks have become the most popular supplement in the sports population, with a prevalence of 73 percent in American college athletes, 75 percent in Canadian Varsity athletes and 42 percent in British elite athletes [12274].

Caffeine is the most commonly consumed psychoactive drug in the world, and some of its behavioral effects (such as arousal) may resemble those produced by cocaine, amphetamines, and other stimulants. Coffee consumption accounts for about 75 percent of the adult intake of caffeine in the United States, although that might be changing among younger adults with the growing popularity of energy drinks. The caffeine content of coffee varies greatly, depending on the beans, how they're roasted, and other factors, but the average for an 8-ounce cup is about 100 milligrams (mg). Tea has about half as much caffeine as coffee. Decaffeinated coffee has some caffeine, but the 2 to 4 mg in an 8-ounce cup is a smidgen compared with the caffeinated version. The lethal dose of caffeine is about 10 grams, which is equivalent to the amount of caffeine in 100 cups of coffee. Caffeine gets absorbed in the stomach and small intestine and then distributed throughout the body, including the brain. The amount circulating in the blood peaks 30 to 45 minutes after it’s
ingested and only small amounts are around eight to 10 hours later. In between, the amount circulating declines as caffeine gets metabolized in the liver [12275].

**Epidemiology of use**

Caffeine is a proven ergogenic aid, increasing athletic performance, endurance, and mental chronometry at doses as low as 1-3 mg/kg. As coffee is a readily available and commonly ingested form of caffeine, the two are often equated. However, coffee also contains hundreds of other biologically active compounds, many of which are metabolically distinct from caffeine. The purpose of one review was to examine the prevalence of coffee and (or) caffeine consumption among elite Canadian athletes, and to delineate the effects of coffee and caffeine on physical activity, weight maintenance, performance, and metabolism. A total of 270 self-reported 3-day food records were examined for caffeine intake from athletes registered with Canadian Sport Centres in 2005 and 2006. Athletes ranged in age from 16-45 years, and competed in 38 different sports. Results showed that 30 percent of athletes ingested >1 mg/kg per day from a variety of sources. Average daily intake was 0.85 ± 13 mg/kg. Caffeine intake was not correlated with any one sport; the 10 highest caffeine users were athletes from 9 different sports, including skill, endurance, and power sports. No differences were noted for average caffeine ingestion between summer and winter sports. High caffeine intakes corresponded to coffee ingestion, with the 25 highest individual intakes (193-895 mg/day) from coffee drinkers. In summary, it could be concluded that the majority of high-level Canadian athletes consume dietary caffeine primarily in the form of coffee. However, levels consumed are insufficient to elicit performance enhancement. Potential detrimental effects of caffeine consumption on exercise performance include gastric upset, withdrawal, sleep disturbance, and interactions with other dietary supplements [08305].

**Self-reported consumption**

One study was undertaken to examine self-reported caffeine consumption and reasons for its use, amongst UK athletes, following its removal from the 2004 World Anti-Doping Agency (WADA) Prohibited List. A convenience sample of track and field athletes (n=193) and cyclists (n=287) completed a postal or Web-based questionnaire. Messages were posted on athletics and cycling club Web sites and mailing lists to direct athletes to the Web-based questionnaire. Postal questionnaires were distributed at domestic sporting events. A higher proportion of cyclists (60 %) compared with track and field athletes (33 %) consumed caffeine to enhance performance. A higher proportion of elite as opposed to sub-elite athletes representing cycling and athletics used caffeine to enhance performance. Of all caffeine containing products used, coffee, energy drinks, pharmaceutical preparations and caffeinated sports supplements were most prevalent. Results revealed that amongst UK athletes, the intention to use caffeine as an ergogenic aid was high, and that use was more widespread and accepted in competitive sport, especially at elite level, when compared to recreational sport [07164].

**Emergency medicine residents’ use of psychostimulants**

It was evaluated the frequency that emergency medicine house staff report use of stimulants and sedatives to aid in shift work and circadian transitions. It was surveyed residents from 12 regional emergency medicine programs inviting them to complete a voluntary, anonymous electronic questionnaire regarding their use of stimulants and sedatives. Out of 485 eligible residents invited to participate in the survey, 226 responded (47 % response frequency). The reported use of prescription stimulants for shift work is uncommon (3 % of respondents.) In
contrast, 201 residents (89%) report use of caffeine during night shifts, including 118 residents (52%) who use this substance every night shift. Eighty-six residents (38%) reported using sedative agents to sleep following shift work with the most common agents being anti-histamines (31%), nonbenzodiazepine hypnotics such as zolpidem (14%), melatonin (10%), and benzodiazepines (9%). It was concluded that emergency medicine residents report substantial use of several classes of hypnotics to aid in shift work. Despite anecdotal reports, use of prescription stimulants appears rare, and is notably less common than use of sedatives and non-prescription stimulants [11495].

Athletes’ knowledge of effects of caffeine

One descriptive cross-sectional study assessed the perceptions, knowledge, and experiences of caffeine use by athletes competing at the 2005 Ironman Triathlon World Championships. Questionnaires were distributed to 140 athletes (105 men and 35 women, 40 ± 11 years old) representing 16 countries during prerace registration. A large proportion (73%) of these endurance athletes believe that caffeine is ergogenic to their endurance performance, and 84 percent believe it improves their concentration. The most commonly reported positive caffeine experiences related to in-competition use of cola drinks (65%) and caffeineated gels (24%). The athletes’ ability to accurately quantify the caffeine content of common food items was limited. The most popular sources of caffeine information were self-experimentation (16%), fellow athletes (15%), magazines (13%), and journal articles (12%). Over half the athletes (53%) could not identify an amount of caffeine required to improve their triathlon performance. Mean (+/- standard deviation) suggested doses were 3.8 ± 3.0 mg/kg body weight. Few side effects associated with taking caffeine during exercise were reported [07165].

Factors influencing serum caffeine concentrations

To determine whether differences in training status, body composition and/or habitual caffeine intake influenced serum caffeine concentrations following caffeine ingestion trained cyclists/triathletes (n=14) and active (n=14) males consumed 6 mg/kg anhydrous caffeine were studied. Peak, total and time to peak serum caffeine concentrations were determined from venous blood samples at baseline and 6 time-points over 4 h following intake. Body composition was assessed by dual energy X-ray absorptiometry and habitual caffeine intake by a questionnaire. Trained cyclists/triathletes had 16 percent lower peak caffeine concentrations following caffeine ingestion compared to active individuals, although this was not statistically significant. There was no significant difference between trained cyclists/triathletes and active males in total or time to peak serum caffeine concentrations. Fat mass was significantly associated with total but not peak or time to peak serum caffeine concentration. There were no associations between habitual caffeine intake and peak, total or time to peak serum caffeine concentrations. It was concluded that following caffeine ingestion three findings from the study it was evident that endurance-trained athletes trended towards lower peak caffeine concentrations compared to active males; that higher fat mass was associated with higher concentrations of caffeine in the blood over 4h, and that habitual caffeine intake does not appear to influence serum caffeine concentrations. Identification of the optimal conditions to ensure peak availability of caffeine within the blood and/or overcoming some of the variation in how individuals respond to caffeine requires consideration of the training status and body composition of the athlete [0458].
Bioavailability of coffee

Coffee and green tea are two of the most widely consumed hot beverages in the world. Their respective bioavailability has been studied separately, but absorption of their respective bioactive phenolics has not been compared. In a randomised cross-over design, nine healthy subjects drank instant coffee and green tea. Blood samples were collected over 12 h and at 24 h to assess return to baseline. After green tea consumption, (-)-epigallocatechin (EGC) was the major catechin, appearing rapidly in the plasma; (-)-EGC gallate (EGCg) and (-)-epicatechin (EC) were also present, but (-)-EC gallate and C were not detected. Dihydroferulic acid and dihydrocaffeic acid were the major metabolites that appeared after coffee consumption with a long time needed to reach maximum plasma concentration, suggesting metabolism and absorption in the colon. Other phenolic acid equivalents (caffeic acid (CA), ferulic acid (FA) and isoferulic acid (iFA)) were detected earlier, and they peaked at lower concentrations. Summations of the plasma area under the curves (AUC) for the measured metabolites showed 1.7-fold more coffee-derived phenolic acids than green tea-derived catechins. Furthermore, it was found a significant correlation between coffee metabolites based on AUC. Inter-individual differences were observed, but individuals with a high level of CA also showed a correspondingly high level of FA. However, no such correlation was observed between the tea catechins and coffee phenolic acids. Correlation between AUC and maximum plasma concentration was also significant for CA, FA and iFA and for EGCg. This implies that the mechanisms of absorption for these two classes of compounds are different, and that a high absorber of phenolic acids is not necessarily a high absorber of catechins [10501].

Caffeine dosages versus effects

Athletes are among the groups of people who are interested in the effects of caffeine on endurance and exercise capacity. Although many studies have investigated the effect of caffeine ingestion on exercise, not all are suited to draw conclusions regarding caffeine and sports performance. Characteristics of studies that can better explore the issues of athletes include the use of well-trained subjects, conditions that reflect actual practices in sport, and exercise protocols that simulate real-life events. There is a scarcity of field-based studies and investigations involving elite performers. Researchers are encouraged to use statistical analyses that consider the magnitude of changes, and to establish whether these are meaningful to the outcome of sport. The available literature that follows such guidelines suggests that performance benefits can be seen with moderate amounts (~3 mg/kg body mass) of caffeine. Furthermore, these benefits are likely to occur across a range of sports, including endurance events, stop-and-go events (e.g. team and racquet sports), and sports involving sustained high-intensity activity lasting from 1-60 min (e.g. swimming, rowing, and middle and distance running races). The direct effects on single events involving strength and power, such as lifts, throws, and sprints, are unclear. Further studies are needed to better elucidate the range of protocols (timing and amount of doses) that produce benefits and the range of sports to which these may apply. Individual responses, the politics of sport, and the effects of caffeine on other goals, such as sleep, hydration, and refuelling, also need to be considered [08304].

The most common form of caffeine consumption is coffee. One cup of coffee contains approximately 100 mg of caffeine. This is the same dosage of caffeine that is contained in most over the counter preparations that are used for promoting wakefulness. Soft drinks are another common form of caffeine consumption. Levels vary depending on the type of soft drink. A 12-oz Coca-Cola can contains approximately 45.6 mg of caffeine, a 12-oz glass of
iced tea contains approximately 70 mg of caffeine. The average American consumes about 200 mg of caffeine daily; adults average about 2.4 mg/kg/d whereas children average about 1.1 mg/kg/d. Interestingly, studies have shown that caffeine can produce its ergogenic potential at doses much less than 800 mg, probably as low as 250 mg. Most studies that show ergogenic potential of caffeine have used dosages of around 400 to 600 mg of caffeine which is equivalent to the amount of caffeine in four to six cups of coffee. A dose of 100 mg of caffeine (equivalent to one cup of coffee) will produce a urine concentration of approximately 1.5 mg/mL. Caffeine content (mg) of common substances in 2005 [06183]:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Caffeine content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coffee</td>
<td>7.5 oz 100</td>
</tr>
<tr>
<td>Coca-Cola</td>
<td>12 oz 46</td>
</tr>
<tr>
<td>Diet Coke</td>
<td>12 oz 46</td>
</tr>
<tr>
<td>Mountain Dew</td>
<td>12 oz 54</td>
</tr>
<tr>
<td>Dr. Pepper</td>
<td>12 oz 40</td>
</tr>
<tr>
<td>Sprite</td>
<td>12 oz 0</td>
</tr>
<tr>
<td>Iced tea</td>
<td>12 oz 70</td>
</tr>
<tr>
<td>Over-the-counter stimulants</td>
<td>1 capsule 100</td>
</tr>
</tbody>
</table>

The content (mg) of an “ordinary” cup (8.4 ounce) of caffeine is [12275]:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Caffeine content (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starbucks coffee</td>
<td>180</td>
</tr>
<tr>
<td>Red bull</td>
<td>80</td>
</tr>
<tr>
<td>Lipton green tea (1 bag)</td>
<td>35</td>
</tr>
</tbody>
</table>

**Duration of effect**

It appears that the ergogenic effect of caffeine occurs regardless of the timing of intake (either before or during the event). The effect of caffeine intake appears to be prolonged to as much as 6 hours following ingestion. This ergogenic effect is seen at a similar magnitude with both a one-time ingestion prior to exercise as well as with multiple smaller, but equal dosages given throughout a period of prolonged exercise. An interesting finding is that the ergogenic effect of caffeine is more pronounced in nonusers (< 50 mg daily) compared with regular users (> 300 mg daily) of caffeine. This is most likely explained by the upregulation of adenosine receptors with the regular consumption of caffeine [06183].

**Administration mode**

In the majority of studies that look at caffeine as an ergogenic aid, the caffeine is consumed in capsule form. It was looked at the ergogenic potential of caffeine ingested in the form of coffee. The plasma concentrations of caffeine were similar whether ingested in the form of coffee or capsule. However, enhancement of endurance was seen only when caffeine was consumed independent of coffee. Likely, there are substances in coffee that antagonize the ergogenic potential of caffeine. Another source of caffeine that has been studied in terms of its ergogenic potential is caffeinated soft drinks. There is a practice among some endurance athletes to use a “defizzed” soft drink as a replacement for sports drinks during the latter stages of such events, believing that the caffeine intake produces an ergogenic effect. A study out of Australia suggests that the use of Coca-Cola produces an ergogenic effect similar to that of more conventional forms of caffeine intake. These findings are of undetermined significance, however, as the dose of caffeine consumed through Coca-Cola in this study are less than dosages of caffeine previously proven to be ergogenic [06183].

**Dosage versus endurance cycle time**
One study investigated the effects of two different doses of caffeine on endurance cycle time trial performance in male athletes. Using a randomised, placebo-controlled, double-blind crossover study design, sixteen well-trained and familiarised male cyclists (age 32 years) completed three experimental trials, following training and dietary standardisation. Participants ingested either a placebo, or 3 or 6 mg/kg body mass of caffeine 90 min prior to completing a set amount of work equivalent to 75 percent of peak sustainable power output for 60 min. Exercise performance was significantly improved with both caffeine treatments as compared to placebo (4.2 % with 3 mg/kg body mass and 2.9 % with 6 mg/kg body mass). The difference between the two caffeine doses was not statistically significant. Caffeine ingestion at either dose resulted in significantly higher heart rate values than the placebo conditions, but no statistically significant treatment effects in ratings of perceived exertion (RPE) were observed. A caffeine dose of 3 mg/kg body mass appears to improve cycling performance in well-trained and familiarised athletes. Doubling the dose to 6 mg/kg body mass does not confer any additional improvements in performance [12291].

Use as a flavor

Over 60 percent of soft-drinks sold in the United States contain caffeine, a mildly addictive psycho-active chemical, as a flavor additive. Using sweeteners as controls, we assessed whether caffeine has flavor activity in a cola soft-drink. A forced-choice triangle discrimination methodology was used to determine detection thresholds of caffeine in sweeteners and a cola beverage. The subjects (n=30, 28 female, 23 years old) were trained tasters and completed over 1600 discrimination tests during the study. The mean detection thresholds for caffeine in the sweet solutions were: 0.333 ± 0.1mM sucrose; 0.467 ± 0.29 mM aspartame; 0.462+/−0.3mM sucralose, well below the concentration in common cola beverages (0.55-0.67 mM). A fixed concentration of caffeine, corresponding to the concentration of caffeine in a common cola beverage (0.67 mM) was added to the sweeteners and a non-caffeinated cola beverage. Subjects could distinguish between caffeinated and non-caffeinated sweeteners, but all subjects failed to distinguish between caffeinated and non-caffeinated cola beverage. Caffeine has no flavor activity in soft-drinks yet will induce a physiologic and psychologic desire to consume the drink [06184].

Potential adverse effects

Caffeine can potentially cause some adverse effects. Reported effects at moderate doses include locomotor agitation, tachycardia, diuresis, insomnia, irritability, and increased anxiety. Severe caffeine toxicity has been linked to seizures and arrhythmias. In addition, it has been well documented that caffeine produces a withdrawal syndrome with cessation of repeated use. This can occur even with repeated usage at low dosages. Studies have demonstrated that withdrawal symptoms can occur with cessation of caffeine use for a short time period as soon as 3 days after administration in novel users and as soon as 12 hours in habitual users. Common symptoms of caffeine withdrawal include headache, irritability, increased fatigue, drowsiness, decreased alertness, difficulty concentrating, and decreased energy and activity levels. Symptoms can be mild to moderate in severity. Fortunately, withdrawal symptoms are generally short-lived. Several studies have shown that caffeine can increase core body temperature. Increased diuresis with a concomitant decrease in body weight has also been demonstrated after administration of caffeine [06183].

Addiction

The common-sense use of the term addiction is that regular consumption is irresistible and that it creates problems. Caffeine use does not fit this profile. Its intake does no harm to the
individual or to society and its users are not compelled to consume it. Though cessation of regular use may result in symptoms such as headache and lethargy, these are easily and reliably reversed by ingestion of caffeine. Some have argued that continued caffeine use is an attempt to suppress low grade withdrawal symptoms such as sleepiness and lethargy. In some moderate users, this is possible; however, in experimental contexts, the phenomenon is too inconsistent to constitute a reliably valid syndrome [06185].

Caffeine and ephedra
Unfortunately, there have been multiple deaths linked to the use of caffeine in combination with ephedra. It is felt that, when used in combination, the potential deadly effects are secondary to ephedra, rather than caffeine. However, the cardiovascular effects of ephedra are likely increased with concomitant stimulant use (e.g. caffeine). In fact, based on this evidence, ephedra was banned by the US Food and Drug Administration in 2004. None of the reported deaths were linked to the use of caffeine alone [06183].

Acceleration of caffeine metabolism of tobacco and cannabis
Tobacco and marijuana accelerate caffeine metabolism, which reduces the time caffeine circulates in the body. Oral contraceptives slow it down, so they have the opposite effect. Researchers have identified genes that influence a person’s natural risk of caffeine metabolism, which might explain why some people are exquisitely sensitive to caffeine while others are not [12275].

Overview of physiological effects of caffeine in health and disease
Caffeine probably has multiple targets in the brain, but the main one seems to be adenosine receptors. Adenosine is a brain chemical that dampens brain activity. By hogging adenosine’s receptors, caffeine sets off a chain of events that affects the activity of dopamine, another important brain chemical, and the areas of the brain involved in arousal, pleasure, and thinking. A part of the brain affected by Parkinson’s disease, called the striatum, has many adenosine receptors; by docking on them, caffeine seems to have some protective effects. Outside the brain, caffeine can be a performance enhancer, boosting the strength of muscle contraction and offsetting some of the physiological and psychological effects of physical exertion. But, especially in the short term, it also has negative effects, which include raising blood pressure, making arteries stiffer, and increasing levels of homocysteine, insulin, and possibly cholesterol. Habitual use may cause some of these effects to wear off. For some conditions, though, coffee may have some benefit despite, rather than because of, caffeine [12275].

Caffeine has been studied more than any other ingredient in coffee, and it tends to get credit if the body part benefited is the brain. But coffee contains literally a thousand different substances, and some of the lesser lights are thought to be responsible for healthful effects in other parts of the body. Some studies show caffeinated and decaffeinated coffee as having the same effect, which suggests that something else in coffee is involved. It gets complicated, though. Caffeine and some of these other substances in coffee seem to have their good and bad sides, and coffee’s overall effect may depend on how much they cancel each other out [12275]:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alzheimer's disease</td>
<td>Human and animal studies show hints of protection. Some preliminary evidence suggests activity against beta-amyloid plaque that may have a causative role in Alzheimer's.</td>
</tr>
<tr>
<td>Cancer</td>
<td>Studies suggest a lower risk for some cancers (endometrial, aggressive prostate, estrogen-negative breast), but not others (esophageal). Antioxidant</td>
</tr>
</tbody>
</table>
and anti-inflammatory substances could be responsible for possible anticancer activity.

**Diabetes**

Effects on insulin and blood sugar levels that would promote diabetes seem to be temporary. Regular use is associated with lower risk, and high intake (3-6 cups a day) seems to have a greater effect. Protection may come from increases in the hormone adiponectin and other factors that affect insulin and blood sugar levels.

Coffee drinking increases some factors (homocysteine) associated with higher risk. But moderate consumption (1-3 cups a day) has been linked to a small decrease in risk. The evidence for a possible protective effect is stronger for women.

Coffee drinking is associated with lower levels of enzymes that indicate liver damage and inflammation. Coffee may improve response to some treatments for hepatitis C. Findings suggest some protection against liver cancer. Cafestol and kahweol, substances found in unfiltered coffee, may be responsible for liver benefits.

**Heart attack**

Coffee drinking increases some factors (homocysteine) associated with higher risk. But moderate consumption (1-3 cups a day) has been linked to a small decrease in risk. The evidence for a possible protective effect is stronger for women.

Coffee drinking is associated with lower levels of enzymes that indicate liver damage and inflammation. Coffee may improve response to some treatments for hepatitis C. Findings suggest some protection against liver cancer. Cafestol and kahweol, substances found in unfiltered coffee, may be responsible for liver benefits.

**Liver disease**

Coffee drinking is associated with lower levels of enzymes that indicate liver damage and inflammation. Coffee may improve response to some treatments for hepatitis C. Findings suggest some protection against liver cancer. Cafestol and kahweol, substances found in unfiltered coffee, may be responsible for liver benefits.

**Parkinson's disease**

Studies show a moderate (25%) decrease in risk for coffee drinkers. The effect is less in women. Research has found evidence of activity in the part of the brain affected by Parkinson's.

Moderate consumption (3-4 cups a day) is associated with lower risk. But chance of a stroke may increase immediately after intake, particularly among infrequent consumers.

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**Physiological effects of caffeine**

Caffeine's physiologic effects on the body have been well studied. Caffeine is metabolized through the liver via the cytochrome P450 enzyme system. It is rapidly absorbed through the gastrointestinal tract, with approximately 90 percent cleared from the stomach within 20 minutes. Peak plasma concentrations of caffeine are achieved in 40 to 60 minutes. Its half-life is approximately 3 to 5 hours. Caffeine is lipophilic and readily crosses most bodily membranes. It crosses to blood-brain barrier (BBB) and also crosses the placenta. Caffeine has been theorized to exert its effects through fat oxidation, central nervous system (CNS) stimulation, and direct action at skeletal muscle. There are several purported mechanisms for these effects including antagonism of adenosine receptors, inhibition of cyclic AMP, phosphodiesterase activity, increased calcium mobilization, and antagonism of benzodiazepine receptors. Caffeine has been reported to increase lipolysis throughout the body with a resultant increase in plasma free fatty acid levels. It is thought that by having increased levels of fatty acids available for use by the body, muscle glycogen can be spared. Current research suggests that the main mechanism responsible for the physiologic effects of caffeine is blockade of CNS adenosine receptors. Because it easily crosses the BBB, caffeine can readily have an effect on the CNS. The dosage of caffeine required to block adenosine receptors is much less than that required for caffeine to perform most of the other theorized physiologic mechanisms. A study using laboratory animals compared the results of centrally administered caffeine (adenosine antagonist) with 5'-N-ethylcarboxamidoadenosine (NECA), an adenosine agonist. Caffeine improved run time to fatigue whereas NECA reduced it. Administration of caffeine peripherally failed to produce the same effect. Adenosine is a modulator of CNS neurotransmission. It is a potent vasodilator. It also decreases catecholamine release and inhibits lipolysis. Caffeine nonselectively blocks adenosine receptors, thus competitively inhibiting the action of adenosine. Upregulation of these receptors occurs with regular consumption of caffeine [06183].
Caffeine produces its effect on various systems throughout the body. In the cardiovascular system, caffeine acts to increase heart rate and blood pressure. There is a positive relationship with caffeine consumption (in the form of coffee intake) and elevated systolic blood pressure. The extent of blood pressure elevation appears to depend on the individual's baseline blood pressure. Studies have shown that individuals with diagnosed hypertension have a greater increase in blood pressure in response to caffeine than do normotensive individuals. These effects are additive to that of other pressor agents, including cigarette smoking and psychologic stress. Caffeine may also increase the incidence of premature ventricular contractions (PVCs). Although these hemodynamic effects are similar despite gender, the underlying mechanism appears to differ slightly when comparing men and women. Men given caffeine show an increase in vascular resistance with no effect on cardiac output. However, women given similar amounts of caffeine show no difference in vascular resistance. There is an increase in stroke volume, which results in an increased cardiac output, which accounts for the hemodynamic changes that are seen. There is speculation that this effect may be due to estrogen effects, but has not been proven. In the respiratory system, caffeine produces bronchial dilation and relaxation of pulmonary smooth muscle. It also increases respiratory rate. In neonates, caffeine is used as a respiratory stimulant to prevent apneic episodes. Interestingly, caffeine ingestion potentiates the slowed reaction time that is induced by alcohol consumption. Therefore, the popularly held belief that coffee is an "antidote" for alcohol intoxication is false [06183].

**Effect on EMG frequency variables**

The ergogenic effect of caffeine and its mechanism of action on short-term, high-intensity exercise are controversial. One proposed mechanism is caffeine's stimulatory effect on the central nervous system and thus, motor-unit excitation. The latter is non-invasively determined from surface electromyographic signal (EMG) frequency measures. The purpose of this study was to determine if power output and surface EMG frequency variables during high-intensity cycling were altered following caffeine ingestion. Eighteen recreationally active college males (mean age, 22) performed the Wingate test (WG) after ingestion of gelatin capsules containing either placebo (PL; dextrose) or caffeine (CAFF; 5 mg/kg body mass). The trials were separated by 1 week and subjects were asked to withdraw from all caffeine-containing products for 48 h before each trial. From the resulting power-time records, peak power (PP; highest power output in 5 s), minimum power (MP; lowest power output in 5 s), and the percent decline in power (Pd) were calculated. Surface EMG records of the right vastus lateralis (VL) and the gastrocnemius (GA) muscles corresponding to the PP and MP periods were collected and used to determine the integrated electromyogram (IEMG), the mean (MNPF), and the median (MDPF) of the signal's power spectrum. A 2-way repeated measures analysis of variance (ANOVA) (treatment x time) was conducted to determine the effect of caffeine on these variables across levels of time. Caffeine ingestion had no effect on PP, MP, or the Pd compared with the placebo. For both muscles, MNPF and MDPF diminished significantly across time and to a similar degree in both the CAFF and PL trials. Regardless of muscle, CAFF had no effect on the percent change in IEMG from the first 5 s to the last 5 s. For both treatments, the GA displayed a significantly greater pre versus post percent decline in the EMG signal amplitude compared with the VL. These results indicate that caffeine does not impact power output during a 30 s high-intensity cycling bout. Furthermore, these data suggest that caffeine does not impact the neuromuscular drive as indicated by the similar IEMG scores between treatments. Similarly, caffeine does not seem to impact the frequency content of the surface EMG signal and thus the nature of recruited motor units before and after the expression of fatigue. The lack of decline in the IEMG in the VL despite the decline in power output over the course of the WG suggests a peripheral as
opposed to a neural mechanism of fatigue in this muscle. The significant difference in the pre vs. post percent decline in the GA IEMG score further supports this notion. The pre versus post decline in the IEMG noted in the GA may suggest a fatigue-triggered change in pedaling mechanics that may promote dominance of knee extensors with less reliance on plantar flexors [06186].

Effect on energy expenditure

PA energy expenditure (PAEE) is the most variable component of Total Energy Expenditure (TEE) and largely due to the balance of sedentary time (SedT) and low intensity physical activity (LIPA). There has been an emergence for seeking an understanding of factors which determine variations in SedT, LIPA, and PAEE. Sedentary behavior and physical activity are relatively resistant to change by experimental dietary treatments and significant body weight changes. Although caffeine (Caf) is by far the most heavily used nutritional agent ingested to promote a sense of vigor/alertness, it is still unknown if Caf is effective in increasing PAEE and physical activity. The aim of the study was to test the hypothesis that 2 daily doses of Caf (as a capsule to blind the treatment and divided equally during breakfast and lunch) increase PAEE and TEE, and it would do so through increasing the frequent and brief bouts of physical activity (1-5 min long) through the day as measured by accelerometry. In 21 low Caf users (<100 mg/day), it was used a double-blind crossover trial with two conditions (4-day each with a 3-day washout period) randomly ordered as 5 mg/kg/day of Caf and maltodextrin as placebo (Plc). Resting energy expenditure (REE) by indirect calorimetry, total energy expenditure (TEE) from doubly labeled water, PAEE calculated as TEE-(REE+0.1TEE), and accelerometry measurements of both LIPA and MVPA were not different between conditions. However, regardless of caffeine or placebo, there were several significant relationships between brief bouts of LIPA and MVPA with PAEE. In conclusion, this double-blind study found that low and moderate-vigorous activity as well as the total volume of PAEE in free-living conditions is resistant to dietary caffeine intake that was equivalent to 5 cups of espresso or 7 cups of tea [13449].

Effect on glucose homeostasis

Caffeine is a substance that has been used in our society for generations, primarily for its effects on the central nervous system that causes wakefulness. Caffeine supplementation has become increasingly more popular as an ergogenic aid for athletes and considerable scientific evidence supports its effectiveness. Because of their potential to alter energy metabolism, the effects of coffee and caffeine on glucose metabolism in diabetes have also been studied both epidemiologically and experimentally. Predominantly targeting the adenosine receptors, caffeine causes alterations in glucose homeostasis by decreasing glucose uptake into skeletal muscle, thereby causing elevations in blood glucose concentration. Caffeine intake has also been proposed to increase symptomatic warning signs of hypoglycemia in patients with type 1 diabetes and elevate blood glucose levels in patients with type 2 diabetes. Other effects include potential increases in glucose counterregulatory hormones such as epinephrine, which can also decrease peripheral glucose disposal. Despite these established physiological effects, increased coffee intake has been associated with reduced risk of developing type 2 diabetes in large-scale epidemiological studies. One review highlighted the known effects of caffeine on glucose homeostasis and diabetes metabolism during rest and exercise [13450].

Molecular effects
Caffeine activates 5’AMP-activated protein kinase (AMPK), a signalling intermediary implicated in the regulation of glucose, lipid, and energy metabolism in skeletal muscle. Skeletal muscle expresses two catalytic alpha subunits of AMPK, alpha1 and alpha2, but the isoform specificity of caffeine-induced AMPK activation is unclear. The aim of one study was to determine which alpha isoform is preferentially activated by caffeine in vitro and in vivo using rat skeletal muscle. Rat epitrochlearis muscle was isolated and incubated in vitro in the absence or presence of caffeine. In another experiment, the muscle was dissected after intravenous injection of caffeine. Isoform-specific AMPK activity, the phosphorylation status of AMPKalpha Thr(172) and acetyl-CoA carboxylase (ACC) Ser(79), the concentrations of ATP, phosphocreatine (PCr), and glycogen, and 3-O-methyl-d-glucose (3MG) transport activity were estimated. Incubation of isolated epitrochlearis muscle with 1 mM of caffeine for 15 min increased AMPKalpha1 activity, but not AMPKalpha2 activity; concentrations of ATP, PCr and glycogen were not affected. Incubation with 3 mM of caffeine activated AMPKalpha2 and reduced PCr and glycogen concentrations. Incubation with 1 mM of caffeine increased the phosphorylation of AMPK and ACC and enhanced 3MG transport. Intravenous injection of caffeine (5 mg/kg) predominantly activated AMPKalpha1 and increased 3MG transport without affecting energy status. The results suggest that of the two alpha isoforms of AMPK, AMPKalpha1 is predominantly activated by caffeine via an energy-independent mechanism and that activation of AMPKalpha1 increases glucose transport and ACC phosphorylation in skeletal muscle [10388].

Studies have proposed that caffeine-induced activation of glucose transport in skeletal muscle is independent of AMP-activated protein kinase (AMPK) because alpha-AMPK Thr172 phosphorylation was not increased by caffeine. However, previous studies, as well as the present, show that AMPK phosphorylation measured in whole muscle lysate is not a good indicator of AMPK activation in rodent skeletal muscle. In lysates from incubated rat soleus muscle, a predominant model in previous caffeine studies, both acetyl-CoA carboxylase-beta (ACCbeta) Ser221 and immunoprecipitated alpha1-AMPK activity increased with caffeine incubation, without changes in AMPK phosphorylation or immunoprecipitated alpha2-AMPK activity. This pattern was also observed in mouse soleus muscle, where only ACCbeta and alpha1-AMPK phosphorylation were increased following caffeine treatment. Preincubation with the selective CaMKK inhibitor STO-609 (5 microM), the CaM-competitive inhibitor KN-93 (10 microM), or the SR Ca\(^{2+}\) release blocking agent dantrolene (10 microM) all inhibited ACCbeta phosphorylation and alpha1-AMPK phosphorylation, suggesting that SR Ca\(^{2+}\) release may work through a CaMKK-AMPK pathway. Caffeine-stimulated 2-deoxyglucose (2DG) uptake reflected the AMPK activation pattern, being increased with caffeine and inhibited by STO-609, KN-93, or dantrolene. The inhibition of 2DG uptake is likely causally linked to AMPK activation, since muscle-specific expression of a kinase-dead AMPK construct greatly reduced caffeine-stimulated 2DG uptake in mouse soleus. It was concluded that a SR Ca\(^{2+}\)-activated CaMKK may control alpha1-AMPK activation and be necessary for caffeine-stimulated glucose uptake in mouse soleus muscle [07166].

Caffeine most likely exerts its performance enhancing effect on the human body mainly by five mechanisms:

- Antagonism of adenosine. Due to its close chemical resemblance of adenosine, caffeine blocks adenosine receptors (mainly A\(_1\) and A\(_2A\) receptor subtypes), thereby competitively inhibiting its action. Caffeine can decrease cerebral blood flow as well as antagonize A\(_1\), A\(_2A\) and A\(_2B\) adenosine receptors in blood vessels, thereby reducing adenosine-mediated vasodilation and consequently decrease myocardial blood flow.
- Increased fatty acid oxidation: increased lipolysis leads to decreased reliance on glycogen use. Caffeine switches the substrate preference from glycogen to fat by...
increasing hormone sensitive lipase (HSL) activity and inhibition of glycogen phosphorylase activity.

- Caffeine acts as a nonselective competitive inhibitor of the phosphodiesterase enzymes. Phosphodiesterases hydrolyze the phosphodiesterase bond in molecules such as cyclic adenosine monophosphate (cAMP), inhibiting the breakdown of cAMP. cAMP activates lipolysis by activating HSL and is an important molecule in the epinephrine cascade. It further activates protein kinase A, which in turn can phosphorylate a number of enzymes involved in glucose and lipid metabolism.

- Increased post-exercise muscle glycogen accumulation: enhanced recovery by increased rate of glycogen resynthesis following exercise. It has been reported that caffeine ingestion has no effect on glycogen accumulation during recovery in recreationally active individuals, but it has also been reported that caffeine (8 mg/kg body weight) co-ingested with carbohydrates (CHO) increases rates of postexercise muscle glycogen accumulation compared with consumption of CHO alone in well-trained athletes after exercise-induced glycogen depletion. Although this issue needs further study in different populations (untrained, trained) and at different time points (during exercise or recovery), caffeine added to postexercise CHO feeding seems to have the potential to improve glycogen resynthesis.

- Mobilization of intracellular calcium: It has been shown that caffeine can enhance calcium release from the sarcoplasmic reticulum and can also inhibit its reuptake. Via this mechanism, caffeine can enhance contractile force during submaximal contractions in habitual and nonhabitual caffeine consumers. Intracellular calcium favors the activation of endothelial nitric oxide synthase, which increases nitric oxide. Some of the ergogenic effects of caffeine might therefore as well be mediated partly by effecting the neuromuscular system and increasing contractile force. There is, however, still controversy about the translation of results from in vitro studies on muscle preparations to caffeine dose and calcium release in vivo.

As for many pharmacological substances, there is generally more than one potential mechanism explaining the ergogenic effects. This is also true for caffeine which might affect both the central nervous system (CNS) and skeletal muscle. Although questionable, a potential downside is that caffeine also has diuretic properties which can exert ergolytic effects during prolonged endurance events. Caffeine intake at very high doses (>500-600 mg or four to seven cups per day) can cause restlessness, tremor and tachycardia [13008].

**Blocking of adenosine receptors**

Caffeine, the most widely used psychoactive compound, is an adenosine receptor antagonist. It promotes wakefulness by blocking adenosine A(2A) receptors (A(2A)Rs) in the brain, but the specific neurons on which caffeine acts to produce arousal have not been identified. Using selective gene deletion strategies based on the Cre/loxP technology in mice and focal RNA interference to silence the expression of A(2A)Rs in rats by local infection with adeno-associated virus carrying short-hairpin RNA, it was reported that the A(2A)Rs in the shell region of the nucleus accumbens (NAc) are responsible for the effect of caffeine on wakefulness. Caffeine-induced arousal was not affected in rats when A(2A)Rs were focally removed from the NAc core or other A(2A)R-positive areas of the basal ganglia. The observations suggest that caffeine promotes arousal by activating pathways that traditionally have been associated with motivational and motor responses in the brain [13343].

**Coffee polyphenol caffeic acid**
Chlorogenic acid is an ester of caffeic and quinic acids, and is one of the most widely consumed polyphenols because it is abundant in foods, especially coffee. We explored whether chlorogenic acid and its metabolite, caffeic acid, act directly on skeletal muscle to stimulate 5’-adenosine monophosphate-activated protein kinase (AMPK). Incubation of rat epitrochlearis muscles with Krebs buffer containing caffeic acid (≥0.1 mM, ≥30 min) but not chlorogenic acid increased the phosphorylation of AMPKαThr(172), an essential step for kinase activation, and acetyl CoA carboxylase Ser(79), a downstream target of AMPK, in a dose- and time-dependent manner. Analysis of isoform-specific AMPK activity revealed that AMPKα2 activity increased significantly, whereas AMPKα1 activity did not change. This enzyme activation was associated with a reduction in phosphocreatine content and an increased rate of 3-O-methyl-d-glucose transport activity in the absence of insulin. These results suggest that caffeic acid but not chlorogenic acid acutely stimulates skeletal muscle AMPK activity and insulin-independent glucose transport with a reduction of the intracellular energy status [12287].

**Analgesic effect**

Another reason for the widespread use of caffeine within the exercise community might be its small but significant analgesic effect, possibly mediated by augmenting plasma endorphin concentrations. It is also established that caffeine reduces the rate of perceived exertion during exercise, suggesting that athletes are able to sustain higher intensities but do not perceive this effort to be different from placebo conditions [13008].

**Users versus non-users**

Some studies used caffeine-naïve whereas others used caffeine-habituated subjects. There seems to be a higher increase in plasma adrenalin in caffeine-naïves compared to caffeine habituated subjects after caffeine ingestion. However, no differences between habitual caffeine intake and 1500 m running performance or force of contraction could be observed. For both caffeine-naïve as well as caffeine-habituated subjects, moderate to high doses of caffeine are ergogenic during prolonged moderate intensity exercise. Although there is clearly the need to study caffeine habituation further, the differences between users and non-users do not seem to be major [13008].

**Metabolism**

Caffeine, theophylline, theobromine, and paraxanthine administered to animals and humans distribute in all body fluids and cross all biological membranes. They do not accumulate in organs or tissues and are extensively metabolized by the liver, with less than 2 percent of caffeine administered excreted unchanged in human urine. Dose-independent and dose-dependent pharmacokinetics of caffeine and other dimethylxanthines may be observed and explained by saturation of metabolic pathways and impaired elimination due to the immaturity of hepatic enzyme and liver diseases. While gender and menstrual cycle have little effect on their elimination, decreased clearance is seen in women using oral contraceptives and during pregnancy. Obesity, physical exercise, diseases, and particularly smoking and the interactions of drugs affect their elimination owing to either stimulation or inhibition of CYP1A2. Their metabolic pathways exhibit important quantitative and qualitative differences in animal species and man. Chronic ingestion or restriction of caffeine intake in man has a small effect on their disposition, but dietary constituents, including broccoli and herbal tea, as
well as alcohol were shown to modify their plasma pharmacokinetics. Using molar ratios of metabolites in plasma and/or urine, phenotyping of various enzyme activities, such as cytochrome monooxygenases, N-acetylation, 8-hydroxylation, and xanthine oxidase, has become a valuable tool to identify polymorphisms and to understand individual variations and potential associations with health risks in epidemiological surveys [11200].

**M mobilization of fat**

Part of caffeine's intrigue is that we still do not know the exact mechanism by which it enhances performance, with a number of effects on different body tissues being possible. Furthermore, individuals respond differently to caffeine (as with many drugs), across a range from positive to negative outcomes, and some tissues become tolerant to repeated caffeine use, while others do not. The potentially beneficial effects of caffeine include the mobilisation of fat from adipose tissue and the muscle cell, stimulation of the release and activity of adrenaline, effects on cardiac muscle, direct changes to muscle contractility and alterations to the central nervous system to change perceptions of effort or fatigue. Most scientists believe that the last factor is the most important and consistent factor in explaining performance enhancement. Recent evidence has changed our perspective on two of the widely promoted effects of caffeine. Whereas caffeine was believed to enhance endurance performance via increased utilisation of fat as an exercise fuel and reduced use of the limited muscle stores of glycogen, studies now show that the effect of caffeine on "glycogen sparing" during submaximal exercise is short-lived and inconsistent. It is often warned that caffeine-containing drinks have a diuretic effect and will cause an athlete to become dehydrated. In fact, small to moderate doses of caffeine have minor effects on urine losses or the overall hydration in people who are habitual caffeine users. In addition, caffeine-containing drinks such as tea, coffee and cola drinks provide a significant source of fluid in the everyday diets of many people [10174].

**Effects on performance after a fat meal**

One study examined the effects of caffeine, co-ingested with a high fat meal, on perceptual and metabolic responses during incremental (experiment 1) and endurance (experiment 2) exercise performance. Trained participants performed three constant-load cycling tests at approximately 73 percent of maximal oxygen uptake (VO$_{2\text{max}}$) for 30 min at 20 degrees C (experiment 1, n=8) and to the limit of tolerance at 10 degrees C (experiment 2, n=10). The 30 min constant-load exercise in experiment 1 was followed by incremental exercise (15 W/min) to fatigue. Four hours before the first test, the participants consumed a 90 percent carbohydrate meal (control trial); in the remaining two tests, the participants consumed a 90% fat meal with (fat + caffeine trial) and without (fat-only trial) caffeine. Caffeine and placebo were randomly assigned and ingested 1 h before exercise. In both experiments, ratings of perceived leg exertion were significantly lower during the fat + caffeine than fat-only trial. Ratings of perceived breathlessness were significantly lower in experiment 1 and heart rate higher in experiment 1 on the fat + caffeine than fat-only trial. In the two experiments, oxygen uptake, ventilation, concentration on blood glucose, lactate and plasma glycerol were significantly higher on the fat + caffeine than fat-only trial. In experiment 2, concentration of plasma free fatty acids, blood pyruvate and the lactate to pyruvate ratio were significantly higher on the fat + caffeine than fat-only trial. Time to exhaustion during incremental exercise and constant-load exercise was not different between the fat-only and fat + caffeine trials. In conclusion, while a number of metabolic responses were increased during exercise after caffeine ingestion, perception of effort was reduced and this may be attributed to the direct stimulatory effect of caffeine on the central nervous system. However,
this caffeine-induced reduction in effort perception did not improve exercise performance [06199].

Effects on blood pressure

The effect of coffee and caffeine on blood pressure (BP) and cardiovascular disease (CVD) in hypertensive persons is uncertain. The objective of one study was to summarize the evidence on the acute and longer-term effects of caffeine and coffee intake on BP and on the association between habitual coffee consumption and risk of CVD in hypertensive individuals. A systematic review and meta-analysis of publications identified in a PubMed and EMBASE search up to 30 April 2011 was undertaken. Data were extracted from controlled trials on the effect of caffeine or coffee intake on BP change and from cohort studies on the association between habitual coffee consumption and CVD. In 5 trials, the administration of 200–300 mg caffeine produced a mean increase of 8.1 mm Hg (95 % confidence interval 5.7 to 10.6 mm Hg) in systolic BP and of 5.7 mm Hg (95 % confidence interval: 4.1 to 7.4 mm Hg) in diastolic BP. The increase in BP was observed in the first hour after caffeine intake and lasted ≥3 h. In 3 studies of the longer-term effect (2 weeks) of coffee, no increase in BP was observed after coffee was compared with a caffeine-free diet or was compared with decaffeinated coffee. Last, 7 cohort studies found no evidence of an association between habitual coffee consumption and a higher risk of CVD. It was concluded that in hypertensive individuals, caffeine intake produces an acute increase in BP for ≥3 h. However, current evidence does not support an association between longer-term coffee consumption and increased BP or between habitual coffee consumption and an increased risk of CVD in hypertensive subjects [11491].

Effect on cardiac blood flow

Caffeine consumption has been receiving increased interest from both the medical and lay press, especially given the increased amounts now available in energy products. Acute ingestion of caffeine usually increases cardiac work; however, caffeine impairs the expected proportional increase in myocardial blood flow to match this increased work of the heart, most notably during exercise. This appears to be mainly due to caffeine’s effect on blocking adenosine-induced vasodilatation in the coronary arteries in normal healthy subjects. This review summarizes the available medical literature specifically relating to pure caffeine tablet ingestion and reduced exercise coronary blood flow, and suggests possible mechanisms. Further studies are needed to evaluate this effect for other common caffeine-delivery systems, including coffee, energy beverages, and energy gels, which are often used for exercise performance enhancement, especially in teenagers and young athletes [13444].

Caffeine-reduced myocardial blood flow during exercise

Caffeine consumption has been receiving increased interest from both the medical and lay press, especially given the increased amounts now available in energy products. Acute ingestion of caffeine usually increases cardiac work; however, caffeine impairs the expected proportional increase in myocardial blood flow to match this increased work of the heart, most notably during exercise. This appears to be mainly due to caffeine’s effect on blocking adenosine-induced vasodilatation in the coronary arteries in normal healthy subjects. This review summarizes the available medical literature specifically relating to pure caffeine tablet ingestion and reduced exercise coronary blood flow, and suggests possible mechanisms. Further studies are needed to evaluate this effect for other common caffeine-delivery systems, including coffee, energy beverages, and energy gels, which are often used for exercise performance enhancement, especially in teenagers and young athletes [13444].
systems, including coffee, energy beverages, and energy gels, which are often used for exercise performance enhancement, especially in teenagers and young athletes [13445].

**Dose-dependent neuromuscular effects**

The purpose of one study was to determine the oral dose of caffeine needed to increase muscle force and power output during all-out single multijoint movements. Thirteen resistance-trained men underwent a battery of muscle strength and power tests in a randomized, double-blind, crossover design, under four different conditions: (a) placebo ingestion (PLAC) or with caffeine ingestion at doses of (b) 3 mg/kg body weight (CAFF 3 mg), (c) 6 mg/kg (CAFF 6 mg), and (d) 9 mg/kg (CAFF 9 mg). The muscle strength and power tests consisted in the measurement of bar displacement velocity and muscle power output during free-weight full-squat (SQ) and bench press (BP) exercises against four incremental loads (25 %, 50 %, 75 %, and 90 % one-repetition maximum, 1RM). Cycling peak power output was measured using a 4-s inertial load test. Caffeine side effects were evaluated at the end of each trial and 24 h later. Mean propulsive velocity at light loads (25-50 % 1RM) increased significantly above PLAC for all caffeine doses (5.4-8.5 %). At the medium load (75 % 1RM), CAFF 3 mg did not improve SQ or BP muscle power or BP velocity. CAFF 9mg was needed to enhance BP velocity and SQ power at the heaviest load (90 % 1RM) and cycling peak power output (6.8-11.7 %). The CAFF 9 mg trial drastically increased the frequency of the adverse side effects (15-62 %). The ergogenic dose of caffeine required to enhance neuromuscular performance during a single all-out contraction depends on the magnitude of load used. A dose of 3 mg/kg is enough to improve high-velocity muscle actions against low loads, whereas a higher caffeine dose (9 mg/kg) is necessary against high loads, despite the appearance of adverse side effects [13467].

**Effect of caffeine on delayed onset muscle soreness**

The beneficial effects of caffeine on aerobic activity and resistance training performance are well documented. However, less is known concerning caffeine's potential role in reducing perception of pain and soreness during exercise. In addition, there is no information regarding the effects of caffeine on delayed onset muscle soreness (DOMS). The primary purpose of this study was to examine the effect of caffeine ingestion on muscle soreness, blood enzyme activity, and performance after a bout of elbow flexion/extension exercise. Nine low-coffeine-consuming males were randomly assigned to ingest either caffeine or placebo 1 hour before completing 4 sets of 10 bicep curls on a preacher bench, followed by a fifth set in which subjects completed as many repetitions as possible. Soreness and soreness on palpation intensity were measured using three 0-10 visual analog scales before exercise, and 24, 48, 72, 96, and 120 hours after exercise. After a washout period, subjects crossed over to the other treatment group. Caffeine ingestion resulted in significantly lower levels of soreness on day 2 and day 3 compared with placebo. Total repetitions in the final set of exercise increased with caffeine ingestion compared with placebo. The study demonstrates that caffeine ingestion immediately before an upper-body resistance training out enhances performance. A further beneficial effect of sustained caffeine ingestion in the days after the exercise bout is an attenuation of DOMS. This decreased perception of soreness in the days after a strenuous resistance training workout may allow individuals to increase the number of training sessions in a given time period [13469].

**Effects on performance**
In summary, caffeine, even at physiological doses (3-6 mg/kg), as well as coffee are proven ergogenic aids and as such – in most exercise situations, especially in endurance-type events – clearly work-enhancing. It most likely has a peripheral effect targeting skeletal muscle metabolism as well as a central effect targeting the brain to enhance performance, especially during endurance events. Also for anaerobic tasks, the effect of caffeine on the CNS might be most relevant. Further, post-exercise caffeine intake seems to benefit recovery by increasing rates of glycogen resynthesis [13008].

<table>
<thead>
<tr>
<th>Acute effect</th>
<th>Effect on performance</th>
<th>Caffeine dose</th>
</tr>
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<tbody>
<tr>
<td>Greater reliance on fat metabolism; increased FFAs; lower respiratory exchange ratio (RER)</td>
<td>Increased time trial performance</td>
<td>6 mg/kg body mass</td>
</tr>
<tr>
<td>Countercraft central fatigue, maintenance of MVC directed effect on CNS</td>
<td>3 % P_{MAX} increase, increase in voluntary activation</td>
<td>6 mg/kg body mass</td>
</tr>
<tr>
<td>No clear mechanism; effect on CNS (greater motor unit recruitment and altered neurotransmitter function) or direct effect on skeletal muscle</td>
<td>Enhanced time trial performance</td>
<td>6 mg/kg caffeine 1 h pre-exercise and about 1.5 mg/kg after 2 h of exercise</td>
</tr>
<tr>
<td>Direct effect on skeletal muscle; interaction with ryanodine receptor; potentiated calcium release from the SR</td>
<td>Increase of contraction force at low frequency stimulation (20 Hz)</td>
<td>6 mg/kg 100 min before stimulation</td>
</tr>
<tr>
<td>Blunted pain response</td>
<td>Significantly higher reps during leg press set 3 with caffeine, same RPE</td>
<td>6 mg/kg 1 h prior to 10 RM bench and leg press</td>
</tr>
<tr>
<td>Glycogen-sparing effect and increased utilization of intramuscular TGs and plasma FFAs with caffeine</td>
<td>Increased cycling time trial performance with caffeine</td>
<td>9 mg/kg body mass 1 h before exercise</td>
</tr>
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</table>

Most of the beneficial effects of caffeine have been shown in relation to alertness and neurocognitive performance, particularly in periods of sleep deprivation. Caffeine has also been implicated with having an analgesic as well as antinociceptive effect. Several studies have been conducted looking at caffeine as an ergogenic aid. Those looking at caffeine use during periods of sleep deprivation have shown a benefit to caffeine administration in terms of alertness and neurocognitive performance. Time to fall asleep on the multiple sleep latency test (MSLT) following periods of sleep deprivation was increased with administration of caffeine. Similarly, performance on a driving simulator following a period of sleep deprivation was found to be improved with caffeine administration [06183].

*Effect versus measurable levels*
Because some were afraid that the misuse of caffeine might increase after its removal from the doping list, the urinary concentrations have been monitored since. Data presented show that the misuse did not increase, although in some sport disciplines such as cycling some misuse of caffeine was still found. Some advocated that if unfair play should be prevented it might be reconsidered to add caffeine to the list again with a urinary threshold level of 12 microg/mL. However, it should be emphasized that research has shown that caffeine has performance enhancing properties up to approximately 5 mg/kg, above which no additional effect on performance was found. However, when dosages up to 5 mg/kg are taken the urine levels generally stay below 12 microg/mL. So, there is no scientific basis to support the statement that re-establishing a threshold of 12 micro/mL would avoid unfair competition. The misuse found in some sports may be attributed to lack of knowledge and education which appears to be supported by the facts in several doping cases. An important, but too often underscored aspect of the fight against doping is education of athletes and people around the athlete. The recommendation to add caffeine to the doping list again ignores the reasons why it was removed some years ago. Proper education in those sports where caffeine is still abused may be a more effective approach [07167].

**Effects on the brain**

Caffeine can improve exercise performance when it is ingested at moderate doses (3-6 mg/kg body mass). Caffeine also has an effect on the central nervous system (CNS), and it is now recognized that most of the performance-enhancing effect of caffeine is accomplished through the antagonism of the adenosine receptors, influencing the dopaminergic and other neurotransmitter systems. Adenosine and dopamine interact in the brain, and this might be one mechanism to explain how the important components of motivation (i.e. vigor, persistence and work output) and higher-order brain processes are involved in motor control. Caffeine maintains a higher dopamine concentration especially in those brain areas linked with “attention”. Through this neurochemical interaction, caffeine improves sustained attention, vigilance, and reduces symptoms of fatigue. Other aspects that are localized in the CNS are a reduction in skeletal muscle pain and force sensation, leading to a reduction in perception of effort during exercise and therefore influencing the motivational factors to sustain effort during exercise. Because not all CNS aspects have been examined in detail, one should consider that a placebo effect may also be present. Overall, it appears that the performance-enhancing effects of caffeine reside in the brain, although more research is necessary to reveal the exact mechanisms through which the CNS effect is established [13456].

**Caffeine, exercise and the brain**

Caffeine can improve exercise performance when it is ingested at moderate doses (3-6 mg/kg body mass). Caffeine also has an effect on the central nervous system (CNS), and it is now recognized that most of the performance-enhancing effect of caffeine is accomplished through the antagonism of the adenosine receptors, influencing the dopaminergic and other neurotransmitter systems. Adenosine and dopamine interact in the brain, and this might be one mechanism to explain how the important components of motivation (i.e. vigor, persistence and work output) and higher-order brain processes are involved in motor control. Caffeine maintains a higher dopamine concentration especially in those brain areas linked with “attention”. Through this neurochemical interaction, caffeine improves sustained attention, vigilance, and reduces symptoms of fatigue. Other aspects that are localized in the CNS are a reduction in skeletal muscle pain and force sensation, leading to a reduction in perception of effort during exercise and therefore influencing the motivational factors to sustain effort during exercise. Because not all CNS aspects have been examined in detail, one should consider that a placebo effect may also be present. Overall, it appears that the
performance-enhancing effects of caffeine reside in the brain, although more research is necessary to reveal the exact mechanisms through which the CNS effect is established [13457].

**Effects on mood and vigilance performance**

One study investigated the impact of pre-existent expectancy regarding the effects of the caffeine load of a drink and the perception of the caffeine content on subjective mood and vigilance performance. Caffeine deprived participants (n=25) were tested in four conditions (within subjects design), using a 2×2 design, with caffeine load and information regarding the caffeine content of the drink. In two sessions, they were given caffeinated coffee and in two were given decaffeinated coffee. Within these two conditions, on one occasion they were given accurate information about the drink and on the other they were given inaccurate information about the drink. Mood and vigilance performance were assessed post ingestion. Caffeine was found to enhance performance, but only when participants were accurately told they were receiving it. When decaffeinated coffee was given, performance was poorer, irrespective of expectancy. However, when caffeine was given, but participants were told it was decaffeinated coffee, performance was as poor as when no caffeine had been administered. There were no easily interpretable effects on mood. The pharmacological effects of caffeine appear to act synergistically with expectancy [10500].

**Effects on endurance**

Although there are a vast number of studies quantifying caffeine's effects, many research studies measure endurance performance using a time-to-exhaustion test (subjects exercise at a fixed intensity to volitional exhaustion). Time-to-exhaustion as a performance measure is not ideal because of the high degree of measurement variability between and within subjects. Also, one must stat that in modern endurance sports there is none in which individuals win by going a longer distance or for a longer amount of time than their competitors. Measuring performance with a time-trial test (set distance or time with best effort) has high reproducibility and is more applicable to sport. Therefore, the purpose of one review was to critically and objectively evaluate studies that have examined the effect of caffeine on time-trial endurance (>5 minutes) performance. A literature search revealed 21 studies with a total of 33 identifiable caffeine treatments that measured endurance performance with a time-trial component. The mean improvement in performance with caffeine ingestion was 3.2 ± 4.3 percent; however, this improvement was highly variable between studies. The high degree of variability may be dependent on a number of factors including ingestion timing, ingestion mode/vehicle, and subject habituation. Further research should seek to identify individual factors that mediate the large range of improvements observed with caffeine ingestion. The authors concluded that caffeine ingestion can be an effective ergogenic aid for endurance athletes when taken before and/or during exercise in moderate quantities (3-6 mg/kg body mass). Abstaining from caffeine at least 7 days before use will give the greatest chance of optimizing the ergogenic effect [08310].

Athletes in Malaysia need to perform in a hot and humid climate. Chronic supplementation of caffeine on endurance performance have been studied extensively in different populations. However, concurrent research on the effects of acute supplementation of caffeine on cardiorespiratory responses during endurance. Nine heat adapted recreational Malaysian male runners who were nonusers of caffeine (24 ± 13 mg per day) were recruited in a placebo-controlled double-blind randomized study. Caffeine (5 mg per kg of body weight) or placebo was ingested in the form of a capsule one hour prior to the running exercise trial at 70 per cent of VO\textsubscript{2}max on a motorised treadmill in a heat-controlled laboratory (31 degrees C, 70% relative humidity). Subjects drank 3 ml of cool water per kg of body weight every 20 min.
during the running trials to avoid the adverse effects of dehydration. Heart rate, core body temperature and rate of perceived exertion (RPE) were recorded at intervals of 10 min, while oxygen consumption was measured at intervals of 20 min. Running time to exhaustion was significantly higher in the caffeine trial compared to the placebo trial. Heart rate, core body temperature, oxygen uptake and RPE did not show any significant variation between the trials but it increased significantly during exercise from their respective resting values in both trials. The study showed that ingestion of 5 mg of caffeine per kg of body weight improved the endurance running performance but did not impose any significant effect on other individual cardiorespiratory parameters of heat-acclimated recreational runners in hot and humid conditions [10390].

The purpose of this work was to determine the effects of caffeine ingestion on cycling time trial performance in well trained male subjects. Eight males undertook three 1 h trial performances on a cycle ergometer, in a double blind, random fashion. The trials were Control, placebo and caffeine. The caffeine was given 60 min prior to exercise in a dose of 6 mg/kg body mass. Prior to ingestion, 60 min post ingestion, and at the end of the trial, subjects gave 10 mL of venous blood which was analysed for lactate, glucose, and free fatty acids. Expired air was collected throughout each test by indirect calorimetry. The cyclists rode significantly further in caffeine trial Control and placebo trials. No significant differences were seen between control and placebo trials. The free fatty acid (FFA) concentrations were significantly higher in the caffeine trials both post ingestion and post exercise than either other trials. It was concluded that performance was improved possibly based upon a greater reliance on fat metabolism, as indicated by increased FFA and a lower respiratory exchange ratio [08309].

It has been demonstrated that caffeine supplementation improved exercise endurance by about 20 percent and significantly reduced the perception of exertion whilst cycling at 18 and 35°C, compared to a placebo. Since no effects were observed on indicators of peripheral metabolic stress, it was concluded that the action was central and, furthermore, since there was no effect on the release of prolactin, it was concluded that caffeine may be acting on central pathways other than those in the hypothalamus which are sensitive to temperature. There is a wealth of literature describing the effects of caffeine on exercise performance [06192].

Most of the studies looking at caffeine in improving exercise or athletic performance of focused on endurance, submaximal exercise activities such as running and cycling. In these situations, caffeine has generally been shown to improve or sustain exercise performance, typically through an increase in the duration of the exercise or a decreased perception of exertion. In cycling, caffeine has been shown to increase time to exhaustion at 85 percent \( V_{O2\max} \) and decrease times to finish a fixed period of activity. Increased times to exhaustion have been seen in running as well as decreased times to run set distances. Other benefits on exercise that have been discovered are improved tennis performance and decreased 1500-meter swim times [06183].

There is little published data in relation to the effects of caffeine upon cycling performance, speed and power in trained cyclists, especially during cycling of approximately 60 s duration. To address this, eight trained cyclists performed a 1 km time-trial on an electronically braked cycle ergometer under three conditions: after ingestion of 5 mg caffeine per kg bodyweight, after ingestion of a placebo, or a control condition. The three time-trials were performed in a randomized order and performance time, mean speed, mean power and peak power were determined. Caffeine ingestion resulted in improved performance time. This change represented a 3.1 percent improvement compared with the placebo condition. Mean speed was also significantly higher in the caffeine than placebo and control conditions. Mean power
increased after caffeine ingestion. Peak power also increased from $864 \pm 107$ W (placebo) and $830 \pm 87$ W (control) to $940 \pm 83$ W after caffeine ingestion. These results provide support for previous research that found improved performance after caffeine ingestion during short-duration high-intensity exercise. The magnitude of the improvements observed in our study could be due to our use of sport-specific ergometry, a tablet form and trained participants [06193].

The aim of one study was to assess the effect of caffeine ingestion on 8 km run performance using an ecologically valid test protocol. A randomized double-blind crossover study was conducted involving eight male distance runners. The participants ran an 8 km race 1 h after ingesting a placebo capsule, a caffeine capsule (3 mg/kg body mass) or no supplement. Heart rate was recorded at 5 s intervals throughout the race. Blood lactate concentration and ratings of perceived exertion were recorded after exercise. A repeated-measures analysis of variance (ANOVA) identified a significant treatment effect for 8 km performance time; caffeine resulted in a mean improvement of 23.8 s in 8 km performance time (1.2 % improvement). In addition, a two-way (time x condition) repeated-measures ANOVA identified a significantly higher blood lactate concentration 3 min after exercise during the caffeine trial. It was concluded that ingestion of 3 mg/kg body mass of caffeine can improve absolute 8 km run performance in an ecologically valid race setting [06194].

The purpose of one study was to investigate if caffeine ingestion improves 5-km time-trial performance in well-trained and recreational runners. Using a double-blind placebo-controlled design, 15 well-trained and 15 recreational runners completed two randomized 5-km time-trials, after ingestion of either 5 mg/kg of caffeine or a placebo. Caffeine ingestion significantly improved 5-km running performance in both the well-trained and recreational runners. In comparison to the placebo trial, the caffeine trial resulted in 1.1 percent (90 % confidence interval 0.4 to 1.6) and 1.0 percent (90 % confidence interval 0.2 to 2.0 %) faster times for the well-trained and recreational runners. Reliability testing of the recreational runners indicated a test-retest error of measurement of 1.4 percent. It was concluded that caffeine ingestion is likely to produce small but significant gains in 5-km running performance for both well-trained and recreational runners [07171].

One double-blind experiment examined the effects of a caffeinated sports drink during prolonged cycling in a warm environment. Sixteen highly trained cyclists completed 3 trials: placebo, carbohydrate-electrolyte sports drink (CES), and caffeinated sports drink (CES+CAF). Subjects cycled for 135 min, alternating between 60 percent and 75 percent $\text{VO}_{2\text{max}}$ every 15 min for the first 120 min, followed by a 15-min performance ride. Maximal voluntary (MVC) and electrically evoked contractile properties of the knee extensors were measured before and after cycling. Work completed during the performance ride was 15-23 percent greater for CES+CAF than for the other beverages. Ratings of perceived exertion were lower with CES+CAF than with placebo and CES. After cycling, the MVC strength loss was two-thirds less for CES+CAF than for the other beverages (5 % vs 15 %). Data from the interpolated-twitch technique indicated that attenuated strength loss with CES+CAF was explained by reduced intrinsic muscle fatigue [07172].

Caffeine reduces fatigue and increases concentration and alertness, and athletes regularly use it as an ergogenic aid. Caffeine-induced increases in performance have been observed in aerobic as well as anaerobic sports. Trained athletes seem to benefit from a moderate dose of 5 mg/kg, however, even lower doses of caffeine (1.0-2.0 mg/kg) may improve performance. Some groups found significantly improved time trial performance or maximal cycling power, most likely related to a greater reliance on fat metabolism and decreased neuromuscular fatigue, respectively. Theophylline, a metabolite of caffeine, seems to be even more effective in doing so. The effect of caffeine on fat oxidation, however, may only be
significant during lower exercise intensities and may be blocked at higher intensities. It was found that ingestion of a high dose of caffeine before exercise reduced muscle glycogenolysis in the initial 15 min of exercise by increasing free fatty acid (FFA) levels which inhibits glycolysis and spares glycogen for later use. Caffeine’s effect of inhibition of glycogen phosphorylase has also been shown in vitro as well as its effect on increasing HSL activity. The effect of caffeine on adipose triglyceride lipase has not been studied and warrants investigation. Following caffeine administration prior to and after the onset of cycling, it was found that plasma free fatty acid levels were increased 30 percent compared to placebo. This action might be mediated by inhibition of the enzyme phosphodiesterase, thereby yielding higher levels of cAMP, which has been identified as an important molecule for glycogen metabolism and lipolysis. Phosphodiesterase inhibition has been observed only at high concentrations. When direct Fick measurements were applied, it was not found altered CHO or fat metabolism, at least in the monitored leg. Further research is needed to evaluate the effect of caffeine on lipolysis, especially during higher exercise intensities [13008].

Augmented post-exercise recovery by increased rates of muscle glycogen resynthesis has been observed. It was found higher rates of muscle glycogen accumulation after the co-ingestion of caffeine with CHO during recovery in highly trained subjects. This might, at least in part, be mediated by the activation of AMP-activated protein kinase (AMPK) as it is involved in the translocation of glucose transporter 4 (GLUT4) to the plasma membrane. This mechanism enables the cell to take up glucose from the plasma and store it as glycogen. Not only does caffeine impact endurance, it has also been reported to benefit cognitive function and fine motor skills. While the performance enhancing effects of caffeine in moderate-to-highly trained endurance athletes are quite clear and well documented, its effects on anaerobic, high-intensity tasks are less well investigated. Whereas caffeine supplementation did not yield significant performance increases in a Wingate test in untrained subjects, it was reported that caffeine ingestion of 3 mg/kg could counter reductions in maximum dynamic strength and muscle power output on the morning (2.5-7.0 %) thereby increasing muscle performance to the levels found in the afternoon. Especially with regard to anaerobic performance caffeine’s adenosine receptor blocking effect in the CNS may be important. A possible explanation for the diverging effect of caffeine on anaerobic performance is that caffeine seems to benefit trained athletes who show specific physiological adaptations whereas performance gains in untrained subjects might be lost or masked by a high variability in performance [13008].

It has been shown that coffee, by containing phenolic compounds such as chlorogenic acids, elicits metabolic effects independent of caffeine. These compounds may have the potential to antagonize the physiological responses of caffeine. The question therefore remains whether ingesting the same amount of caffeine via a food source (e.g. energy bar or coffee) is as effective as ingesting isolated caffeine in the form of a tablet. As mentioned above, the performance enhancing effect of caffeine is very clear. Only a few studies, however, have shown a positive effect of coffee on performance. Whereas some studies found enhanced performance after coffee consumption, others did not. One earlier works reported increases in time trial performance of competitive cyclists only in the coffee trial group (containing 330 mg caffeine 1 h prior to exercise) but not in the decaffeinated coffee trial. It was also studied exercise endurance in runners after ingestions of a caffeine (4.45 mg/kg BW) or placebo capsule with water or either decaffeinated coffee, decaffeinated coffee with added caffeine or regular coffee. The authors found that only caffeine significantly improved running time to exhaustion at 75 percent VO\textsubscript{2}\text{max} but neither did regular coffee or decaffeinated coffee plus caffeine. Based on these results, the authors speculated that some component(s) in coffee possibly interfere with the ergogenic response of caffeine alone [13008].
The use of caffeine as an ergogenic aid to promote endurance has been widely studied, with human literature showing the greatest benefit during submaximal muscle activities. Recent evidence suggests that the acute treatment of skeletal muscle with physiological concentrations of caffeine (70 microM maximum) will directly potentiate force production. The aims of the present study are: firstly, to assess the effects of a physiological concentration (70 microM) of caffeine on endurance in maximally activated mouse soleus (relatively slow) muscle; and secondly, to examine whether endurance changes when muscle is activated submaximally during caffeine treatment. Maximally stimulated soleus muscle treated with 70 microM caffeine resulted in a significant (18 %) decrease in endurance. In contrast, at a submaximal stimulation frequency, caffeine treatment significantly prolonged endurance (by 19 %). Findings are activation-dependent such that, during high frequency stimulation, caffeine accelerates fatigue, whereas, during low frequency stimulation, caffeine delays fatigue [0462].

**Effects on biking**

The purpose of one work was to determine the effects of caffeine on high intensity time trial cycling performance in well-trained subjects. Six male cyclists undertook three 1-h performances, control, placebo and caffeine, on a Velotron cycle ergometer conducted in a double-blind, random fashion. Subjects rested for 60 min and were then given caffeine or placebo in a dose of 6 mg/kg body mass and then commenced exercise after another 60 min of rest. Before ingestion, 60 min postingestion, and at the end of the test, finger-prick blood samples were analyzed for lactate. The cyclists rode significantly further in the caffeine trial (28.0 ± 1.3 km) than they did in the control performance (26.3 ± 1.5 km) or placebo (26.4 ± 1.5 km trials). No differences were seen in heart rate data throughout the tests. Blood lactate levels were significantly higher at the end of the trials than either at rest or postingestion, but there were no differences between the three trial groups. The authors concluded that on the basis of the data, it was concluded that performance was improved with the use of a caffeine supplement [09255].

The purpose of one experiment was to learn whether low doses of caffeine have ergogenic, perceptual, and metabolic effects during cycling. To determine the effects of 1, 2, and 3 mg/kg caffeine on cycling performance, differentiated ratings of perceived exertion (D-RPE), quadriceps pain intensity, and metabolic responses to cycling exercise, 13 cyclists exercised on a stationary ergometer for 15 min at 80 percent VO2, then, after 4 min of active recovery, completed a 15-min VO2peak performance ride 60 min after ingesting caffeine or placebo. Work done (kJ/kg) during the performance ride was used as a measure of performance. D-RPE, pain ratings, and expired-gas data were obtained every 3 min, and blood lactate concentrations were obtained at 15 and 30 min. Compared with placebo, caffeine doses of 2 and 3 mg/kg increased performance by 4 percent (95% confidence interval 1.0-6.8%) and 3 percent (95% confidence interval -0.4 % to 6.8 %), respectively. These effects were ergogenic, on average, but varied considerably in magnitude among individual cyclists. There were no effects of caffeine on D-RPE or pain throughout the cycling task. Selected metabolic variables were affected by caffeine, consistent with its known actions. The authors conclude that caffeine preparations of 2 and 3 mg/kg enhanced performance, but future work should aim to explain the considerable interindividual variability of the drug's ergogenic properties [08307].

The purpose of one study was to determine if improved supramaximal exercise performance in trained cyclists following caffeine ingestion was associated with enhanced O2 uptake (VO2 kinetics), increased anaerobic energy provision (accumulated O2-AO2-deficit), or a reduction in the accumulation of metabolites (for example, K+) associated with muscular fatigue. Six highly trained male cyclists (VO2peak 68 ± 8 mL/kg/min) performed supramaximal (120% VO2peak) exercise bouts to exhaustion on an electronically braked cycle ergometer, following
double-blind and randomized ingestion of caffeine/placebo (5 mg/kg). Time to exhaustion (TE), VO\(_2\) kinetics, AO\(_2\) deficit, blood lactate, plasma potassium, caffeine and paraxanthine concentrations were measured. Caffeine ingestion elicited significant increases in TE and AO\(_2\) deficit (7%). In contrast, no changes were observed in AO\(_2\) deficit at isotime, VO\(_2\) kinetics, blood lactate at exhaustion or peak potassium following caffeine ingestion. However, potassium was significantly reduced (13%) during warm-up cycling immediately prior to the onset of the supramaximal bout for the caffeine trials, compared with placebo. It appears that caffeine ingestion is beneficial to supramaximal cycling performance in highly trained men. The reduced plasma potassium during submaximal warm-up cycling may prolong the time taken to reach critical potassium at exhaustion, thus delaying fatigue. Considering caffeine ingestion did not change VO\(_2\) kinetics or isotime AO\(_2\) deficit, increases in absolute AO\(_2\) deficit may be a consequence of prolonged TE, rather than causal [10179].

Caffeine is thought to act as a central stimulant and to have effects on physical, cognitive, and psychomotor functioning. Twenty-four well-trained cyclists consumed the products (a performance bar containing 45 g of carbohydrate and 100 mg of caffeine, an isocaloric noncaffeine performance bar, or 300 mL of placebo beverage) immediately before performing a 2.5-h exercise at 60 percent VO\(_{2\text{max}}\) followed by a time to exhaustion trial at 75 percent VO\(_{2\text{max}}\). Additional products were taken after 55 and 115 min of exercise. Cognitive function measures were performed before exercise and while cycling after 70 and 140 min of exercise and again 5 min after completing the time to exhaustion ride. Participants were significantly faster after caffeine when compared with carbohydrate on both the computerized complex information processing tests, particularly after 140 min and after the time to exhaustion ride. On the beverage trial, performance was significantly slower than after both other treatments. There were no speed-accuracy tradeoffs. Time to exhaustion was significantly longer after caffeine consumption compared with both carbohydrates and beverage trials, and time to exhaustion was longer after carbohydrates than after beverages. No differences were found in the ratings of perceived exertion, mean heart rate, and relative exercise intensity (% VO\(_{2\text{max}}\)). The authors concluded that caffeine in a performance bar can significantly improve endurance performance and complex cognitive ability during and after exercise. These effects may be salient for sports performance in which concentration plays a major role [08311].

The purpose of one study was to investigate the effects of caffeine ingestion on the performance of an intermittent sprint cycling test (ISCT) with different rest intervals. Fourteen males with team sport experience consumed 6 mg/kg of caffeine or a placebo 60 min prior to completing two sets of an ISCT with 4-min rest intervals. Each set consisted of 12 × 4-s sprints with 20- or 90-s active recovery intervals at 60-70 rpm. Blood lactate was collected at baseline and immediately following the completion of six sprints in each set. At 20-s recovery intervals, peak power and total work were not significantly different between conditions during the ISCT; but caffeine reduced 6 percent effort for mean power in Sprint 10 of the later stage, as well as an increased fatigue index and elevated blood lactate levels during the ISCT. At 90-s recovery intervals, peak power, mean power, and total work under caffeine conditions were significantly higher than under placebo conditions during the ISCT, but no differences were apparent in fatigue index and blood lactate levels. In conclusion, caffeine ingestion may be ergolytic, affecting performance and fatigue development in the later stage during a prolonged and intermittent sprint test with a short recovery interval. However, caffeine produces an ergogenic effect in the initial stage of an intermittent sprint performance with a longer recovery interval [12280].

Caffeine has been reported to alter perceptions of exertion, muscle pain, and mood, yet the majority of existing data were obtained in resting volunteers or during steady-state exercise. The primary aim of one study was to examine the effects of caffeine on rating of perceived
exertion (RPE) and perceptions of leg pain, arousal, and pleasure/displeasure during a simulated cycling time trial. Endurance-trained (n=8, VO$_{2_{\text{max}}}$ 58 ± 4 mL/kg/min) and active (n=8) men initially completed two familiarization trials separated by at least 48 h. Over the next three trials, they completed a 10 km time trial preceded by ingestion of drinks containing caffeine (5 mg/kg ingested on 2 separate days) or placebo. Treatments were ingested using a single-blind, crossover design, and participants were deceived as to the content of all drinks. During exercise, RPE (6-20 scale), leg pain (0-10 scale), arousal (Felt Arousal Scale), and pleasure/displeasure (Feeling Scale) were recorded using various categorical scales. Repeated measures analysis of variance was used to assess differences in all variables across time and treatments, with fitness level used as a between-subjects variable. Pleasure/displeasure was altered with caffeine compared to placebo, although leg pain, RPE, and arousal were similar across treatments. Caffeine increased cycling performance by 0.3-2.0 percent versus placebo, with no effect of fitness level. Only in trained men; however, was there a significant caffeine-mediated improvement in cycling performance, which was consequent with diminished mood in trained and improved mood in active individuals [12281].

Both caffeine (CAF) and pseudoephedrine (PSE) are proposed to be central nervous system stimulants. However, during competition, CAF is a permitted substance, whereas PSE is a banned substance at urinary levels >150 microg/L. As a result, this study aimed to compare the effect of CAF versus PSE use on cycling time trial (TT) performance to explore whether the legal stimulant was any less ergogenic that the banned substance. Here, 10 well-trained male cyclists and/or triathletes were recruited for participation. All athletes were required to attend the laboratory on four separate occasions, inclusive of a familiarisation trial and three experimental trials which required participants to complete a simulated 40 km (1200 kJ) cycling TT, after the ingestion of either 200 mg CAF, 180 mg PSE or a non-nutritive placebo (PLA). The results showed that the total time taken and the mean power produced during each TT was not significantly different between trials, despite a 1.3 percent faster overall time (about 57 sec) after CAF consumption. Interestingly, the time taken to complete the second 50 percent of the TT was significantly faster in CAF as compared to PSE (by 99 sec), with magnitude based inferences suggesting a 91% beneficial effect of CAF during the second half of the TT. This investigation further confirms the ergogenic benefits of CAF use during TT performances, and further suggests this legal CNS stimulant has a better influence than a supra-therapeutic dose of PSE [13443].

**Cross-country skiing**

Caffeine (CAF) improves performance of both short and long duration in running and cycling where performance relies on power output, and endurance capacity of leg muscles. No studies have so far tested effects of CAF while using the double poling (DP) technique in cross-country skiing (XCS). When DP arm muscles provide the speed generating force, and therefore play an important role to performance outcome. The metabolism of arm muscles differs from that of leg muscles. Thus, results from studies on leg muscles and CAF may not be directly applicable to exercises while DP in XCS. The purpose of our study was therefore to investigate effects of CAF on exercise performance in DP. Ten highly trained male cross-country skiers performed a placebo (PLA) and CAF trial using a randomized, double-blinded, cross-over design. Performance was assessed by time to complete an 8 km cross country DP performance test (C-PT). CAF (6 mg/kg) or PLA was ingested 75 min before the C-PT. Results: CAF ingestion significantly reduced times to complete the 8 km C-PT. The subjects maintained higher speed and heart rate throughout the C-PT, and lactate was higher immediately after the C-PT with CAF exposure compared to PLA. Subjects reported lower rating of perceived exertion at submaximal intensities during CAF compared to PLA, although heart rate was similar. Conclusion: CAF intake enhances endurance performance in an 8 km C-PT where arm muscles limit performance. CAF ingestion allowed the participants
to exercise with a higher heart rate, and work intensity, possibly by reducing perception of effort or facilitating motor unit recruitment [13440].

**Effects on short term performance**

The efficacy of caffeine ingestion in enhancing aerobic performance is well established. However, despite suggestions that caffeine may enhance resistance exercise performance, research is equivocal on the effect of acute caffeine ingestion on resistance exercise performance. It has also been suggested that dampened perception of perceived exertion and pain perception might be an explanation for any possible enhancement of resistance exercise performance due to caffeine ingestion. Therefore, the aim of one study was to examine the acute effect of caffeine ingestion on repetitions to failure, rating of perceived exertion (RPE) and muscle pain perception during resistance exercise to failure. Eleven resistance trained individuals (9 males, 2 females, mean age 26 years), took part in this double-blind, randomised cross-over experimental study whereby they ingested a caffeinated (5 mg/kg) or placebo solution 60 minutes before completing a bout of resistance exercise. Experimental conditions were separated by at least 48 hours. Resistance exercise sessions consisted of bench press, deadlift, prone row and back squat exercise to failure at an intensity of 60 percent 1 repetition maximum. Results indicated that participants completed significantly greater repetitions to failure, irrespective of exercise, in the presence of caffeine. Mean of repetitions to failure was 20 and 19 in caffeine and placebo conditions, respectively. There were no differences in peak heart rate or peak blood lactate values across conditions. RPE was significantly lower in the caffeine compared to the placebo condition and was significantly higher during lower body exercises compared to upper body exercises irrespective of substance ingested. For muscle pain perception, a significant condition by exercise interaction revealed that muscle pain perception was lower in the caffeine condition, irrespective of exercise. With caffeine, pain perception was significantly higher in the deadlift and back squat compared to the bench press. However, with placebo, pain perception was significantly higher for the deadlift and back squat compared to the prone row only. Therefore, acute caffeine ingestion not only enhances resistance exercise performance to failure but also reduces perception of exertion and muscle pain [13463].

The efficacy of caffeine ingestion in enhancing aerobic performance is well established. The evidence for caffeine's effects on resistance exercise is mixed and has not fully examined the associated psychological and psychophysiological changes. One study examined acute effects of ingesting a caffeine-containing energy drink on repetitions to failure, the rating of perceived exertion (RPE), and the readiness to invest physical effort (RTIPE) and mental effort during resistance exercise to failure. Thirteen resistance-trained men took part in this double-blind, randomized cross-over experimental study whereby they ingested a caffeinated (179 mg) energy drink or placebo solution 60 minutes before completing a bout of resistance exercise comprising bench press, deadlift, prone row, and back squat exercise to failure at an intensity of 60 percent 1-repetition maximum. Experimental conditions were separated by at least 48 hours. Participants completed significantly greater repetitions to failure, irrespective of exercise, in the energy drink condition. Rating of perceived exertion was significantly higher in the placebo condition and was significantly higher during lower-body exercises compared with upper-body exercises irrespective of the substance ingested. Readiness to invest mental effort was greater with the energy drink condition, irrespective of time. A significant time × substance interaction for RTIPE indicated that RTIPE increased for both placebo and energy drink conditions preingestion to pre-exercise, but the magnitude of increase was greater with the energy drink condition compared with placebo. This resulted in higher RTIPE postexercise for the energy drink condition. These results suggest that acute ingestion of a caffeine-containing energy drink can enhance resistance exercise performance
to failure and positively enhance psychophysiological factors related to exertion in trained men [12276].

Whereas the outcomes of caffeine ingestion (from natural sources and pills) are well known, the effects of caffeine-containing energy drinks on sports performance have been the object of fewer studies. The first report concerning the effects of energy drinks on physical performance was carried out by Alford and co-workers in 2001. These authors found that about 1 mg of caffeine per kg of body weight (one 250-mL serving of an energy drink) improved reaction time, alertness and aerobic and anaerobic performance. In contrast, subsequent investigations using energy drinks have shown that about 1 mg/kg of caffeine is not enough to enhance maximal oxygen uptake, peak power during three repetitions of the Wingate test, or running velocity during 24 “all-out” sprints. In addition, the ingestion of about 2 mg/kg of caffeine in the form of an energy drink was ergogenic during a cycling time trial but did not prolong time-to-exhaustion during a running test at 80 percent VO2max. The ergogenic effect of caffeine on endurance activities has been typically demonstrated with doses from 3 to 9 mg/kg, while the ingestion of 1 mg/kg of caffeine did not improve performance. Similar results have been found in team-sports specific activities: the ingestion of 6 mg/kg of caffeine increased repeated sprint ability, while the ingestion of 1 mg/kg of caffeine did not alter repeated sprint performance. Since the ingestion of one serving of an energy drink (typically 250-mL that contains 80 mg of caffeine) provides a dose of about 1 mg/kg of caffeine in a man of average weight, previous results about the inefficacy of energy drinks to improve performance may be explained by the low dose of caffeine provided for the subjects. During a soccer match, players combine periods of high-intensity exercise interspersed with periods of lower-intensity exercise or recovery. Thus, the ability to perform repeated sprints with minimal recovery between sprint bouts is one of the most crucial capacities for team sport athletes. In addition, an enhanced ability to repeat sprints is related with playing at a higher competitive level, especially in soccer [12274].

The aims of one study were to evaluate the effects of caffeine supplementation on sprint cycling performance and to determine if there was a dose-response effect. Using a randomized, double-blind, placebo-controlled design, 17 well-trained men (age: 24) completed 7 maximal 10-second sprint trials on an electromagnetically braked cycle ergometer. Apart from trial 1 (familiarization), all the trials involved subjects ingesting a gelatine capsule containing either caffeine or placebo (maltodextrin) 1 hour before each sprint. To examine dose-response effects, caffeine doses of 2, 4, 6, 8, and 10 mg/kg bm were used. There were no significant differences in baseline measures of plasma caffeine concentration before each trial. There was, however, a significant supplement × time interaction, with larger caffeine doses producing higher postsupplementation plasma caffeine levels. In comparison with placebo, caffeine had no significant effect on peak power, mean power, or time to peak power. There was also no significant effect of supplementation on pretrial blood lactate, but there was a significant time effect, with blood lactate reducing over the 50 minute postsupplementation rest period from 1.29 ± 0.36 to 1.06 ± 0.33 mmol/L. The results of this study show that caffeine supplementation has no effect on short-duration sprint cycling performance, irrespective of the dosage used [12277].

One study aimed to determine whether caffeine ingestion would increase the workload voluntarily chosen by athletes in a limited-sleep state. In a double-blind, crossover study, 16 professional rugby players ingested either a placebo or 4 mg/kg caffeine 1 hr before exercise. Athletes classified themselves into nondeprived (8 hr+) or sleep-deprived states (6 hr or less). Exercise comprised 4 sets of bench press, squats, and bent rows at 85 percent 1-repetition maximum. Athletes were asked to perform as many repetitions on each set as possible without failure. Saliva was collected before administration of placebo or caffeine and again before and immediately after exercise and assayed for testosterone and cortisol. Sleep
deprivation produced a very large decrease in total load. Caffeine ingestion in the nondeprived state resulted in a moderate increase in total load, with a larger effect in the sleep-deprived state, resulting in total load similar to those observed in the nondeprived placebo condition. Eight of the 16 athletes were identified as caffeine responders. Baseline testosterone was higher and cortisol trended lower in non-sleep-deprived athletes. Changes in hormones from predose to preexercise correlated to individual workload responses to caffeine. Testosterone response to exercise increased with caffeine compared with placebo, as did cortisol response. It was concluded that caffeine increased voluntary workload in professional athletes, even more so under conditions of self-reported limited sleep. Caffeine may prove worthwhile when athletes are tired, especially in those identified as responders [12278].

Caffeine is the most widely used drug in the world, commonly ingested in coffee, tea, soda, and energy drinks. Its ability to enhance muscular work has been apparent since the early 1900s. Caffeine typically increases endurance performance; however, efficacy of caffeine ingestion for short-term high-intensity exercise is equivocal, which may be explained by discrepancies in exercise protocols, dosing, and subjects' training status and habitual caffeine intake found across studies. The primary aim of one review was to critically examine studies that have tested caffeine's ability to augment performance during exercise dependent on nonoxidative metabolism such as sprinting, team sports, and resistance training. A review of the literature revealed 29 studies that measured alterations in short-term performance after caffeine ingestion. Each study was critically analyzed using the Physiotherapy Evidence Database (PEDro) scale. The mean PEDro score was 7.76 ± 0.87. Eleven of 17 studies revealed significant improvements in team sports exercise and power-based sports with caffeine ingestion, yet these effects were more common in elite athletes who do not regularly ingest caffeine. Six of 11 studies revealed significant benefits of caffeine for resistance training. Some studies show decreased performance with caffeine ingestion when repeated bouts are completed. The exact mechanism explaining the ergogenic effect of caffeine for short-term exercise is unknown [10175].

Multiple studies corroborate the ergogenic properties of caffeine (CAF) for endurance performance, yet fewer investigations document the efficacy of acute caffeine intake for intense, short-term exercise. The aim of one study was to determine the ergogenic potential of caffeine during testing of muscular strength and endurance. Twenty-two resistance-trained men ingested CAF (6 mg/kg) or placebo (PL) 1 h pre-exercise in a randomized, double-blind crossover design. They refrained from caffeine intake and strenuous exercise 48 and 24 h, respectively, pre-visit. Initially, resting heart rate and blood pressure were obtained followed by one-repetition maximum (1-RM) testing on the barbell bench press and leg press. Upon determination of 1-RM, participants completed repetitions to failure at 60 percent 1-RM. Heart rate, blood pressure, and rating of perceived exertion (RPE) were measured after the final repetition. Compared to PL, there was no effect of caffeine on muscular strength, as 1-RM bench press and leg press were similar. Total weight lifted during the 60 percent 1-RM trial was 11 and 12 percent higher for the bench press and leg press with caffeine compared to placebo, yet did not reach significance. RPE was similar at the end of resistance exercise with CAF versus placebo. Acute caffeine intake does not significantly alter muscular strength or endurance during intense bench press or leg press exercise, yet the practical importance of the increased muscular endurance remains to be explored [07174].

**Effect of caffeine on upper-body anaerobic performance**

Peak power (PP) and mean power (MP) attained in upper body sprint performance test are considered important factors for competitive success in wrestling. This study aimed to determine whether acute caffeine ingestion would better maintain PP and MP across a simulated competition day in wrestling. In a double-blind, counterbalanced, crossover study,
14 trained wrestlers ingested either placebo or 5 mg/kg caffeine and completed four 6-min upper body intermittent sprint performance tests with 30-min recovery periods between consecutive tests. PP and MP were recorded during and blood lactate concentration was measured before and after each test. Ratings of perceived fatigue (RPF) and exertion (RPE) were recorded before and after each test, respectively. Heart rate (HR) was monitored across the whole testing period. Mean power decreased across four tests in both trials, but the reduction in PP (from 277 ± 35 W to 257 ± 45 W) only occurred in caffeine trial. Both pretest blood lactate concentration and HR were higher in caffeine than in placebo trial in the third and fourth tests. No between-trial differences occurred in RPF or RPE. It was concluded that acute caffeine ingestion has a partially detrimental effect on upper body intermittent sprint performance in trained wrestlers. Elevated HR and blood lactate levels observed between tests after caffeine ingestion suggest that caffeine may impair recovery between consecutive maximal efforts [13439, 13446].

**Effect on morning reduction in neuromuscular performance**

To investigate whether caffeine ingestion counteracts the morning reduction in neuromuscular performance associated with the circadian rhythm pattern 12 highly resistance-trained men underwent a battery of neuromuscular tests under three different conditions; i) morning (10:00 a.m.) with caffeine ingestion (i.e. 3 mg/kg; AM(CAFF) trial); ii) morning (10:00 a.m.) with placebo ingestion (AM(PLAC) trial); and iii) afternoon (18:00 p.m.) with placebo ingestion (PM(PLAC) trial). A randomized, double-blind, crossover, placebo controlled experimental design was used, with all subjects serving as their own controls. The neuromuscular test battery consisted in the measurement of bar displacement velocity during free-weight full-squat (SQ) and bench press (BP) exercises against loads that elicit maximum strength (75 % 1RM load) and muscle power adaptations (1 m/s load). Isometric maximum voluntary contraction (MVC(LEG)) and isometric electrically evoked strength of the right knee (EVOK(LEG)) were measured to identify caffeine's action mechanisms. Steroid hormone levels (serum testosterone, cortisol and growth hormone) were evaluated at the beginning of each trial (PRE). In addition, plasma norepinephrine (NE) and epinephrine were measured PRE and at the end of each trial following a standardized intense (85 % 1RM) 6 repetitions bout of SQ (POST). In the PM(PLAC) trial, dynamic muscle strength and power output were significantly enhanced compared with AM(PLAC) treatment (3.0-7.5 %). During AM(CAFF) trial, muscle strength and power output increased above AM(PLAC) levels (4.6-5.7 %) except for BP velocity with 1 m/s load. During AM(CAFF), EVOK(LEG) and NE (a surrogate of maximal muscle sympathetic nerve activation) were increased above AM(PLAC) trial. These results indicate that caffeine ingestion reverses the morning neuromuscular declines in highly resistance-trained men, raising performance to the levels of the afternoon trial. The electrical stimulation data, along with the NE values, suggest that caffeine increases neuromuscular performance having a direct effect in the muscle [12279].

**Effects on sprint**

One study examined the effects of 6 mg/kg caffeine ingestion in team-sport players (n=10) on repeated-sprint running performance (5 sets of 6 x 20 m) and reaction times, 60 min after caffeine or placebo ingestion. Best single sprint and total set sprint times, blood lactate and simple and choice reaction times were measured. Total sprint times across sets 1, 3 and 5 (departure every 25 s) were significantly faster after caffeine than placebo. Similarly, total sprint times across sets 2 and 4 (departure every 60 s), were significantly faster after caffeine than placebo. Significantly higher blood lactates were recorded in caffeine compared to placebo after set 3 and set 5. There were no significant effects on simple or choice reaction time, although effect sizes suggested improved post-exercise times after caffeine. It was concluded that caffeine ingestion 60 min prior to exercise can enhance repeated sprint running performance and is not detrimental to reaction times [08308].
Using a randomized double-blind research design, 21 physically active men ingested a gelatin capsule containing either caffeine (5 mg per kg body mass) or placebo (maltodextrin) 1 h before completing an indoor multiple sprint running trial (12 x 30 m; repeated at 35 second intervals). Venous blood samples were drawn to evaluate plasma caffeine and primary metabolite concentrations. Sprint times were recorded via twin-beam photocells, and earlobe blood samples were drawn to evaluate pretest and posttest lactate concentrations. Relative to placebo, caffeine supplementation resulted in a 0.06 second (1.4 %) reduction in fastest sprint time (95 % confidence interval 0.04 to 0.09 s), which corresponded with a 1.2 percent increase in fatigue (95 % confidence interval 0.3 to 2.2%). Caffeine supplementation also resulted in a 3.4 beat per minute increase in mean heart rate (95 % confidence interval 0.1 to 6.6) and elevations in pretest (+0.7 mmol per L; 95 % confidence interval 0.1 to1.3) and posttest (+1.8 mmol per L; 95 % confidence interval 0.3 to 3.2) blood lactate concentrations. It was concluded that although the effect of recovery duration on caffeine-induced responses to multiple sprint work requires further investigation, the results of the study showed that caffeine has ergogenic properties with the potential to benefit performance in both single and multiple sprint sports [08312].

Effect on anaerobe performance

One study investigated the effects of caffeine on repeated, anaerobic exercise using a double-blind, randomized crossover design. Seventeen subjects (five female) underwent cognitive (reaction time, number recall) and blood (glucose, potassium, catecholamines, lactate) testing before and after consuming caffeine (6 mg/kg), placebo, or nothing (control). An exercise test (two 60 s maximal cycling bouts) was conducted 90 min after caffeine/placebo consumption. Plasma caffeine concentrations significantly increased after caffeine ingestion, however, there were no positive effects on cognitive or blood parameters except a significant decrease in plasma potassium concentrations at rest. Potentially negative effects of caffeine included significantly higher blood lactate compared to control and significantly slower time to peak power in exercise bout 2 compared to control and placebo. Caffeine had no significant effect on peak power, work output, RPE, or peak heart rate. In conclusion, caffeine had no ergogenic effect on repeated, maximal cycling bouts and may be detrimental to anaerobic performance [06191].

Effects on upper-body resistance exercise

One double-blind, within-subjects experiment examined the effects of acute caffeine ingestion on perceptions of muscle pain following a bout of high-intensity, upper-body resistance exercise to failure. Moderately trained males (n=18) ingested a dose of caffeine (5 mg/kg) or placebo in a randomised and counterbalanced order and 1 hour later completed bench press exercise to failure at an intensity of 60 percent 1 repetition maximum. Repetitions completed was taken as a measure of performance, peak heart rate was determined via heart rate telemetry during the exercise bout, rating of perceived exertion (RPE) and upper body muscle pain was recorded immediately upon failure of the exercise task and peak blood lactate concentration was determined post-exercise. Caffeine resulted in improved repetitions to failure, greater peak blood lactate, and lower RPE compared to placebo. Muscle pain perception was also significantly lower in the caffeine condition compared to placebo. These results support prior studies using aerobic based exercise modes in suggesting that caffeine ingestion can dampen exercise-induced muscle pain. Specifically, caffeine ingestion enhances muscular strength performance and reduces upper body muscle pain perception immediately following a bout of high-intensity resistance exercise to failure [12282].

Effects of multi-task performance

1215
The purpose of one study was to examine the acute effects of a caffeine-containing supplement on upper- and lower-body strength and muscular endurance as well as anaerobic capabilities. Thirty-seven resistance-trained men (mean age: 21 years) volunteered to participate in this study. On the first laboratory visit, the subjects performed 2 Wingate Anaerobic Tests (WAnTs) to determine peak power (PP) and mean power (MP), as well as tests for 1 repetition maximum (1RM), dynamic constant external resistance strength, and muscular endurance (TOTV; total volume of weight lifted during an endurance test with 80 % of the 1RM) on the bilateral leg extension (LE) and free-weight bench press (BP) exercises. Following a minimum of 48 hours of rest, the subjects returned to the laboratory for the second testing session and were randomly assigned to 1 of 2 groups: a supplement group (SUPP; n=17), which ingested a caffeine-containing supplement, or a placebo group (PLAC; n=20), which ingested a cellulose placebo. One hour after ingesting either the caffeine-containing supplement or the placebo, the subjects performed 2 WAnTs and were tested for 1RM strength and muscular endurance on the LE and BP exercises. The results indicated that there was a significant increase in BP 1RM for the SUPP group, but not for the PLAC group. The caffeine-containing supplement had no effect, however, on LE 1RM, LE TOTV, BP TOTV, PP, and MP. Thus, the caffeine-containing supplement may be an effective supplement for increasing upper-body strength and, therefore, could be useful for competitive and recreational athletes who perform resistance training [06189].

Intermittent-sprint ability in team-sport
Caffeine can be a powerful ergogenic aid for the performance of prolonged, submaximal exercise. Little evidence, however, supports an ergogenic effect of caffeine on intermittent-sprint performance. Hence, one study was conducted to examine the effects of acute caffeine ingestion on prolonged intermittent-sprint performance. Using a double-blind, placebo-controlled design, 10 male team-sport athletes (amateur level) completed two exercise trials, separated by 7 d, 60 min after ingestion of either 6 mg/kg caffeine or placebo. The exercise trial was performed on a front-access cycle ergometer and consisted of 2 x 36-min halves, each composed of 18 x 4-s sprints with 2-min active recovery at 35 percent VO2peak between each sprint. Urinary caffeine levels were measured after exercise. The total amount of sprint work performed during the caffeine trial was 8.5 percent greater than that performed during the placebo trial in the first half, and was 7.6 percent greater in the second half. Similarly, the mean peak power score achieved during sprints in the caffeine trial was 7.0 percent greater than that achieved during the placebo trial in the first half, and was 6.6 percent greater in the second half. Urinary caffeine levels following the caffeine trial ranged from 3.5 to 9.1 microg/mL. The study revealed that acute caffeine ingestion can significantly enhance performance of prolonged, intermittent-sprint ability in competitive, male, team-sport athletes [06190].

Effects on skill performance
Popular use of caffeine is often at high concentrations (4-9 mg/kg) on the basis that these are more efficacious, but the proof of this is low with individual variability and consumption habits being the more dominant factors. While the ability of acute caffeine to address cognitive related sleep deficits is reasonably established, it is only recently that creatine has demonstrated similar properties. It has been suggested that sleep deprivation is associated with an acute reduction in high energy phosphates that in turn produces some degree of cognitive processing deficit. If sleep deprivation is associated with an energy deficit then errors in performance are perhaps more likely to occur when concentration demands are high and/or for prolonged periods of repeated task execution. Some evidence suggests that it is tasks of this nature that are most affected by acute sleep deprivation. Sleep deprivation is not uncommon around competition in sport particularly with the frequent demands of international travel. Assessing its effects on performance is however difficult, especially in
team sports where multiple physical and skill components are involved. While overt physical components such as power don't appear affected by acute deprivation a few studies do however suggest acute deprivation can affect certain sport skill and physical performance. Caffeine, for example, has been shown to improve both mood and mental function following sleep deprivation. The psychostimulant effects of caffeine appear to be related to the pre and post synaptic brakes that adenosine imposes on dopaminergic neurotransmission by acting on different adenosine receptor heteromers, although numerous mechanisms are likely to be involved. It is not known how much mood and other cognitive function, particularly motivation on repeat skill tasks, interact. The absorption of caffeine in plasma following consumption has been estimated at between 30 and 90 min with half life of several hours. Effective doses of caffeine (and their dose response nature) remain contentious in literature possibly reflecting larger inter-subject variability in responses and different sensitivities of various physical and behavioural expressions. It was thus investigated the effects of sleep deprivation with or without acute supplementation of caffeine or creatine on the execution of a repeated rugby passing skill. Ten elite rugby players completed 10 trials on a simple rugby passing skill test (20 repeats per trial), following a period of familiarisation. The players had between 7-9 h sleep on 5 of these trials and between 3-5 h sleep (deprivation) on the other 5. At a time of 1.5 h before each trial, they undertook administration of either: placebo tablets, 50 or 100 mg/kg creatine, 1 or 5 mg/kg caffeine. Saliva was collected before each trial and assayed for salivary free cortisol and testosterone. Sleep deprivation with placebo application resulted in a significant fall in skill performance accuracy on both the dominant and non-dominant passing sides. No fall in skill performance was seen with caffeine doses of 1 or 5 mg/kg, and the two doses were not significantly different in effect. Similarly, no deficit was seen with creatine administration at 50 or 100 mg/kg and the performance effects were not significantly different. Salivary testosterone was not affected by sleep deprivation, but trended higher with the 100 mg/kg creatine dose, compared to the placebo treatment. Salivary cortisol was significantly elevated with the 5 mg/kg dose of caffeine versus placebo. Thus, acute sleep deprivation affects performance of a simple repeat skill in elite athletes and this was ameliorated by a single dose of either caffeine or creatine. At the doses and administration time of caffeine use in this study there was, however, no evidence of an effect in non-sleep deprived subjects. Acute creatine use may help to alleviate decrements in skill performance in situations of sleep deprivation, such as transmeridian travel, and caffeine at low doses appears as efficacious as higher doses, at alleviating sleep deprivation deficits in athletes with a history of low caffeine use. Both options are without the side effects of higher dose caffeine use [11197].

*Effect on athletic agility*

Caffeine has been shown to improve sprint time, anaerobic power, and reaction time, all integral aspects of agility. The purpose of this study was to determine whether an acute caffeine dose would enhance agility and anaerobic power. Sixteen subjects participated in a randomized, double-blind experiment and performed the proagility run and the 30-second Wingate test 60 minutes after ingestion of caffeine (6 mg/kg) or placebo. No significant change was observed in the proagility run after caffeine ingestion compared with placebo. Also, no significant change was observed in peak power, mean power, or percent power decrease. Agility is an integral component of athletic skill and any reasonable method for enhancing agility would benefit active individuals. However, results from this study indicate that a 6 mg/kg dose of caffeine does not impact agility as measured by the proagility run test or power output as measured by the 30-second Wingate test in recreationally active young adult males who are not habituated to caffeine [06196].

*Effects on resistance training*
One study examined the placebo effect of caffeine on number of repetitions, rating of perceived exertion (RPE), blood pressure (BP), and peak heart rate (PHR) during resistance-training exercise with repetitions performed to volitional failure. Following determination of 1-rep maximum in single-leg leg extension, 15 males performed reps to failure at 60 percent 1-RM in 3 conditions: control, perceived caffeine condition, and perceived placebo condition presented in a randomized order. Participants were informed they would ingest 250 mL of solution that contained either 3 mg/kg body weight caffeine or 3 mg/kg body weight placebo 1 h before each exercise trial. A deceptive protocol was employed and subjects consumed a placebo solution in both conditions. During each condition, total reps, RPE for the active muscle and overall body, and PHR were recorded. Subjects completed 2 more repetitions when they perceived they had ingested caffeine. RPE was significantly lower in the perceived caffeine and control conditions and RPE for the active muscle was significantly higher across all conditions compared with RPE for the overall body. No substantial differences were evident in PHR across conditions. Results of this study are similar to studies of actual caffeine ingestion. However, the perception of consuming a substance that purportedly enhances performance is sufficient enough to enable individuals to complete a greater number of reps to failure during short-term resistance exercise [09253].

The purpose of one study was to evaluate the effects of caffeine ingestion before a resistance exercise session on markers of muscle damage (CK, LDH, ALT, AST) and leukocyte levels. Fifteen soccer athletes completed two resistance exercise sessions that differed only in the ingestion of caffeine or a placebo preworkout. CK concentration increased significantly following the caffeine session and the placebo session, with no significant differences between sessions. Similarly, LDH concentration increased significantly following the caffeine session and the placebo session, with no significant differences between sessions. Both sessions resulted in significant increases in the total leukocyte count, neutrophils, lymphocytes, and monocytes, with no significant differences between sessions. It was concluded that ingestion of caffeine at 4.5 mg/kg did not augment markers of muscle damage or leukocyte levels above that which occurs through resistance exercise alone [10181].

The primary aim of one study was to determine the efficacy of acute caffeine intake to enhance intense resistance training performance. Fourteen resistance-trained men (age and body mass = 23 ± 1 years and 83 ± 13 kg, respectively) who regularly consumed caffeine ingested caffeine (6 mg·kg) or placebo 1 hour before completion of 4 sets of barbell bench press, leg press, bilateral row, and barbell shoulder press to fatigue at 70-80 percent 1-repetition maximum. Two minutes of rest was allotted between sets. Saliva samples were obtained to assess caffeine concentration. The number of repetitions completed per set and total weight lifted was recorded as indices of performance. Compared to placebo, there was a small but significant effect of acute caffeine intake on repetitions completed for the leg press but not for upper-body exercise. Total weight lifted across sets was similar with caffeine vs placebo yet there were 9 “responders” to caffeine, represented by a meaningful increase in total weight lifted with caffeine versus placebo. Any ergogenic effect of caffeine on performance of fatiguing, total-body resistance training appears to be of limited practical significance. Additional research is merited to elucidate interindividual differences in caffeine-mediated improvements [11202].

**Effects of performance of chronic use**

The purpose of one study was to examine the effects of daily administration of a supplement that contained caffeine in conjunction with 8 weeks of aerobic training on VO\textsubscript{2peak}, time to running exhaustion at 90 percent VO\textsubscript{2peak}, body weight, and body composition. Thirty-six college students (14 men and 22 women, age 22 years) volunteered for this investigation and were randomized into either a placebo (n=18) or supplement group (n=18). The subjects
ingested 1 dose (3 pills = 201 mg of caffeine) of the placebo or supplement per day during the study period. In addition, the subjects performed treadmill running for 45 minutes at 75 percent of the heart rate at VO$_{2peak}$, three times per week for 8 weeks. All subjects were tested pretraining and posttraining for VO$_{2peak}$, time to running exhaustion (TRE) at 90 percent VO$_{2peak}$, body weight (BW), percentage body fat (%FAT), fat weight (FW), and fat-free weight (FFW). The results indicated that there were equivalent training-induced increases in VO$_{2peak}$ and TRE for the supplement and placebo groups, but no changes in BW, %FAT, FW, or FFW for either group. These findings indicated that chronic use of the caffeine-containing supplement in the present study, in conjunction with aerobic training, provided no ergogenic effects as measured by VO$_{2peak}$ and TRE, and the supplement was of no benefit for altering body weight or body composition [06195].

**Effects on cycling**

The primary aim of one study was to determine the repeatability of caffeine's ergogenic effects on cycling performance. It was hypothesized that improvements in performance would be similar when caffeine was ingested on 2 separate days. Nine endurance-trained men and women (mean age and maximal oxygen uptake, 27 years and 58 mL/kg/min) initially completed two familiarization trials. During 3 subsequent sessions separated by at least 48 hours, the subjects completed a 10-km cycling time trial preceded by ingestion of a drink containing caffeine (5 mg/kg) or placebo. Treatments were ingested using a randomized, single-blind, crossover design, and the subjects were deceived as to the specific content of all drinks. During exercise, heart rate, rating of perceived exertion, and time were recorded every 1.6 km. Repeated-measures analysis of variance was used to compare the differences in variables across distance and treatment. In both caffeine trials, caffeine increased, cycling performance by 1.6 and 1.9 percent versus placebo, and 7 of 9 subjects revealed improved performance. The mean performance improvement in the caffeine trials was similar across days. Heart rate during exercise was higher with caffeine versus placebo, although the rating of perceived exertion was similar. Data reveal that caffeine's ergogenic effects on cycling performance are repeatable across days, yet some individuals did not exhibit improved performance with caffeine [12293].

To investigate whether coinciding peak serum caffeine concentration with the onset of exercise enhances subsequent endurance performance it was performed a randomised, placebo-controlled, double-blind crossover study, with 14 male trained cyclists and triathletes who consumed 6 mg/kg caffeine or a placebo either 1h (C(1h)) prior to completing a 40 km time trial or when the start of exercise coincided with individual peak serum caffeine concentrations (C(peak)). C(peak) was determined from a separate 'caffeine profiling' session that involved monitoring caffeine concentrations in the blood every 30 min over a 4h period. Following caffeine ingestion, peak serum caffeine occurred 120 min in 12 participants and 150 min in 2 participants. Time to complete the 40 km time trial was significantly faster (2.0 %) in C(1h) compared to placebo. No statistically significant improvement in performance was noted in the C(peak) trial versus placebo (1.1 %). Whilst no differences in metabolic markers were found between C(peak) and placebo conditions, plasma concentrations of glucose, norepinephrine and epinephrine were higher in the C(1h) trial 6 min post-exercise versus placebo. Thus, in contrast to coinciding peak serum caffeine concentration with exercise onset, caffeine consumed 60 min prior to exercise resulted in significant improvements in 40 km time trial performance. The ergogenic effect of caffeine was not found to be related to peak caffeine concentration in the blood at the onset of endurance exercise [13465].

**Effects on muscles**
Caffeine (1,3,7-trimethylxanthine) has been implicated in the regulation of glucose and lipid metabolism including actions such as insulin-independent glucose transport, glucose transporter 4 expression, and fatty acid utilization in skeletal muscle. These effects are similar to the exercise-induced and 5’adenosine monophosphate-activated protein kinase (AMPK)-mediated metabolic changes in skeletal muscle, suggesting that caffeine is involved in the regulation of muscle metabolism through AMPK activation. It was explored whether caffeine acts on skeletal muscle to stimulate AMPK. Incubation of rat epitrochlearis and soleus muscles with Krebs buffer containing caffeine (≥3 mmol/L, ≥15 minutes) increased the phosphorylation of AMPKα Thr(172), an essential step for full kinase activation, and acetyl-coenzyme A carboxylase Ser(79), a downstream target of AMPK, in dose- and time-dependent manners. Analysis of isoform-specific AMPK activity revealed that both AMPKα1 and α2 activities increased significantly. This enzyme activation was associated with a reduction in phosphocreatine content and an increased rate of 3-O-methyl-d-glucose transport activity in the absence of insulin. These results suggest that caffeine has similar actions to exercise by acutely stimulating skeletal muscle AMPK activity and insulin-independent glucose transport with a reduction of the intracellular energy status [09252].

Effects of neural recovery

The purpose of one study was to test the hypothesis that prior caffeine ingestion would enhance neural recovery after isometric fatiguing maximal intermittent plantar flexions, and thus would enhance the recovery of voluntary muscle strength. After a familiarisation session, 13 males randomly participated in two experimental trials where they ingested either caffeine (approximately 6 mg/kg) or identical placebo pills 1 h prior to testing. Subjects were tested for electromyogram (EMG) activity and evoked V-waves in the soleus and gastrocnemius medialis muscles. These measurements were obtained during brief plantar flexion maximum voluntary isometric contractions (MVICs), and normalised by the superimposed maximal M-wave (EMG/M(SUP) and V/M(SUP), respectively), before and after (20 s, 10 min and 20 min) a fatigue protocol (seven 25 s MVICs, 5 s rest). There were no effects (P > 0.05) of caffeine ingestion on EMG/M(SUP), V/M(SUP), MVIC or M(SUP). The central neural modulation (EMG/M(SUP) and V/M(SUP)) and voluntary strength changes followed a similar time-course with a substantial reduction 20 s post-fatigue and a gradual return towards baseline values. Thus, there was no effect of prior caffeine ingestion on neuromuscular recovery after maximal fatiguing contractions [10178].

Effects in children

Both cognitive and physical performance can be viewed as potentially enhanceable, and arguments can be made that enhancement can serve two purposes: gaining an edge or keeping up with others (who may or may not have used performance-enhancing substances). Caffeine, a central nervous system and cardiac stimulant, is frequently used by children for both academic and athletic performance enhancement. In fact, the marketplace contains a plethora of caffeinated products marketed directly to children. One article examines safety and ethical issues associated with the use of caffeine by children and explores the question: Can cognitive performance enhancement be ethically permissible if sports performance enhancement is not? [07170].

Effects on tennis

To determine the effects of prolonged simulated tennis on performance and the ergogenic potential of caffeine, carbohydrates, and cooling. Twelve highly trained male tennis players
performed 4 simulated matches (2 h 40 min) against a ball machine on an indoor hard court. The counterbalanced experimental trials involved caffeine supplementation (3 mg/kg), carbohydrate supplementation (6 % solution), precooling and intermittent cooling, and placebo control. Physiological markers (core temperature, heart rate, blood lactate, and blood glucose), subjective responses (ratings of perceived exertion and thermal sensation), stroke velocity and accuracy, serve kinematics, and tennis-specific perceptual skill quantified the efficacy of interventions. Significant effects of time reflected increased physiological demand, reduced serve velocity and ground-stroke velocity and accuracy, and a slowing of the serve racket-arm acceleration phase. Caffeine increased serve velocity (165 ± 15 km/h) in the final set of the match compared with placebo (159 ± 15 km/h) and carbohydrate (158 ± 13 km/h) conditions. Carbohydrate and cooling conditions afforded physiological advantage (increased blood glucose, and reduced preexercise thermal sensation, but did not affect performance relative to the placebo condition. It was concluded that prolonged simulated tennis induced significant decrements in tennis skills. Caffeine supplementation partly attenuated the effects of fatigue and increased serve velocity. In contrast, carbohydrate and cooling strategies had little ergogenic effect on tennis performance [09257].

Effects on rugby

The aim of one study was to determine the effects of a caffeine-containing energy drink on physical performance during a rugby sevens competition. A second purpose was to investigate the post-competition urinary caffeine concentration derived from the energy drink intake. On two non-consecutive days of a friendly tournament, 16 women from the Spanish National rugby team (mean age and body mass 23 ± 2 years and 66 ± 7 kg) ingested 3 mg of caffeine per kg of body mass in the form of an energy drink (Fure(®)) or the same drink without caffeine (placebo). After 60 min for caffeine absorption, participants performed a 15-s maximal jump test, a 6 × 30 m sprint test, and then played three rugby sevens games against another national team. Individual running pace and instantaneous speed during the games were assessed using global positioning satellite devices. Urine samples were obtained pre and post-competition. In comparison to the placebo, the ingestion of the energy drink significantly increased muscle power output during the jump series, running pace during the games and pace at sprint velocity. However, the energy drink did not affect maximal running speed during the repeated sprint test. The ingestion of the energy drink resulted in a higher post-competition urine caffeine concentration than the placebo (3.3 ± 0.7 vs 0.2 ± 0.1 microg/mL). In summary, 3 mg/kg of caffeine in the form of a commercially available energy drink considerably enhanced physical performance during a women's rugby sevens competition [13441].

The purpose of one study was to investigate the effectiveness of a caffeine-containing energy drink in enhancing rugby players' physical performance during a simulated match. A second purpose was to determine the urinary caffeine excretion derived from the energy drink intake. In a randomized and counterbalanced order, 26 elite rugby players played 2 simulated rugby games (2 × 30 min) 60 min after ingesting 3 mg of caffeine per kilogram of body mass in the form of an energy drink (Fure(®)) or the same drink without caffeine (placebo). During the matches, the individual running distance and the instantaneous speed were measured, and the number of running actions above 20 km/h (i.e. sprints) were determined, using global positioning system devices. The number of impacts above 5 g during the matches was determined by accelerometry. The ingestion of the energy drink, compared with the placebo, increased the total distance covered during the match, the running distance covered at more than 20 km/h, and the number of sprints. The ingestion of the energy drink also resulted in a greater overall number of impacts and a higher postexercise urine caffeine concentration. The use of an energy drink with a caffeine dose
equivalent to 3 mg/kg considerably enhanced the movement patterns of rugby players during a simulated match [13442].

Investigation of various exercise parameters in situations devised to simulate the repeated bouts of exercise required in rugby, a high-intensity team sport. Subjects were given either placebo or a moderate dose of caffeine (6 mg/kg) that previously has been shown to demonstrate ergogenic effects in submaximal exercise situations. Participants were then put through a series of 14 exercise circuits divided into two 40-minute halves. A 10-minute rest period occurred between the two halves. The design was intended to simulate the conditions of an actual rugby game with a first half, halftime, and second half. Subjects taking caffeine showed improvement in a variety of skill tasks including sprint tasks, power tasks, and passing accuracy tasks [06183].

Effects on weight-lifting

The purpose of the present study was to examine the acute effects of a caffeine-containing supplement (SUPP) on 1 repetition maximum (1RM) bench press and leg extension strength, as well as time to exhaustion (TTE), during cycle ergometry at a power output that corresponded to 80 percent of VO$_{2peak}$. The study used a double-blinded, placebo-controlled, crossover design. Twenty-one untrained men were randomly assigned to take either the SUPP or placebo (PLAC) first. The SUPP contained 400 mg of caffeine, 66.7 mg of capsicum extract, 10 mg of bioperine, and 40 mg of niacin, and the PLAC was microcrystalline cellulose. Sixty minutes after taking either the SUPP or PLAC, the subjects were tested for 1RM bench press and leg extension strength, as well as TTE. After 1 week of rest, the subjects ingested the opposite substance (SUPP or PLAC) and were retested for 1RM bench press and leg extension strength, as well as TTE. The results indicated that the SUPP had no effect on 1RM bench press strength, 1RM leg extension strength, or TTE at 80 percent VO$_{2peak}$. These findings did not support the use of the caffeine-containing SUPP in the present study as an ergogenic aid in untrained individuals [10182].

Effects on rowing

To determine whether a dose-response relationship exists between caffeine and 2000-m rowing performance in a randomized, placebo-controlled, double-blind crossover study, 10 competitive male rowers consumed 2, 4, or 6 mg/kg caffeine or a placebo 60 min before completing a 2000-m time trial on a rowing ergometer. The trials were preceded by a 24-h standardized diet (including a light preexercise meal of 2 g/kg carbohydrates), and subjects were tested preexercise for hydration, caffeine abstinence, and blood glucose concentrations. Time trial performance was not significantly different across the three caffeine doses or placebo. After the three caffeine trials, postexercise plasma glucose and lactate concentrations were higher compared with the placebo trial. Plasma caffeine concentrations after 60 min of ingestion were lower than the values reported previously by others following the same dose, and there was considerable interindividual variation in plasma caffeine concentrations in response to the various caffeine doses. It was concluded that the large interindividual response to the caffeine doses suggests that individual characteristics need to be considered when administering caffeine for performance enhancement. In addition, preexercise feeding may significantly affect plasma caffeine concentrations and the potential for caffeine to improve performance [10180].

Effects on iron-man

One study assessed the knowledge, prevalence, and quantity of caffeine use by athletes
competing at the 2005 Ironman Triathlon World Championships. Caffeine-related questionnaires were self-administered to 140 (105 male and 35 female, 40 ± 11 years) athletes representing 16 countries. Fifty of these athletes further consented to immediate post-race blood samples for analysis of plasma caffeine and paraxanthine using high-performance liquid chromatography (HPLC). Seventy-two percent of 70 athletes correctly identified caffeine as being an unrestricted substance in triathlon. The majority of athletes (89 %) were planning on using a caffeinated substance immediately prior to or throughout the race. Cola drinks (78 %), caffeinated gels (42 %), coffee (usually pre-race) (37 %), energy drinks (13 %), and NoDoz tablets (9 %) were the most popular caffeinated choices. Mean ± standard deviation post race plasma caffeine and paraxanthine levels were 22 ± 20 micromol/L and 9 ± 6 micromol/L, respectively. Seven athletes (14 %) finished with plasma caffeine levels > 40 micromol/L. Plasma values from elite athletes did not differ from age group competitors. Despite the prevalence of its consumption and the training experience of this athletic group, over one quarter of athletes remained either confused or uninformed about caffeine’s legality. Levels of plasma caffeine taken immediately post race indicated that athletes typically finish with quantities of caffeine that have been shown to improve endurance performance (i.e. approximately 20 micromol/L or a dose of ± 3 mg/kg body weight) [06198].

Effect on field hockey

One study examined the impact of caffeine ingestion on field hockey skill performance following high-intensity fatigue. Thirteen male hockey players (mean age 21 years) performed hockey sprint dribble and ball handling tests at rest and after a bout of total body fatigue (90 % maximal capacity) following caffeine (5 mg/kg) or placebo ingestion. Sprint dribble times were slower postfatigue compared with rest but were significantly faster postfatigue with caffeine compared with postfatigue with placebo ingestion. Ball handling scores were higher at rest compared with postfatigue, but scores postfatigue were higher following caffeine than placebo ingestion. Rating of perceived exhaustion (RPE) was lower and readiness to invest physical and mental effort were significantly higher in the caffeine condition. Caffeine ingestion may therefore be effective in offsetting decrements in skilled performance associated with fatigue [12289].

Effect on sedentary men

It is not known if ergogenic effects of caffeine ingestion in athletic groups occur in the sedentary. To investigate this, it was used a counterbalanced, double-blind, crossover design to examine the effects of caffeine ingestion (6 mg/kg body-mass) on exercise performance, substrate utilisation and perceived exertion during 30 minutes of self-paced stationary cycling in sedentary men. Participants performed two trials, one week apart, after ingestion of either caffeine or placebo one hour before exercise. Participants were instructed to cycle as quickly as they could during each trial. External work (J/kg) after caffeine ingestion was greater than after placebo. Further, heart rate, oxygen uptake and energy expenditure during exercise were greater after caffeine ingestion; whereas ratings of perceived exertion and respiratory exchange ratio values did not differ between trials. The ability to do more exercise after caffeine ingestion, without an accompanying increase in effort sensation, could motivate sedentary men to participate in exercise more often and so reduce adverse effects of inactivity on health [12288].

Effects on basket
One study investigated whether performance enhancement from caffeine described by other researchers transfers to male basketball players. The effects of caffeine ingestion were studied in a maximal-effort test on a treadmill that was followed by a vertical-jump test. Five elite-level male basketball players completed a graded treadmill test that measured maximal oxygen uptake, blood lactate profiles, respiratory exchange ratio, and rating of perceived exertion at each 3-minute stage. After a 15-minute warm-down, the subjects performed 10 vertical rebound jumps. Each subject completed the test twice – once with a 3 mg/kg of body weight dose of caffeine and once with a placebo, with the dosage administered 60 minutes before commencement of exercise. The test was thus administered according to a double-blind protocol. No substantial trends were found between caffeine and control trials, regardless of trial order. The study showed that the specified dosage had negligible effects on the players' power and endurance performance and had no efficacy as an ergogenic aid for male basketball players [13464].

Coffeine in central fatigue

Strong or sustained contractions can fatigue muscles and reduce their capacity to generate maximal voluntary force. This impairment may result not only from reduced force from the muscle but also from reduced output from the spinal motoneuron pool. This is known as “central fatigue.” Demonstration of the suboptimal volitional output from the motoneuron pool is easily achieved using methods that interpolate an extra motor impulse via an external agency, such as a nerve stimulator. If a twitchlike increment in force is obtained with stimulation of the nerve innervating part of the contracting muscles, suboptimal motoneuron output is obvious. However, quantitation of the level of output to the muscle group is less easy. For example, whereas many motor nerves innervate the bulk of synergists in a particular task, they also innervate antagonists (e.g., stimulation of the common peroneal nerve contracts ankle dorsiflexors but also some plantar flexors, stimulation of the femoral nerve activates knee extensors but also two weak knee flexors), and hence the size of the superimposed twitch can be contaminated by unwanted force produced by antagonists, and voluntary activation is spuriously high. As supraspinal fatigue develops with exercise, there are dramatic changes within the motor cortex and also in corticospinal “connectivity” with motoneurons. For example, the silent period in the electromyogram (EMG) after cortical stimulation lengthens (a reflection of intracortical inhibition) and the initial excitatory EMG response (motor evoked potential, MEP) increases during fatiguing contractions, and there are depressions in MEPs and cervicomedullary evoked potentials (a test of corticomotoneuronal function) after exercise. It has been used caffeine ingestion to probe links between force and motor cortical output. It is argued that, if a decrease in “central excitability” causes central fatigue with its associated failure in voluntary activation of the muscle, then caffeine, which increases central excitability (as represented by the size of the MEP), should reduce the fatiguing decline in voluntary force. Sets of repeated knee extensor contractions reduced force by about 35 percent, and performance was measured in two sessions: one with, and one without prior caffeine administration (6 mg/kg). TMS was delivered during weak contractions between exercise sets. Femoral nerve stimulation was applied during and after maximal voluntary contractions (MVCs) to assess changes in the M wave and in voluntary activation. Before fatigue, caffeine increased voluntary activation in brief maximal efforts (by 2-3 %) and increased the baseline MEP during very weak contractions (3 % MVC). During fatiguing exercise, MEP size was elevated by caffeine compared with placebo, whereas maximal voluntary activation and force recovery were unaffected. That is, increased central excitability did not ameliorate the fatigue-related falls in voluntary activation and voluntary force. Furthermore, after caffeine, the MEP at the end of fatiguing exercise was not decreased compared with control values, so the impairment of
voluntary activation seen at this moment is not due to impaired central excitability [06197].

**Ratings of perceived exertion**

One study examined effects of caffeine on session ratings of perceived exertion (RPE) following 30 min constant-load cycling. Individuals (n=15) of varying aerobic fitness completed a max trial and two 30 min cycling bouts (double-blind, counterbalanced) following ingestion of 6 mL/kg of caffeine or matched placebo. RPE overall, legs and breathing were estimated every 5 min and session RPE was estimated 30 min post-exercise using the OMNI pictorial scale. Session RPE for caffeine and placebo trials were compared using paired t test. Between-trial comparisons of HR, RPE overall, RPE legs and RPE breathing were analyzed using an independent 2 (trial) × 6 (time point) repeated measures analysis of variance (ANOVA) for each dependent variable. Caffeine resulted in a significantly lower session RPE for caffeine versus placebo. Acute perceptual responses were significantly lower for caffeine for RPE overall (15, 20, 25, and 30 min), RPE breathing (15, 20, 25, and 30 min) and RPE legs (20 and 30 min). Survey responses post-exercise revealed greater feelings of nervousness, tremors, restlessness and stomach distress following caffeine versus placebo. Blunted acute RPE and survey responses suggest participants responded to caffeine ingestion. Caffeine decreased acute RPE during exercise which could partially account for lower session RPE responses. However, decreased session RPE could also reveal a latent analgesic affect of caffeine extending into recovery. Extending the understanding of session RPE could benefit coaches in avoiding overtraining when adjusting training programs [13447].

Caffeine containing energy drinks is commonly consumed in the belief that it will enhance the quality of an exercise session and enhance mood. However, studies examining their efficacy are sparse. The aim of this study was to examine the effect of a caffeinated energy drink on leg pain perception, perceived exertion, mood state and readiness to invest effort pre, during and post 60 min cycling exercise. Fourteen active individuals (7 males, 7 females, mean age 24 years), completed two 60 min cycling trials at an intensity of 60 percent VO$_2$ max preceded by ingestion of solutions containing either a caffeinated energy drink or placebo using a double-blind, deceptive, crossover design. During exercise, RPE (6-20 scale), leg pain (0-10 scale), heart rate (HR) and blood lactate (Bla) were recorded. Participants also completed measures of mood state and readiness to invest physical effort (RTIPE) pre- and post-exercise. Repeated measures analysis of variance was used to assess differences in all variables and across time and treatments, with gender used as a between subjects variable. Results indicate that HR was significantly higher from 30 to 60 min and RPE and pain perception were significantly lower from 20 to 60 min in the energy drink condition compared to placebo. Lactate was significantly higher in the last 15 min of the energy drink trial and RTIPE increased significantly more from pre-ingestion to pre-exercise post-ingestion in the energy drink condition compared to placebo. No gender differences were evident. The data revealed positive effects of energy drink ingestion on perception of exertion, leg muscle pain perception and readiness to invest effort during submaximal cycling in active adults [13448].

**Caffeine levels before and after the removal of caffeine from the doping list**

Caffeine concentrations were measured in the urine of 4633 athletes tested for doping control in the Ghent Doping Control Laboratory in 2004. Determination of these concentrations was done using an alkaline extraction with a mixture of dichloromethane and methanol (9 : 1; v/v) followed by high performance liquid chromatography and ultraviolet detection (HPLC-UV). The method was validated according to ISO 17 025 standards.
(International Organisation for Standardisation). Quantification was done by using a linear calibration curve in the range from 0 to 20 microg/mL. The limit of quantification (LOQ) was 0.10 microg/ml. Because the results were not normally distributed, transformation of the data was done to evaluate the difference in detected concentrations in several sports. This resulted in an overall average concentration of 1.12 ± 2.68 microg/mL. Comparison of the most frequently tested sports in 2004 demonstrated that caffeine concentrations in samples originating from power lifters are significantly higher in comparison to urines taken in other sports. Also, a significant difference between caffeine concentrations found in cycling and concentrations found in other sports, including athletics and some ball sports, was observed. A comparison was made between results obtained in 2004 and results obtained before the removal of caffeine from the WADA (World Anti-Doping Agency) doping list indicating that average caffeine concentrations decreased after the withdrawal of caffeine from the list of prohibited substances. The overall percentage of positive samples between the two periods remained the same although the percentage of positive samples noticed in cycling increased after the removal of caffeine from the doping list [06187].

Caffeine in diabetics

Caffeine is a substance that has been used in our society for generations, primarily for its effects on the central nervous system that causes wakefulness. Caffeine supplementation has become increasingly more popular as an ergogenic aid for athletes and considerable scientific evidence supports its effectiveness. Because of their potential to alter energy metabolism, the effects of coffee and caffeine on glucose metabolism in diabetes have also been studied both epidemiologically and experimentally. Predominantly targeting the adenosine receptors, caffeine causes alterations in glucose homeostasis by decreasing glucose uptake into skeletal muscle, thereby causing elevations in blood glucose concentration. Caffeine intake has also been proposed to increase symptomatic warning signs of hypoglycemia in patients with type 1 diabetes and elevate blood glucose levels in patients with type 2 diabetes. Other effects include potential increases in glucose counterregulatory hormones such as epinephrine, which can also decrease peripheral glucose disposal. Despite these established physiological effects, increased coffee intake has been associated with reduced risk of developing type 2 diabetes in large-scale epidemiological studies. One review paper highlighted the known effects of caffeine on glucose homeostasis and diabetes metabolism during rest and exercise [13437].

Several prospective epidemiologic studies over the past 4 years concluded that ingestion of caffeinated and decaffeinated coffee can reduce the risk of diabetes. This finding is at odds with the results of trials in humans showing that glucose tolerance is reduced shortly after ingestion of caffeine or caffeinated coffee and suggesting that coffee consumption could increase the risk of diabetes. This review discusses epidemiologic and laboratory studies of the effects of coffee and its constituents, with a focus on diabetes risk. Weight loss may be an explanatory factor, because one prospective epidemiologic study found that consumption of coffee was followed by lower diabetes risk but only in participants who had lost weight. A second such study found that both caffeine and coffee intakes were modestly and inversely associated with weight gain. It is possible that caffeine and other constituents of coffee, such as chlorogenic acid and quinides, are involved in causing weight loss. Caffeine and caffeinated coffee have been shown to acutely increase blood pressure and thereby to pose a health threat to persons with cardiovascular disease risk. One short-term study found that ground decaffeinated coffee did not increase blood pressure. Decaffeinated coffee, therefore, may be the type of coffee that can safely help persons decrease diabetes risk. However, the ability of decaffeinated coffee to achieve these effects is based on a limited number of studies, and the underlying biological mechanisms have yet to be elucidated [06188].
Combination with creatinine

Caffeine and creatine are 2 of the most widely available and used compounds in sport. Although the use of either is not considered a doping infraction, the evidence does suggest ergogenic potential in certain sports. The purpose of one paper was to review the pharmacology and potential mechanism(s) of action of caffeine and creatine as they pertain to possible use as an ergogenic aid in sport. Previous review articles on caffeine and creatine use in sport were screened for relevant information and references, and studies for review and recent articles (2007 onwards) were obtained and reviewed using a PUBMED search with the terms “caffeine AND exercise”, “creatine and creatine monohydrate AND exercise”, and appropriate linked articles were evaluated. Caffeine taken before (3-6 mg/kg) or during (1-2 mg/kg) endurance exercise enhances performance, through central nervous system and direct muscle effects. Creatine monohydrate supplementation at higher (approx. 20 g/day × 3-5 days) or lower (approx. 5 g/day × 30 days) doses increases skeletal muscle total and phosphocreatine by 10-20 percent. Creatine supplementation appears to minimally but significantly enhance high-intensity sport performance and the mass and possibly strength gains made during resistance exercise training over the first few months. Although caffeine and creatine appear to be ergogenic aids, they do so in a sport-specific context and there is no rationale for their simultaneous use in sport. Higher doses of caffeine can be toxic and appear to be ergolytic. There is no rationale for creatine doses in excess of the recommendations, and some athletes can get stomach upset, especially at higher creatine doses [11201].

Combination with ephedrine

Caffeine and ephedrine-related alkaloids recently have been removed from International Olympic Committee banned substances lists, whereas ephedrine itself is now permissible at urinary concentrations less than 10 mug.mL. The changes to the list may contribute to an increased use of caffeine and ephedra as ergogenic aids by athletes. Consequently, It was investigated the effects of ingesting caffeine (C) or a combination of ephedra and caffeine (C + E) on muscular strength and anaerobic power using a double-blind, crossover design. Forty-five minutes after ingesting a glucose placebo (P: 300 mg), C (300 mg) or C + E (300 mg + 60 mg), 9 resistance-trained male participants were tested for maximal strength by bench press (1 repetition maximum) and latissimus dorsi pull down (1 repetition maximum). Subjects also performed repeated repetitions at 80 percent of 1 repetition maximumon both activities until exhaustion. After this test, subjects underwent a 30-second Wingate test to determine peak anaerobic cycling power, mean power, and fatigue index. Although subjects reported increased alertness and enhanced mood after supplementation with caffeine and ephedra, there were no significant differences between any of the treatments in muscle strength, muscle endurance, or peak anaerobic power. The results do not support the contention that supplementation with ephedra or caffeine will enhance either muscle strength or anaerobic exercise performance [08316].

Caffeine versus pseudoephedrine

Caffeine (CAF) improves performance in both short- and long-duration running and cycling where performance relies on power output and endurance capacity of leg muscles. No studies have so far tested the effects of CAF while using the double-poling (DP) technique in
cross-country skiing. When using the DP technique, arm muscles provide the speed-generating force and therefore play an important role in performance outcome. The metabolism of arm muscles differs from that of leg muscles. Thus, results from studies on leg muscles and CAF may not be directly applicable to exercises while using the DP technique in cross-country skiing. The purpose of one study was therefore to investigate the effects of CAF on exercise performance in DP. Ten highly trained male cross-country skiers performed a placebo (PLA) and CAF trial using a randomized, double-blind, crossover design. Performance was assessed by measuring the time to complete an 8-km cross-country DP performance test (C-PT). CAF (6 mg/kg) or PLA was ingested 75 min before the C-PT. CAF ingestion reduced the time to complete the 8-km C-PT. The subjects maintained higher speed and HR throughout the C-PT, and lactate was higher immediately after the C-PT with CAF exposure compared with PLA. Subjects reported lower RPE at submaximal intensities during CAF compared with PLA, although HR was similar. It was concluded that CAF intake enhances endurance performance in an 8-km C-PT, where arm muscles limit performance. CAF ingestion allowed the participants to exercise with a higher HR and work intensity possibly by reducing perception of effort or facilitating motor unit recruitment [13473].

Both caffeine (CAF) and pseudoephedrine (PSE) are proposed to be central nervous system stimulants. However, during competition, CAF is a permitted substance, whereas PSE is a banned substance at urinary levels >150 microg/mL. As a result, one study aimed to compare the effect of CAF versus PSE use on cycling time trial (TT) performance to explore whether the legal stimulant was any less ergogenic than the banned substance. Here, 10 well-trained male cyclists or triathletes were recruited for participation. All athletes were required to attend the laboratory on four separate occasions – including a familiarization trial and three experimental trials, which required participants to complete a simulated 40 km (1,200 kJ) cycling TT after the ingestion of either 200 mg CAF, 180 mg PSE or a nonnutritive placebo (PLA). The results showed that the total time taken and the mean power produced during each TT was not significantly different between trials, despite a 1.3 percent faster overall time (57 s) after CAF consumption. Interestingly, the time taken to complete the second half of the TT was significantly faster in CAF as compared with PSE (by 99 s), with magnitude based inferences suggesting a 91 percent beneficial effect of CAF during the second half of the TT. This investigation further confirms the ergogenic benefits of CAF use during TT performances and further suggests this legal CNS stimulant has a better influence than a supra-therapeutic dose of PSE [13474].

Combination with albuterol

The main aim of one study was to evaluate the comparative and additive effects of caffeine and albuterol (short-acting beta2-agonist) on the severity of exercise induced bronchoconstriction (EIB). Ten asthmatic subjects with exercise-induced bronchoconstriction participated in a randomized, double-blind, double-dummy crossover study. One hour before an exercise challenge, each subject was given 0, 3, 6, or 9 mg/kg of caffeine or placebo mixed in a flavored sugar drink. Fifteen minutes before the exercise bout, an inhaler containing either albuterol (180 microg) or placebo was administered to each subject. Pulmonary function tests were conducted pre- and post-exercise. Caffeine at a dose of 6 and 9 mg/kg significantly reduced the mean maximum percentage fall in post-exercise FEV1 compared to the double-placebo and baseline. There was no significant difference in the post-exercise % fall in FEV1 between albuterol and the 9 mg/kg dose of caffeine. Interestingly, there was no significant difference in the post-exercise percentage fall in FEV1 between albuterol and albuterol with 3, 6 or 9 mg/kg of caffeine. Similar changes were observed for the post-exercise percentage fall in FVC, FEF (25-75 %) and PEF. These data
indicate that moderate (6 mg/kg) to high doses (9 mg/kg) of caffeine provide a significant protective effect against EIB. It is feasible that the negative effects of daily use of short-acting beta-agonists by asthmatic athletes could be reduced simply by increasing caffeine consumption prior to exercise [10177].

Combination with sodium bicarbonate

To determine the effects of ingesting caffeine (CAFF) and sodium bicarbonate (SB), taken individually and simultaneously, on 3-km cycling time-trial (TT) performance 10 well-trained cyclists, age 24 years, participated in this acute-treatment, double-blind, crossover study that involved four 3-km cycling TTs performed on separate days. Before each TT, participants ingested either 3 mg/kg body mass (BM) of CAFF, 0.3 g/kg BM of SB, a combination of the two (CAFF+SB), or a placebo (PLAC). They completed each 3-km TT on a laboratory-based cycle ergometer, during which physiological, perceptual, and performance measurements were determined. For statistical analysis, the minimal worthwhile difference was considered about 1 percent based on previous research. Pretrial pH and HCO₃ were higher in SB and CAFF+SB than in the CAFF and PLAC trials. Differences across treatments for perceived exertion and gastric discomfort were mostly unclear. Compared with PLAC, mean power output during the 3-km TT was higher in CAFF, SB, and CAFF+SB trials (2.4 %, 2.6 %, 2.7 % respectively), resulting in faster performance times (-0.9, -1.2, -1.2 % respectively). Effect sizes for all trials were small (0.21 - 0.24). Thus, when ingested individually, both CAFF and SB enhance high-intensity cycling TT performance in trained cyclists. However, the ergogenic effect of these 2 popular supplements was not additive, bringing into question the efficacy of coingesting the 2 supplements before short-duration high-intensity exercise. In this study there were no negative effects of combining CAFF and SB, 2 relatively inexpensive and safe supplements [12292].

The purpose of this study was to investigate the effects of sodium bicarbonate (NaHCO₃), caffeine, and their combination on repeated 200-m freestyle performance. Six elite male freestyle swimmers ingested sodium bicarbonate (0.3 g/kg), caffeine (6.2 ± 0.3 mg/kg), a combination of both, and placebo on 4 separate occasions before completing 2 maximal 200-m freestyle time trials separated by 30 min. No significant differences were observed for performance but drop-off in performance time from first to second trial, however, was significantly greater when caffeine was ingested than with bicarbonate or the combination. This is likely because of the lower blood pH and slower recovery of blood HCO₃ after caffeine ingestion. These findings suggest that the ergogenic benefit of taking caffeine alone for repeated 200-meter swimming performance appears limited. When combined with sodium bicarbonate, however, its negative impact on repeated maximal exercise performance is reversed [08317].

The purpose of this investigation was to determine the effect of ingested caffeine, sodium bicarbonate, and their combination on 2,000-m rowing performance, as well as on induced alkalosis (blood and urine pH and blood bicarbonate concentration, HCO₃⁻, blood lactate concentration, gastrointestinal symptoms, and rating of perceived exertion (RPE). In a double-blind, crossover study, 8 well-trained rowers performed 2 baseline tests and 4 × 2,000-m rowing-ergometer tests after ingesting 6 mg/kg caffeine, 0.3 g/kg body mass (BM) sodium bicarbonate, both supplements combined, or a placebo. Capillary blood samples were collected at preingestion, pretest, and posttest time points. Pairwise comparisons were made between protocols, and differences were interpreted in relation to the likelihood of exceeding the smallest worthwhile-change thresholds for each variable. A likelihood of >75 percent was considered a substantial change. Caffeine supplementation elicited a substantial
improvement in 2,000-m mean power, with mean (± SD) values of 354 ± 67 W versus placebo with 346 ± 61 W. Pretest bicarbonate reached 29.2 ± 2.9 mmol/L with caffeine + bicarbonate and 29.1 ± 1.9 mmol/L with bicarbonate. There were substantial increases in pretest bicarbonate and pH and posttest urine pH after bicarbonate and caffeine + bicarbonate supplementation compared with placebo, but unclear performance effects. It was concluded that rowers' performance in 2,000-m efforts can improve by about 2 percent with 6 mg/kg BM caffeine supplementation. When caffeine is combined with sodium bicarbonate, gastrointestinal symptoms may prevent performance enhancement, so further investigation of ingestion protocols that minimize side effects is required [11493].

### Combination with sodium citrate

The aim of one study was to investigate whether caffeine and/or sodium citrate have an ergogenic effect on the 1500 m exercise performance in elite wheelchair athletes. A placebo-controlled, randomized, cross-over and double-blind study design was conducted with the four treatments placebo, caffeine, sodium citrate and the combination of caffeine and sodium citrate. Nine healthy, elite wheelchair racing athletes (category T53/54) completed the study. All athletes were national team members, including several Paralympic Games, World and European Championship medalists. The athletes performed a 1500 m time trial four times on a wheelchair training roller. Time to complete 1500 m, pH, bicarbonate and sodium concentration as well as lactate concentration were measured. The time to complete 1500 m was not significantly different between the four treatments (placebo; caffeine; sodium citrate; combination. However, pH and bicarbonate concentrations were significantly increased with sodium citrate ingestion compared to placebo. Moreover, maximal lactate concentrations were significantly higher in the caffeine and the combination treatment compared to placebo. The supplementation with sodium citrate and/or caffeine did not provide an ergogenic effect on the 1500 m exercise performance in wheelchair elite athletes [13468].

### Combination with carbohydrates

Carbohydrate (CHO) and caffeine (CAF) both improve endurance performance. To determine by systematic literature review coupled with meta-analysis whether CAF ingested with CHO (CHO+CAF) improves endurance performance more than CHO alone databases were searched using the keywords caffeine, endurance, exercise, carbohydrate, and performance. Criteria for inclusion were studies that used human subjects performing an endurance-exercise performance task and included both a CHO and CHO+CAF condition. Effect sizes were calculated as the standardized mean difference. Twenty-one studies met the criteria for analysis. Effect sizes for individual studies ranged from -0.08 (trivial effect favoring CHO) to 1.01 (large effect favoring CHO+CAF). The overall ES equaled 0.26 (95% confidence interval 0.15 to 0.38), indicating that CHO+CAF provides a small but significant performance benefit over CHO. Effect size was not significantly related to CAF dose, exercise duration, or performance-assessment method. To determine whether effect size of CHO+CAF versus CHO was different than CAF compared with water (placebo), a subgroup meta-analysis compared 36 CAF versus placebo studies against the 21 CHO+CAF versus CHO studies. The overall effect sizes for the former group of studies was nearly 2-fold greater than in CHO+CAF versus CHO studies. CHO+CAF ingestion provides a significant but small effect to improve endurance performance compared with CHO alone. However, the magnitude of the performance benefit that CAF provides is less when added to CHO than when added to placebo [11204].
The aim of this study was to evaluate the effect of co-ingesting carbohydrate and caffeine (CHO+CAF) in comparison to carbohydrate (CHO) and placebo (PLA), during a reliable soccer-specific test. Eight university-standard soccer players ingested a PLA, a 6.4 percent CHO or 6.4 percent CHO and 160 mg CAF (CHO+CAF) solution on three occasions, in a double-blind randomized cross-over design, with each trial separated by 7 days. The protocol was 90 min in duration, made up of ten 6 min exercise blocks, each followed by soccer-specific skills tests (agility, dribbling, heading and kicking accuracy). Dependant variables (agility, dribbling, heading, kicking accuracy, glucose, lactate, HR and RPE) were analysed using one-way repeated measures ANOVA. Significant difference was found between CHO+CAF, CHO and PLA for each of the soccer-specific skill tests. Significant improvement was observed in agility time in CHO versus PLA trials, although no significant difference was reported for dribbling, heading and kicking accuracy. Blood glucose and lactate were significantly elevated with CHO+CAF supplementation over PLA, but there was no difference compared to CHO. Blood glucose increased significantly in the CHO trial compared to PLA, with no difference between CHO+CAF and CHO. No significant difference was reported for HR and RPE values across all trial conditions. Skill performance during simulated soccer activity improved with CHO+CAF supplementation in comparison to both CHO and PLA. CHO+CAF co-ingestion had no ergogenic benefit over CHO in the maintenance and availability of blood glucose however, CHO+CAF co-ingestion did allow players to sustain a higher work intensity as opposed to CHO and PLA beverages as shown by elevated blood lactate levels [11487].

The aim of one study was to test the hypothesis that adding caffeine to postexercise carbohydrate (CHO) feedings improves subsequent high-intensity interval-running capacity compared with CHO alone. In a repeated-measures design, 6 men performed a glycogen-depleting exercise protocol until volitional exhaustion in the morning. Immediately after and at 1, 2, and 3 hr postexercise, participants consumed 1.2 g/kg body mass CHO of a 15 percent CHO solution, a similar CHO solution but with addition of 8 mg/kg body mass of caffeine (CHO+CAFF), or an equivalent volume of flavored water only (WAT). After the 4-hr recovery period, participants performed the Loughborough Intermittent Shuttle Test (LIST) to volitional exhaustion as a measure of high-intensity interval-running capacity. Average blood glucose values during the 4-hr recovery period were higher in the CHO conditions than in the WAT trial (4.6 ± 0.3 mmol/L), although there was no difference between CHO (6.2 ± 0.8 mmol/L) and CHO+CAFF (6.7 ± 1.0 mmol/L). Exercise capacity during the LIST was significantly longer in the CHO+CAFF trial (48 ± 15 min) than in the CHO (32 ± 15 min) and WAT conditions (19 ± 6 min). All 6 participants improved performance in CHO+CAFF compared with CHO. The study provides novel data by demonstrating that adding caffeine to postexercise CHO feeding improves subsequent high-intensity interval-running capacity, a finding that may be related to higher rates of postexercise muscle glycogen resynthesis previously observed under similar feeding conditions [11488].

The aim of one study was to test the hypothesis that adding caffeine to postexercise carbohydrate (CHO) feedings improves subsequent high-intensity interval-running capacity compared with CHO alone. In a repeated-measures design, 6 men performed a glycogen-depleting exercise protocol until volitional exhaustion in the morning. Immediately after and at 1, 2, and 3 hr postexercise, participants consumed 1.2 g/kg body mass CHO of a 15 percent CHO solution, a similar CHO solution but with addition of 8 mg/kg body mass of caffeine (CHO+CAFF), or an equivalent volume of flavored water only (WAT). After the 4-hr recovery period, participants performed the Loughborough Intermittent Shuttle Test to volitional exhaustion as a measure of high-intensity interval-running capacity. Average blood glucose values during the 4-hr recovery period were higher in the CHO conditions than in the WAT trial (4.6 ± 0.3 mmol/L), although there was no difference between CHO (6.2 ± 0.8 mmol/L) and CHO+CAFF (6.7 ± 1.0 mmol/L). Exercise capacity during the study was significantly
longer in the CHO+CAFF trial (48 ± 15 min) than in the CHO (32 ± 15 min) and WAT conditions (19 ± 6 min). All 6 participants improved performance in CHO+CAFF compared with CHO. The study provides novel data by demonstrating that adding caffeine to postexercise CHO feeding improves subsequent high-intensity interval-running capacity, a finding that may be related to higher rates of postexercise muscle glycogen resynthesis previously observed under similar feeding conditions [13348].

The importance of endogenous carbohydrate (CHO) availability for high-intensity exercise performance has been well described in the literature. Several studies have shown that performance during high-intensity exercise is impaired when endogenous CHO availability (i.e. muscle and liver glycogen stores) is reduced. For example, it was reported that after three days of a low-CHO diet (about 5 % CHO) the average power output measured during a 30 s Wingate test in healthy men not engaged in any competitive sport was significantly reduced (from 581 ± 7 to 533 ± 7 W) when compared with a normal diet (about 50 % CHO). According to these authors, the reduction in performance (9 %) with low CHO availability was due to a lower contribution of the anaerobic energy system. Similarly, it was found a reduction in the anaerobic work capacity of healthy, non-athletic men when exercise was performed after a muscle-glycogen-depletion protocol compared to a control condition (10.33 ±2. 41 vs 12.83 ± 2.21 kJ, respectively), suggesting that low CHO availability can reduce the anaerobic contribution to total energy expenditure during high-intensity exercise. In addition, reduction in self-selected power output during high-intensity interval training when performed with low endogenous CHO availability has been also reported in well-trained subjects, and it may be associated with a reduction in the anaerobic contribution. While low CHO availability seems to reduce the anaerobic contribution and impair performance during high-intensity exercise, acute ingestion of caffeine seems to have the opposite effect. Taken together, several studies suggest that acute ingestion of caffeine may improve performance during high-intensity exercise via an increase in the anaerobic contribution. The purpose one study was therefore to examine the effects of caffeine ingestion on performance and energy expenditure (anaerobic and aerobic contribution) during a 4 km cycling time trial (TT) performed after a carbohydrate (CHO) availability-lowering exercise protocol. After preliminary and familiarization trials, seven amateur cyclists performed three 4-km cycling TT in a double-blind, randomized and crossover design. The trials were performed either after no previous exercise (CON), or after a CHO availability-lowering exercise protocol (DEP) performed in the previous evening, followed by either placebo (DEP-PLA) or 5 mg/kg of caffeine intake (DEP-CAF) 1 hour before the trial. Performance was reduced (-2.1 %) in DEP-PLA versus CON. However, performance was restored in DEP-CAF (404.6±17.1 s) compared with DEP-PLA, while no differences were found between DEP-CAF and CON. The anaerobic contribution was increased in DEP-CAF compared with both DEP-PLA and CON, and this was more pronounced in the first 3 km of the trial. Similarly, total anaerobic work was higher in DEP-CAF than in the other conditions. The integrated electromyographic activity, plasma lactate concentration, oxygen uptake, aerobic contribution and total aerobic work were not different between the conditions. The reduction in performance associated with low CHO availability is reversed with caffeine ingestion due to a higher anaerobic contribution, suggesting that caffeine could access an anaerobic "reserve" that is not used under normal conditions [13459].

The mechanisms by which caffeine increases the anaerobic contribution and performance during high-intensity exercise is not fully understood, but it has been proposed that caffeine intake would promote an inhibitory action on adenosine receptors, which would increase the activity of the enzyme phosphofructokinase, thereby increasing anaerobic glycolysis. Alternatively, caffeine may act on the central nervous system leading to an increase in motivational drive and neuromuscular excitability, which, in turn, results in a lowered rating of perceived exertion (RPE) for a given workload and improved neuromuscular function, as
measured via electromyography activity (EMG). In addition, it has also been suggested that caffeine attenuates muscle sensory signals to the brain and decreases the threshold of activation of motor neurons. All of these central alterations could lead to an ability to produce more work anaerobically [13459].

Although several studies have investigated the isolated effects of both CHO availability and caffeine intake on anaerobic contribution and performance, no study has examined whether acute caffeine ingestion could counteract the negative effects of low CHO availability on both the anaerobic contribution and performance. This seems to be particularly important since many athletes perform two training sessions in the same day, or participate in multi-stage event races (e.g. tour de France), where the time to replenish endogenous CHO stores between sessions or races may not be sufficient. Furthermore, most studies with either caffeine or CHO availability have focused on investigating their effects during time-to-exhaustion tests. However, time trials (TT) appear to be more reliable and to have greater external validity compared to constant-workload tests until exhaustion. Furthermore, during a high-intensity TT, where athletes are free to vary power output, anaerobic metabolism seems to exert a decisive effect on both performance and the distribution of work [13459].

**Caffeine at low muscle glycogen availability**

Commencing selected workouts with low muscle glycogen availability augments several markers of training adaptation compared with undertaking the same sessions with normal glycogen content. However, low glycogen availability reduces the capacity to perform high-intensity (>85 % of peak aerobic power, VO$_2$ peak) endurance exercise. We determined whether a low dose of caffeine could partially rescue the reduction in maximal self-selected power output observed when individuals commenced high-intensity interval training with low (LOW) compared with normal (NORM) glycogen availability. Twelve endurance-trained cyclists/triathletes performed four experimental trials using a double-blind Latin square design. Muscle glycogen content was manipulated via exercise-diet interventions so that two experimental trials were commenced with LOW and two with NORM muscle glycogen availability. Sixty minutes before an experimental trial, subjects ingested a capsule containing anhydrous caffeine (CAFF, 3 mg/kg body mass) or placebo (PLBO). Instantaneous power output was measured throughout high-intensity interval training (8 × 5 min bouts at maximum self-selected intensity with 1min recovery). There were significant main effects for both preexercise glycogen content and caffeine ingestion on power output. LOW reduced power output by approximately 8 percent compared with NORM, whereas caffeine increased power output by 2.8 and 3.5 percent for NORM and LOW, respectively. It was concluded that caffeine enhanced power output independently of muscle glycogen concentration but could not fully restore power output to levels commensurate with that when subjects commenced exercise with normal glycogen availability. However, the reported increase in power output does provide a likely performance benefit and may provide a means to further enhance the already augmented training response observed when selected sessions are commenced with reduced muscle glycogen availability [13460].

**Caffeinated mouth-rinse**

The purpose of one study was to investigate if acute caffeine exposure via mouth-rinse improved endurance cycling time-trial performance in well-trained cyclists. It was hypothesised that caffeine exposure at the mouth would enhance endurance cycling time-trial performance. Ten well-trained male cyclists completed two experimental time-trials following 24h of dietary and exercise standardisation. A randomised, double-blind, placebo-controlled, cross-over design was employed whereby cyclists completed a time-trial in the
fastest time possible, which was equivalent work to cycling at 75 percent of peak aerobic power output for 60min. Cyclists were administered 25 mL mouth-rinses for 10 s containing either placebo or 35 mg of anhydrous caffeine eight times throughout the time-trial. Perceptual and physiological variables were recorded throughout. No significant improvement in time-trial performance was observed with caffeine compared to placebo mouth-rinse. No elevation in plasma caffeine was detected due to the mouth-rinse conditions. Caffeine mouth-rinse had no significant effect on rating of perceived exertion, heart rate, rate of oxygen consumption or blood lactate concentration. Eight exposures of a 35mg dose of caffeine at the buccal cavity for 10s does not significantly enhance endurance cycling time-trial performance, nor does it elevate plasma caffeine concentration [13461].

Effect on individuals with negative energy balance

The ingestion of carbohydrate (+CHO) and caffeine (+CAF) during exercise is a commonly used ergogenic practice. Investigations are typically conducted with subjects who are in a rested state after an overnight fast. However, this state of positive energy balance is not achieved during many work and exercise circumstances. The aim of one study was to evaluate the substrate use and performance effects of caffeine and carbohydrate consumed alone and in combination while participants were in negative energy balance. Male participants (n=9) completed 4 trials in random order: -CAF/-CHO, -CAF/+CHO, +CAF/-CHO, and +CAF/+CHO. Diet and exercise were prescribed for 2 days before each trial to ensure negative energy balance. For each trial, before and after 2 h of cycling at 50 percent of maximal watts, a saliva sample and a muscle biopsy (vastus lateralis) were obtained. A simulated 20 km time trial was then performed. The respiratory exchange ratio was higher in +CHO trials and lower in the +CAF/+CHO trial than in the -CAF/+CHO trial. Salivary cortisol response was significantly higher in the +CAF/-CHO trial than in any of the other trials. Muscle glycogen and heart rates were similar in all trials. Performance in the 20 km time trial was significantly better in the -CAF/+CHO trial than in the -CAF/-CHO trial, but the +CAF/+CHO trial was no better than the +CAF/-CHO trial or any of the other trials. When co-ingested with carbohydrate, caffeine increased fat use and decreased nonmuscle glycogen carbohydrate use over carbohydrate alone when participants are in negative energy balance; however, caffeine had no effect on the 20 km cycling time trial performance [09254].

Combination with epigallocatechin

Different outcomes of the effect of catechin-caffeine mixtures and caffeine-only supplementation on energy expenditure and fat oxidation have been reported in short-term studies. Therefore, a meta-analysis was conducted to elucidate whether catechin-caffeine mixtures and caffeine-only supplementation indeed increase thermogenesis and fat oxidation. First, English-language studies measuring daily energy expenditure and fat oxidation by means of respiration chambers after catechin-caffeine mixtures and caffeine-only supplementation were identified through PubMed. Six articles encompassing a total of 18 different conditions fitted the inclusion criteria. Second, results were aggregated using random/mixed-effects models and expressed in terms of the mean difference in 24 h energy expenditure and fat oxidation between the treatment and placebo conditions. Finally, the influence of moderators such as BMI and dosage on the results was examined as well. The catechin-caffeine mixtures and caffeine-only supplementation increased energy expenditure significantly over 24 h (4.7 % and 4.8 %, respectively). However, 24 h fat oxidation was only increased by catechin-caffeine mixtures. A dose-response effect on 24 h energy expenditure and fat oxidation occurred with a mean increase of 0.53 kJ/mg and 0.02 g/mg for catechin-caffeine mixtures and 0.44 kJ/mg and 0.01 g/mg(-1) for caffeine-only. In conclusion,
catechin-caffeine mixtures or a caffeine-only supplementation stimulates daily energy expenditure dose-dependently by 0.4-0.5 kJ/mg administered. Compared with placebo, daily fat-oxidation was only significantly increased after catechin-caffeine mixtures ingestion [13349].

The aim of one study was to evaluate the combined effects of a 10-week exercise program with ingestion of caffeine and epigallocatechin-3-gallate (EGCG) on body composition, cardiovascular fitness, and strength in overweight and obese women. In a double-blind, placebo-controlled approach, overweight and obese women (n=27) were randomly assigned to treatment groups with exercise (an active-supplementing group with exercise (EX-Act) and a placebo group with exercise (EX-PL)) or without exercise (an active-supplementing group without exercise (NEX-Act) and a placebo group without exercise (NEX-PL)). All participants consumed 1 drink per day for 10 weeks; EX-Act and EX-PL participated in a concurrent endurance and resistance training program. Changes in body composition were assessed using a 4-compartment model. Changes in muscle mass (MM) were evaluated using a DXA-derived appendicular lean-soft tissue equation. There was a significant time × treatment interaction for MM and total cholesterol (TC), and a significant time × training interaction for peak oxygen consumption and upper-body and lower-body strength. Significant differences between the EX groups and NEX groups for percentage change in MM and peak oxygen consumption, and upper-body and lower-body strength, were revealed. Clinical markers for hepatic and renal function revealed no adverse effects. TC significantly decreased for the active-supplementing groups (EX-Act, NEX-Act). The current study suggests that implementing a caffeine-EGCG-containing drink prior to exercise may improve MM, fitness, and lipid profiles in overweight women [10502].

Combination with taurine

Consumption of energy drinks is common among athletes; however, there is a lack of research on the efficacy of these beverages for short-duration, intense exercise. The purpose of one research was to investigate the acute effects of a low-calorie caffeine-taurine energy drink (AdvoCare Spark) on repeated sprint performance and anaerobic power in National Collegiate Athletic Association Division I football players. Twenty football players (age 20) participated in a double-blind, randomized crossover study in which they received the energy drink or an isoenergetic, isovolumetric, non-caffeinated placebo in 2 trials separated by 7 days. The Running Based Anaerobic Sprint Test, consisting of six 35-m sprints with 10 s of rest between sprints, was used to assess anaerobic power. Sprint times were recorded with an automatic electronic timer. The beverage treatment did not significantly affect power or sprint time. However, there was a significant interaction effect between caffeine use and the beverage for sprint times as well as for anaerobic power, indicating a confounding effect. In conclusion, a caffeine-taurine energy drink did not improve the sprint performance or anaerobic power of college football players, but the level of caffeine use by the athletes likely influenced the effect of the drink [12283].

Combination with ecstasy

Concomitant consumption of caffeine with recreational psychostimulant drugs of abuse can provoke severe acute adverse reactions in addition to longer term consequences. The mechanisms by which caffeine increases the toxicity of psychostimulants include changes in body temperature regulation, cardiotoxicity and lowering of the seizure threshold. Caffeine also influences the stimulatory, discriminative and reinforcing effects of psychostimulant...
drugs. In this review, we consider our current understanding of such caffeine-related drug interactions, placing a particular emphasis on an adverse interaction between caffeine and the substituted amphetamine, 3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”), which has been most recently described and characterized. Co-administration of caffeine profoundly enhances the acute toxicity of MDMA in rats, as manifested by high core body temperature, tachycardia and increased mortality. In addition, co-administration of caffeine enhances the long-term serotonergic neurotoxicity induced by MDMA. Observations to date support an interactive model of drug-induced toxicity comprising MDMA-related enhancement of dopamine release coupled to a caffeine-mediated antagonism of adenosine receptors in addition to inhibition of PDE. These experiments are reviewed together with reports of caffeine-related drug interactions with cocaine, d-amphetamine and ephedrine where similar mechanisms are implicated. Understanding the underlying mechanisms will guide appropriate intervention strategies for the management of severe reactions and potential for increased drug-related toxicity, resulting from concomitant caffeine consumption [12284].

Caffeine versus theobromine

The combination of theobromine and caffeine, methylxanthines found in chocolate, has previously been shown to improve mood and cognition. However, it is unknown whether these molecules act synergistically. This study tested the hypothesis that a combination of caffeine and theobromine has synergistic effects on cognition, mood and blood pressure in 24 healthy female subjects. The effects of theobromine (700 mg), caffeine (120 mg) or the combination of both, or placebo were tested on mood (the Bond-Lader visual analog scale), psychomotor performance (the Digit Symbol Substitution Test) and blood pressure before and at 1, 2 and 3 h after administration. Theobromine alone decreased self-reported calmness 3h after ingestion and lowered blood pressure relative to placebo 1 h after ingestion. Caffeine increased self-reported alertness 1, 2 and 3h after ingestion and contentedness 1 and 2 h after ingestion, and increased blood pressure relative to placebo (at 1 h). The combination of caffeine+theobromine had similar effects as caffeine alone on mood, but with no effect on blood pressure. There was no treatment effect on performance. Together these results suggest that theobromine and caffeine could have differential effects on mood and blood pressure. It was tentatively concluded that caffeine may have more CNS-mediated effects on alertness, while theobromine may be acting primarily via peripheral physiological changes [11494].

Caffeine in asthma

The main aim of one study was to evaluate the comparative and additive effects of caffeine and albuterol (short-acting beta2-agonist) on the severity of EIB (exercise-induced bronchoconstriction). Ten asthmatic subjects with EIB participated in a randomized, double-blind, double-dummy crossover study. One hour before an exercise challenge, each subject was given 0, 3, 6, or 9 mg/kg of caffeine or placebo mixed in a flavored sugar drink. Fifteen minutes before the exercise bout, an inhaler containing either albuterol (180 microg) or placebo was administered to each subject. Pulmonary function tests were conducted pre- and post-exercise. Caffeine at a dose of 6 and 9 mg/kg significantly reduced the mean maximum percentage fall in post-exercise FEV₁ compared to the double-placebo and baseline. There was no significant difference in the post-exercise % fall in FEV₁ between albuterol (plus caffeine) and the 9 mg/kg dose of caffeine. Interestingly, there was no significant difference in the post-exercise percentage fall in FEV₁ between albuterol (plus
caffeine) and albuterol with 3, 6 or 9 mg/kg of caffeine. Similar changes were observed for the post-exercise percentage fall in FVC, FEF (25-75 %) and PEF. These data indicate that moderate (6 mg/kg) to high doses (9 mg/kg) of caffeine provide a significant protective effect against EIB. It is feasible that the negative effects of daily use of short-acting beta2-agonists by asthmatic athletes could be reduced simply by increasing caffeine consumption prior to exercise [10183].

Influence on circadian rhythms

Although caffeine alters sleep in many animals, whether or not it affects mammalian circadian clocks remains unknown. It was found that incubating cultured mammalian cell lines, human osteosarcoma U2OS cells and mouse fibroblast NIH3T3 cells, with caffeine lengthened the period of circadian rhythms. Adding caffeine to ex vivo cultures also lengthened the circadian period in mouse liver explants from Per2::Luciferase reporter gene knockin mice, and caused a phase delay in brain slices containing the suprachiasmatic nucleus, where the central circadian clock in mammals is located. Furthermore, chronic caffeine consumption ad libitum for a week delayed the phase of the mouse liver clock in vivo under 12 h light-dark conditions and lengthened the period of circadian locomotor rhythms in mice under constant darkness. The results showed that caffeine alters circadian clocks in mammalian cells in vitro and in the mouse ex vivo and in vivo [13342].

Influence on biomonitoring data

Smoking appears to enhance the body's clearance of dioxins and dioxin-like polychlorinated biphenyls (PCB) by inducing CYP1A2 activity based on studies with a limited number of participants. This hypothesis was evaluated by using data from National Health and Nutrition Examination Survey. Specifically, adult participants were identified and the sums of their serum lipid-adjusted concentrations of 12 polychlorinated dibenzo-p-dioxins/polychlorinated dibenzofurans (PCDD/PCDF) congeners, 33 PCB (total), 26 non-dioxin-like PCB, and 6 mono-ortho (dioxin-like) PCB were determined. In addition to evaluating the association of smoking, the association of caffeine consumption and the interaction between them was evaluated. Data analysis included regression models that were fitted with age, gender, race/ethnicity, and body mass index (BMI). Smokers had significantly lower concentrations of total PCDD/PCDF than nonsmokers. New to this study, a significant interaction between caffeine consumption and smoking for total PCB was found. When caffeine was consumed less than once a day, smokers had higher concentrations of total PCB than nonsmokers. However, when caffeine was consumed at least once a day, smokers had lower concentrations than nonsmokers. A significant interaction between age and caffeine consumption frequency for each of the PCB groups was also observed. The differences in concentration between younger and older age groups were greater when caffeine was consumed at least once a day than when caffeine was consumed less frequently. Smoking and caffeine consumption need to be considered in the interpretation of human biomonitoring data because they appear to affect the serum concentrations of these chemicals [13345].

Impact on pain perception

Caffeine has been shown to reduce leg-muscle pain during submaximal cycle ergometry, as well as in response to eccentric exercise. However, less is known about its analgesic properties during non-steady-state, high-intensity exercise. The primary aim of one study
was to examine the effect of 2 doses of caffeine on leg pain and rating of perceived exertion (RPE) during repeated bouts of high-intensity exercise. Fifteen active men (age 26 ± 4 year) completed 2 bouts of 40 repetitions of "all-out" knee extension and flexion of the dominant leg at a contraction velocity equal to 180°/s. Before each trial, subjects abstained from caffeine intake and intense exercise for 48 hr. Over 3 days separated by 48 hr, subjects ingested 1 of 3 treatments (5 mg/kg or 2 mg/kg of anhydrous caffeine or placebo) in a randomized, single-blind, counterbalanced, crossover design. Leg-muscle pain and RPE were assessed during and after exercise using established categorical scales. Across all treatments, pain perception was significantly increased during exercise, as well as from bout 1 to 2, yet there was no effect of caffeine on pain perception or RPE. Various measures of muscle function were improved with a 5-mg/kg caffeine dose versus the other treatments. In the 5-mg/kg trial, it is plausible that subjects were able to perform better with similar levels of pain perception and exertion [11203].

This experiment examined the effect of a moderate dose of caffeine on quadriceps muscle pain during a bout of high-intensity cycling in low- versus high-caffeine-consuming males. College-age men who were low (< 100 mg/day; n=12) or high (> 400 mg/day; n=13) habitual caffeine consumers ingested caffeine (5 mg/kg body weight) or a placebo in a counterbalanced order and 1 hr later completed 30 min of cycle ergometry at 75-77 percent of peak oxygen consumption. Perceptions of quadriceps muscle pain, as well as oxygen consumption, heart rate, and work rate, were recorded during both bouts of exercise. Caffeine ingestion resulted in a statistically significant and moderate reduction in quadriceps muscle-pain-intensity ratings during the 30-min bout of high-intensity cycle ergometry compared with placebo ingestion in both low and high caffeine consumers. The results suggest that caffeine ingestion is associated with a moderate hypoalgesic effect during high-intensity cycling in college-age men who are low or high habitual caffeine consumers, but future work should consider better defining and differentiating pain and effort when examining the effects of caffeine during acute exercise [09256].

Impact on testosterone levels

Interest in the use of caffeine as an ergogenic aid has increased since the International Olympic Committee lifted the partial ban on its use. Caffeine has beneficial effects on various aspects of athletic performance, but its effects on training have been neglected. To investigate the acute effect of caffeine on the exercise-associated increases in testosterone and cortisol in a double-blind crossover study 24 professional rugby-league players ingested caffeine doses of 0, 200, 400, and 800 mg in random order 1 hr before a resistance-exercise session. Saliva was sampled at the time of caffeine ingestion, at 15-min intervals throughout each session, and 15 and 30 min after the session. Data were log-transformed to estimate percent effects with mixed modeling, and effects were standardized to assess magnitudes. Testosterone concentration showed a small increase of 15 percent (90 % confidence limits ± 19 %) during exercise. Caffeine raised this concentration in a dose-dependent manner by a further small 21 percent (± 24 %) at the highest dose. The 800-mg dose also produced a moderate 52 percent (± 44 %) increase in cortisol. The effect of caffeine on the testosterone:cortisol ratio was a small decline (14 %; ± 21 %). It was concluded that caffeine has some potential to benefit training outcomes via the anabolic effects of the increase in testosterone concentration, but this benefit might be counteracted by the opposing catabolic effects of the increase in cortisol and resultant decline in the testosterone:cortisol ratio [08306].

Impact in hot environments
Caffeine is regarded as a diuretic despite evidence that hydration is not impaired with habitual ingestion. The purpose of one study was to determine whether a caffeinated sports drink impairs fluid delivery and hydration during exercise in warm, humid conditions (29 degrees C, 60 % relative humidity). Sixteen cyclists completed 3 trials: placebo (P), carbohydrate-electrolyte (CE), and caffeinated (195 mg/L) sports drink (CAF+CE). Subjects cycled for 120 min at 60-75 percent VO2max followed by 15 min of maximal-effort cycling. Heart rate and rectal temperature were similar until the final 15 min, when these responses and exercise intensity were higher with CAF+CE than with CE and P. Sweat rate, urine output, plasma-volume losses, serum electrolytes, and blood deuterium-oxide accumulation were not different. Serum osmolality was higher with CAF+CE vs. P but not CE. The authors conclude that CAF+CE appears as rapidly in blood as CE and maintains hydration and sustains cardiovascular and thermoregulatory function as well as CE during exercise in a warm, humid environment [07173].

To investigate the effects of caffeine ingestion on thermoregulation and fluid-electrolyte losses during prolonged exercise in the heat seven endurance-trained (VO2max 61 ± 8 mL/kg.min) heat-acclimated cyclists pedaled for 120 min at 63 percent VO2max in a hot-dry environment (36 degrees C; 29 % humidity) on six occasions: 1) without rehydration (NF); 2) rehydrating 97% of sweat losses with water (WAT); 3) rehydrating the same volume with a 6% carbohydrate-electrolytes solution (CES); or combining these treatments with the ingestion of 6 mg caffeine/kg body weight 45 min before exercise, that is, 4) CAFF + NF; 5) CAFF + WAT; and 6) CAFF + CES. Without fluid replacement (NF and CAFF + NF), final rectal temperature reached 39.4 ± 0.1 degrees C, whereas it remained at 38.7 ± 0.1 degrees C during WAT (CES and CAFF + WAT. Caffeine did not alter heat production, forearm skin blood flow, or sweat rate. However, CAFF + carbohydrate-electrolytes solution tended to elevate rectal temperature above CES alone (38.9 ± 0.1 degrees C vs 38.6 ± 0.1 degrees C). Caffeine ingestion increased sweat losses of sodium, chloride, and potassium (approximately 14 %) and enlarged significantly urine flow (28 %). The authors concluded that caffeine ingested alone or in combination with water or a sports drink was not thermogenic or impaired heat dissipation. Caffeine increased urine flow and sweat electrolyte excretion, but these effects are not enough to affect dehydration or blood electrolyte levels when exercising for 120 min in a hot environment [08313].

**Impact on immunology**

One study investigated the effect of a high and low dose of caffeine on antigen-stimulated natural killer (NK) cell (CD3- CD56+) activation after prolonged, strenuous cycling, as assessed by the early-activation molecule CD69. In a randomized crossover design, 12 healthy male endurance-trained cyclists cycled for 90 min at 70 percent VO2peak 60 min after ingesting either 0 (PLA), 2 (2CAF), or 6 (6CAF) mg/kg body mass of caffeine. Whole blood was stimulated with Pediacel (5 in 1) vaccine. A high dose of caffeine (6CAF) significantly increased the number of CD3-CD56+ cells in the circulation immediately postexercise compared with PLA. For both 2CAF and 6CAF, the geometric mean fluorescence intensity (GMFI) of CD69+ expression on unstimulated CD3-CD56+ cells was significantly higher than with PLA. When cells were stimulated with antigen, the GMFI of CD69 expression remained significantly higher with 2CAF than with PLA 1 hr postexercise. Although not achieving statistical significance, 6CAF also followed a similar trend when stimulated. There were no differences in GMFI of CD69 expression between 2CAF and 6CAF. These results suggest that a high (6 mg/kg) dose of caffeine was associated with the recruitment of NK cells into the circulation and that both a high and low (2 mg/kg) dose of caffeine increased unstimulated and antigen-stimulated NK-cell activation 1 hr after high-intensity exercise.
Furthermore, there does not appear to be a dose-dependent effect of caffeine on NK-cell activation 1 hr after prolonged intensive cycling [11199].

Several studies investigating the effect of caffeine on immune function following exercise have used one large bolus dose of caffeine. However, this does not model typical caffeine consumption. Therefore, the purpose of one study was to investigate whether small repeated doses of caffeine ingested throughout the day would elicit a similar response as one large bolus dose ingested 1 h prior to exercise on antigen-stimulated NK cell CD69 expression following strenuous intermittent exercise. In a randomized cross-over design, 15 healthy males completed six 15 min blocks of intermittent running consisting of maximal sprinting interspersed with less intense running and walking. Participants had ingested either 0 (PLA), 2 mg/kg body mass (BM) caffeine on three separate occasions during the day (3 × CAF) or one dose of 6 (1 × CAF) mg/kg BM caffeine, 1 h before exercise. At 1-h post-exercise, the number of antigen-stimulated CD3(-)CD56(+) cells expressing CD69 was lower on 1 × CAF compared with PLA, with values on 1 × CAF at this time point remaining close to pre-supplement. 1 × CAF tended to attenuate the exercise-induced increase in geometric mean fluorescence intensity of CD69 expression on antigen-stimulated CD3(-)CD56(+) cells 1-h post-exercise. These findings suggest that although one large bolus dose of caffeine attenuated the exercise-induced increase in antigen-stimulated NK cell CD69 expression 1 h following strenuous intermittent exercise, this attenuation at no point fell below pre-supplement values and caffeine does not appear to depress NK cell CD69 expression [13347].

Immunoendocrine effects

One study investigated the effect of caffeine consumed with and without carbohydrate (CHO) on immunoendocrine responses after exercise. On four occasions, 12 recreational male cyclists cycled for 2 h at 65 percent VO$_{2\text{max}}$. Sixty minutes before exercise, participants ingested 6 mg.kg(-1) body mass of caffeine (CAF) or placebo (PLA), then during exercise they consumed a 6 percent CHO or placebo (PLA) drink, providing CAF/CHO, PLA/CHO, CAF/PLA, and PLA/PLA conditions. f-MLP-stimulated neutrophil oxidative burst responses were significantly higher after exercise on CAF/CHO and PLA/CHO than PLA/PLA when expressed as a percentage of baseline value. The response on CAF/PLA tended to be higher than PLA/PLA at this point. No significant differences between CAF/CHO, PLA/CHO, and CAF/PLA were observed after exercise; however, only PLA/CHO showed no significant postexercise decline. Coingestion of CAF/CHO significantly attenuated epinephrine and IL-6 responses that occurred after ingestion of CAF alone (CAF/PLA) and significantly attenuated the transient alterations in circulating leukocyte and neutrophil counts. Plasma cortisol concentration was significantly lower on PLA/CHO than CAF/PLA and PLA/PLA after exercise. Perceived exertion during exercise was significantly lower on CAF/CHO than the other three trials. Taken together, this suggests that coingestion of caffeine and CHO has greater influence on immunoendocrine responses than neutrophil functional responses to prolonged exercise [07169].

Effect on hydration

Acute and chronic caffeine intakes have no impact on hydration status [Maughan RJ, Griffin J. J Hum Nutr Diet 2003; 16: 411-20], although no research has been conducted to analyze the effects using dilution techniques on total-body water (TBW) and its compartments. Therefore, the aim of this study was to investigate the effects of a moderate dose of caffeine on TBW, extracellular water (ECW), and intracellular water (ICW) during a 4-day period in
active males. Thirty men, nonsmokers and low caffeine users (<100 mg/day), aged 20-39 years, participated in this double-blind, randomized, crossover trial. The study included 2 conditions (5 mg/kg/day of caffeine and placebo (malt-dextrin)) of 4 days each, with a 3-day washout period. TBW and ECW were assessed by deuterium oxide and sodium bromide dilution, respectively, whereas ICW was calculated as TBW minus ECW. Body composition was assessed by dual-energy X-ray absorptiometry. Physical activity was assessed by accelerometer and water intake was assessed by dietary records. Repeated-measures analysis of variance (ANOVA) was used to test main effects. No changes in TBW, ECW, or ICW and no interaction between the randomly assigned order of treatment and time were observed. TBW, ECW, and ICW were unrelated to fat-free mass, water ingestion, and PA. These findings indicate that a moderate caffeine dose, equivalent to approximately 5 espresso cups of coffee or 7 servings of tea, does not alter TBW and fluid distribution in healthy men, regardless of body composition, PA, or daily water ingestion [13451].

**Effect on urea formation**

It was investigated the effects of caffeine on the ammonia and amino acid metabolism of elite soccer players. In a double-blind randomized study, athletes (n=19) received 5 mg/kg caffeine or lactose (LEX, control) and performed 45 min of intermittent exercise followed by an intermittent recovery test (Yo-Yo IR2) until exhaustion. The caffeine-supplemented athletes were divided into two groups (CEx and SCEx) depending on their serum caffeine levels (<900 % and >10,000 %, respectively). Data were analyzed by ANOVA and Tukey post hoc test. Caffeine supplementation did not significantly affect the performance. Exercise changed the blood concentrations of several amino acids and increased the serum concentrations of ammonia, glucose, lactate, and insulin. The LEx group showed an exercise-induced increase in valine (29 %), which was inhibited by caffeine. Higher serum caffeine levels abolished the exercise-induced increase (24%-27 %) in glutamine but did not affect the exercise-induced increase in alanine (110 % - 160 %) and glutamate (42 % - 61 %). In response to exercise, the SCEx subjects did not exhibit an increase in urea and showed a significantly lower increase in their serum arginine (15 %), citrulline (16 %), and ornithine concentrations. The data suggest that caffeine might decrease systemic urea by decreasing the glutamine serum concentration, which decreases the transportation of ammonia to the liver and thus urea synthesis [13452].

**Caffeine with carbohydrates**

One study compared the effects of three carbohydrate-hydration strategies on blood glucose concentration, exercise performance and hydration status throughout simulated soccer match-play. A randomized, double-blind and cross-over study design was employed. After familiarization, 14 recreational soccer players completed the soccer match simulation on three separate occasions. Participants consumed equal volumes of 9.6 percent carbohydrate-cafeine-electrolyte (6 mg/kg BW caffeine) solution with carbohydrate-electrolyte gels (H-CHO), 5.6 percent carbohydrate-electrolyte solution with electrolyte gels (CHO) or electrolyte solution and electrolyte gels (PL). Blood samples were taken at rest, immediately before exercise and every 15 min during exercise (first half: 15, 30, 45 min; second half: 60, 75, 90 min). Supplementation influenced blood glucose concentration, however, none of the supplementation regimes were effective in preventing a drop in blood glucose at 60 min. Mean sprint speed was 3 ± 1 percent faster in H-CHO when compared with PL. Supplementation caused a 2.3 ± 0.5 percent increase in plasma osmolality in H-CHO without change in CHO or PL. Similarly, mean sodium concentrations were 2.1 ± 0.4
percent higher in H-CHO when compared with PL. It was concluded that combining high carbohydrate availability with caffeine resulted in improved sprint performance and elevated blood glucose concentrations throughout the first half and at 90 min of exercise; however, this supplementation strategy negatively influenced hydration status when compared with 5.6 percent carbohydrate-electrolyte and electrolyte solutions [13453].

Caffeine with phosphatidylserine

Phosphatidylserine (PS) may attenuate the adverse effects of physical fatigue. Therefore, it was investigated the effects of a multi-ingredient supplement containing 400 mg/d PS and 100 mg/d caffeine (supplement, SUP) for 2 weeks on measures of cognitive function (CF), reaction time (RT), and mood (MD) following an acute exercise stress. It is hypothesized that PS will maintain preexercise CF and RT scores, while attenuating postexercise fatigue. Participants completed 2 acute bouts of resistance exercise (T1 and T2) separated by 2-week ingestion of SUP or control (CON). Outcome measures were assessed pre- and postexercise. When collapsed across groups, a significant decrease in RT performance was seen in the 60-second reaction drill from pre- to postexercise at T1. All other RT tests were similar from pre- to postexercise at T1. Reaction time was not significantly changed by PS. When collapsed across groups, a significant increase in performance of the serial subtraction test was seen. A significant increase (8.9 % and 7.1 %) in the number of correct answers and a significant decrease (8.0 % and 7.5 %) in time to answer were seen from pre- to postworkout at T1 and T2, respectively. A significant increase in total MD score from pre- to postworkout was observed for CON but not for PS at T2. Phosphatidylserine significantly attenuated pre- to postexercise perception of fatigue compared to CON. Ingestion of SUP for 14 days appears to attenuate postexercise MD scores and perception of fatigue, but does not affect CF or RT, in recreationally trained individuals [13454].

Lack of effect on oxidative stress

Coffee has been reported to be rich in antioxidants, with both acute and chronic consumption leading to enhanced blood antioxidant capacity. High-fat feeding is known to result in excess production of reactive oxygen and nitrogen species, promoting a condition of postprandial oxidative stress. It was tested the hypothesis that coffee intake following a high-fat meal would attenuate the typical increase in blood oxidative stress during the acute postprandial period. On 3 different occasions, 16 men and women consumed a high-fat milk shake followed by either 16 ounces of caffeinated or decaffeinated coffee or bottled water. Blood samples were collected before and at 2 and 4 hours following intake of the milk shake and analyzed for triglycerides (TAG), malondialdehyde (MDA), hydrogen peroxide (H2O2), and Trolox equivalent antioxidant capacity (TEAC). Values for TAG and MDA, as well as for H2O2, increased significantly following milk shake consumption, with values higher at 4 hours compared with 2 hours post consumption for TAG and H2O2. TEAC was unaffected by the milk shake consumption. Coffee had no impact on TAG, MDA, H2O2, or TEAC, with no condition or interaction effects noted for any variable. It was concluded that acute coffee consumption following a high-fat milk shake has no impact on postprandial oxidative stress [13472].

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would attenuate the typical increase in blood oxidative stress during the acute postprandial period. On 3 different occasions, 16 men and women consumed a high-fat milk shake followed by either 16 ounces of caffeinated or decaffeinated coffee or bottled water. Blood samples were collected before and at 2 and 4 hours following intake of the milk shake and analyzed for triglycerides (TAG), malondialdehyde (MDA), hydrogen peroxide ($H_2O_2$), and Trolox equivalent antioxidant capacity (TEAC). Values for TAG and MDA, as well as for $H_2O_2$, increased significantly following milk shake consumption, with values higher at 4 hours compared with 2 hours post consumption for TAG and $H_2O_2$. TEAC was unaffected by the milk shake consumption. Coffee had no impact on TAG, MDA, $H_2O_2$, or TEAC, with no condition or interaction effects noted for any variable. Thus, acute coffee consumption following a high-fat milk shake has no impact on postprandial oxidative stress [13470].

**Modulation of oxidative stress markers in the liver of trained rats**

Caffeine has been widely used in sports competitions due to its ergogenic effects. Most of the studies regarding caffeine and exercise have focused on muscle and plasma adaptations, while the impact on the liver is scarcely described. The aim of one study was to analyze the effects of caffeine and exercise training on oxidative stress markers and injury-related parameters in the liver. Rats were divided into sedentary/saline, sedentary/caffeine, exercise/saline, and exercise/caffeine groups. Exercise groups underwent 4 weeks of swimming training, and caffeine (6 mg/kg, p.o.) was supplemented throughout the training protocol. Injury-related liver parameters were assessed in plasma, while redox status and oxidative stress markers were measured on liver homogenates. Exercise training increased muscle citrate synthase activity in the muscle, while in caffeine decreased its activity in both sedentary and trained rats. Aspartate transaminase levels were increased after training, and caffeine intake suppressed this elevation. Caffeine also diminished alanine transaminase levels in both sedentary and exercised rats. Exercise training induced a significant increase on the activity of the enzymes superoxide dismutase and glutathione peroxidase, as an increase on thiobarbituric acid-reactive substances levels was also reached; caffeine intake blunted these alterations. Caffeine intake also suppressed liver catalase activity in both sedentary and exercise groups. The data suggest that caffeine modified the hepatic responses associated to exercise-induced oxidative stress without affecting the performance, exerting different actions according to the tissue. However, further studies are needed to better understand caffeine’s role on liver under exercise training [13475].

**Impact on the inflammatory response**

The objective of the study was to determine the effects of caffeine supplementation on the inflammatory response (IL-6 and IL-10 levels and leukocyte numbers) induced by a 15-km run competition and to examine the effect of caffeine supplementation on the energetic metabolites as well as on the exercise-induced oxidative stress. A double-blinded study of supplementation with caffeine was performed. Athletes participating in the study (n=33) completed a 15 km run competition. Before competition, athletes took 6 mg/kg body weight of caffeine (caffeine group, n=17) or a placebo (placebo group, n=16). Blood samples were taken before and after competition (immediately and after 2-h recovery). Leukocyte numbers were determined in blood. Concentrations of oxidative stress markers, antioxidants, interleukins (IL-6 and IL-10), caffeine, adrenaline, and energetic metabolites were measured in plasma or serum. Caffeine supplementation induced higher increases in circulating total leukocytes and neutrophils, with significant differences between groups after recovery. Adrenaline, glucose, and lactate levels increased after exercise, with higher increases in the
caffeine group. Exercise induced significant increases in IL-6 and IL-10 plasma levels, with higher increases in the caffeine group. Caffeine supplementation induced higher increases in oxidative stress markers after the competition. In conclusion, caffeine supplementation induced higher levels of IL-6 and IL-10 in response to exercise, enhancing the anti-inflammatory response. The caffeine-induced increase in adrenaline could be responsible for the higher increase in IL-6 levels, as well as for the increased lactate levels. Furthermore, caffeine seems to enhance oxidative stress induced by exercise [13471].

**Impact on glycogen accumulation**

It was determined the effect of coinestion of caffeine with carbohydrate (CHO) on rates of muscle glycogen resynthesis during recovery from exhaustive exercise in seven trained subjects who completed two experimental trials in a randomized, double-blind crossover design. The evening before an experiment subjects performed intermittent exhaustive cycling and then consumed a low-CHO meal. The next morning subjects rode until volitional fatigue. On completion of this ride subjects consumed either CHO (4 g/kg body mass) or the same amount of CHO + caffeine (8 mg/kg) during 4 hours of passive recovery. Muscle biopsies and blood samples were taken at regular intervals throughout recovery. Muscle glycogen levels were similar at exhaustion and increased by a similar amount (approximately 80%) after 1 hour of recovery. After 4 hours of recovery caffeine resulted in higher glycogen accumulation. Accordingly, the overall rate of resynthesis for the 4-hour recovery period was 66 percent higher in caffeine compared with CHO. After 1 hour of recovery plasma caffeine levels had increased to $31 \pm 11$ microM, which was a significant difference, and at the end of the recovery reached $77 \pm 11$ microM with caffeine. Phosphorylation of CaMK(Thr286) was similar after exercise and after 1 hour of recovery, but after 4 hour CaMK(Thr286) phosphorylation was significantly higher in caffeine than CHO. Phosphorylation of AMP-activated protein kinase (AMPK)(Thr172) and Akt(Ser473) was similar for both treatments at all time points. It was provided the first evidence that in trained subjects coingestion of large amounts of caffeine (8 mg/kg) with CHO has an additive effect on rates of postexercise muscle glycogen accumulation compared with consumption of CHO alone [08314].

**Impact on ventilation**

The purpose of one project was to determine whether a moderate dosage of caffeine, a common ventilatory stimulant, could augment resting ventilatory responsiveness, exercise ventilation, end-tidal $O_2$ partial pressure ($P_{etO_2}$), and arterial oxyhemoglobin saturation ($HbSaO_2$) in athletes with exercise-induced hypoxemia. Eight highly trained males who demonstrated exercise-induced hypoxemia, ingested in a randomized design a placebo or caffeine (8 mg/kg body wt) 1 hour before testing. Ventilatory responsiveness at rest was assessed via the isocapnic hypoxic and hyperoxic hypercapnic ventilatory responses (HVR and HCVR, respectively). The failure of $HbSaO_2$ to increase at despite an increase in ventilation suggests that mechanisms influencing $HbSaO_2$ other than an inadequate hyperventilatory response may operate to different degrees across individuals as $VO_{2max}$ is approached [08315].

The effects of caffeine on exercise performance have been well documented, with most reviews focusing on the metabolic, hormonal, and/or central nervous system effects. However, caffeine's effects on ventilation and pulmonary function are often overlooked. Studies have shown that caffeine is a strong ventilatory stimulant, increasing the sensitivity of the peripheral chemoreceptors in untrained subjects and increasing exercise ventilation at all
workloads in highly trained endurance athletes. The consequences of increased exercise ventilation could hold either positive or negative effects for exercise performance. Anti-inflammatory and bronchoprotective effects of caffeine are great enough to consider its efficacy as a possible prophylactic antiasthma treatment. Although an upper urinary concentration limit exists for caffeine with international sports doping control agencies, caffeine's universal accessibility in the marketplace has resulted in its daily use being increasingly more socially acceptable as an ergogenic substance for sport and exercise [09250].

Impact on sweating

It was assessed the effect of caffeine on sudomotor activity and sweating sensitivity during physical loading. Both physiological responses could occur due to energy expenditure. Subjects were 13 athletically trained males (22 ± 4 years old, 174 ± 5 cm tall, and weighing 71 ± 5 kg, with maximal oxygen consumption (VO$_{2\text{max}}$) of 54 ± 4 mL/kg/minute). The study involved a within-subject, random, crossover design. Tests were performed following the ingestion of 3 mg/kg caffeine. The physical loading involved running for 30 minutes at 60 percent VO$_{2\text{max}}$ (24 ± 0.5°C, 40 ± 3 % relative humidity). Tympanic temperature (TYMP) was significantly higher in the caffeine-consuming group (Caffe-I) at pre-exercise (40 minutes after caffeine intake and immediately before running). Mean body temperature (mT$_b$) was significantly higher in the Caffe-I group at pre- and post-exercise (30 min after start of running). Onset time of localized sweating was significantly shorter in the Caffe-I group, but localized sweat volume and active sweat gland output (per single gland) was significantly higher in the Caffe-I group. Activated sweat gland density was significantly increased in the Caffe-I group on the abdomen and thigh. In conclusion, caffeine ingestion caused not only increases in TYMP and mT$_b$ through thermogenesis, but also an increased sweating sensitivity via changes in sudomotor activity [11492].

Impact on delayed-onset muscle soreness

One double-blind, placebo-controlled, repeated-measures experiment examined the effects of a 5 mg/kg body weight dose of caffeine on delayed-onset muscle pain intensity and force loss in response to 64 eccentric actions of the dominant quadriceps induced by electrical stimulation. Low caffeine-consuming college-aged females (n=9) ingested caffeine or placebo 24 and 48 hours following electrically stimulated eccentric exercise of the quadriceps. One hour after ingestion, maximal voluntary isometric contractions (MVIC) and submaximal voluntary eccentric actions were used to determine force loss during activation of damaged quadriceps and whether caffeine attenuates muscle pain intensity. Pain intensity was measured using a 0 to 100 visual analog scale. Caffeine produced a large (12.7 raw visual analog scale, VAS, units), statistically significant hypoalgesia during the MVIC. The reduction in pain scores during submaximal voluntary eccentric movements was smaller as was the increase in MVIC force. Thus, eccentric exercise occurs when skeletal muscles produce force while being lengthened. For example, the biceps brachii muscles act eccentrically when a cup of coffee is lowered from the mouth to a tabletop. This experiment found that caffeine (equal to approximately 2 cups of brewed coffee) could produce a large reduction in pain resulting from eccentric exercise-induced, delayed-onset muscle injury. This finding may improve the quality of life of individuals who experience skeletal muscle pain after engaging in unaccustomed, eccentrically biased exercise [06200].
Impact on postexercise oxygen consumption

This study investigated the effect of acute caffeine (CAF) intake on postexercise oxygen consumption (EPOC) after intense resistance training. Fourteen strength-trained men (mean ± SD age and mass =23 ± 4 years and 83 ± 13 kg, respectively) who were caffeine users initially completed one-repetition maximum testing (1-RM) of four exercises: bench press, leg press, lat row, and shoulder press. On each of two days separated by one week, they completed four sets of each exercise to fatigue at 70-80 percent 1-RM, which was preceded by ingestion of CAF (6 mg/kg) or placebo. Pre-exercise, indirect calorimetry was used to assess energy expenditure for 35 min; this was repeated for 75 min postexercise while subjects remained seated in a quiet lab. Results revealed that EPOC was significantly higher with CAF (27 ± 4. L) compared to placebo (23 ± 4 L). With CAF ingestion, oxygen uptake was significantly higher from 10 min pre-exercise to 70 min postexercise. Respiratory exchange ratio was significantly different with CAF versus placebo. Caffeine intake increased total energy expenditure by 15 percent, but the additional calories burned was minimal (+27 kcal). Caffeine ingestion in individuals regularly completing rigorous resistance training significantly increases EPOC and energy expenditure pre- and post-exercise, yet the magnitude of this effect is relatively small [11207].

Impact on potassium levels

There was one report of severe hypokalemia in two young bicycle riders due to massive caffeine intake [10184].

Impact on glutamine acid

It was investigated the effects of caffeine on the ammonia and amino acid metabolism of elite soccer players. In the double-blind, randomized study, the athletes (n=19) received 5 mg·kg caffeine or lactose (LEX, control) and performed 45 min of intermittent exercise followed by an intermittent recovery test (Yo-Yo IR2) until exhaustion. The caffeine-supplemented athletes were divided into two groups (CEx and SCEx) depending on their serum caffeine levels (< 900 % and > 10,000 %, respectively). The data were analyzed by ANOVA and Tukey's post hoc test. Caffeine supplementation did not significantly affect the performance. Exercise changed the blood concentrations of several amino acids and increased the serum concentrations of ammonia, glucose, lactate, and insulin. The LEx group showed an exercise-induced increase in valine (29 %), which was inhibited by caffeine. Higher serum caffeine levels abolished the exercise-induced increase (24-27 %) in glutamine but did not affect the exercise-induced increase in alanine (110-160 %) and glutamate (42-61 %). In response to exercise, the SCEx subjects did not exhibit an increase in uremia and showed a significantly lower increase in their serum arginine (15 %), citrulline (16 %), and ornithine (ND) concentrations. The data suggest that caffeine might decrease systemic urea by decreasing the glutamine serum concentration, which decreases the transportation of ammonia to the liver and thus urea synthesis [12285].

Impact on sex-hormone binding globulin

Findings from observational studies suggest that sex hormone-binding globulin (SHBG) and endogenous sex hormones may be mediators of the putative relation between coffee consumption and lower risk of type 2 diabetes. The objective of one study was to evaluate
the effects of caffeinated and decaffeinated coffee on SHBG and sex hormone levels. After a two-week run-in phase with caffeine abstention, we conducted an 8-week parallel-arm randomized controlled trial. Healthy adults (n=42) were recruited from the Boston community who were regular coffee consumers, nonsmokers, and overweight. Participants were randomized to five 6-ounce cups of caffeinated or decaffeinated instant coffee or water (control group) per day consumed with each meal, mid-morning, and mid-afternoon. The main outcome measures were SHBG and sex hormones [i.e., testosterone, estradiol, dehydroepiandrosterone sulfate]. No significant differences were found between treatment groups for any of the studied outcomes at week 8. At 4 weeks, decaffeinated coffee was associated with a borderline significant increase in SHBG in women, but not in men. At week 4, we also observed several differences in hormone concentrations between the treatment groups. Among men, consumption of caffeinated coffee increased total testosterone and decreased total and free estradiol. Among women, decaffeinated coffee decreased total and free testosterone and caffeinated coffee decreased total testosterone. The data do not indicate a consistent effect of caffeinated coffee consumption on SHBG in men or women, however results should be interpreted with caution given the small sample size. This is the first randomized trial investigating the effects of caffeinated and decaffeinated coffee on SHBG and sex hormones and our findings necessitate further examination in a larger intervention trial [12286].

Effects after a withdrawal period

In one study, it was investigated the impact of a controlled 4-day caffeine withdrawal period on the effect of an acute caffeine dose on endurance exercise performance. Twelve well-trained and familiarized male cyclists, who were caffeine consumers (from coffee and a range of other sources), were recruited for the study. A double-blind placebo-controlled cross-over design was employed, involving four experimental trials. Participants abstained from dietary caffeine sources for 4 days before the trials and ingested capsules (one in the morning and one in the afternoon) containing either placebo or caffeine (1.5 mg/kg body weight/day). On day 5, capsules containing placebo or caffeine (3 mg/kg body weight) were ingested 90 min before completing a time trial, equivalent to one hour of cycling at 75 percent peak sustainable power output. Hence the study was designed to incorporate placebo-placebo, placebo-caffeine, caffeine-placebo, and caffeine-caffeine conditions. Performance time was significantly improved after acute caffeine ingestion by 1:49 ± 1:41 min (3.0 %) following a withdrawal period (placebo-placebo vs placebo-caffeine), and by 2:07 ± 1:28 min (3.6 %) following the non-withdrawal period (caffeine-placebo vs caffeine-caffeine). No significant difference was detected between the two acute caffeine trials (placebo-caffeine vs caffeine-caffeine). Average heart rate throughout exercise was significantly higher following acute caffeine administration compared with placebo. No differences were observed in ratings of perceived exertion between trials. A 3 mg/kg dose of caffeine thus significantly improves exercise performance irrespective of whether a 4-day withdrawal period is imposed on habitual caffeine users [11208].

It has been traditional in caffeine research or in actual competition use to withdraw people from caffeine use for 24-48 h prior to the study or event (i.e. remove their habituation to repeated use). However, there does not seem to be a consistent difference in the performance effects of caffeine between regular users and non-users of caffeine, or as a result of withdrawal from regular caffeine use. Rather there may be several disadvantages to avoiding or withdrawing caffeine prior to a performance trial. Caffeine withdrawal can be associated with side effects such as headaches and fatigue. In fact, it has been suggested that the benefits of caffeine seen in controlled studies may be overstated, and may actually
be explained as the reversal of adverse withdrawal symptoms rather than an ergogenic effect of caffeine per se. It can also increase the risk, with subsequent caffeine intake, of the negative effects often seen with large caffeine doses (irritability, tremor, heart rate increases) [10174].

Effects on arousal

Studies indicate that the change from closed to open eyes in a resting condition results in an increase in skin conductance level (SCL) and a global decrease in EEG alpha activity, both indicative of increased arousal. Other studies show that ingestion of caffeine also produces SCL increase and alpha reduction. One study investigated the additivity of the effects of these two independent arousing variables. EEG activity and SCL were recorded from 22 university students during both eyes-closed and eyes-open resting conditions, under the action of both caffeine and placebo, in a counterbalanced randomised double-blind study. SCL increased significantly from eyes-closed to eyes-open conditions, and from placebo to caffeine, with no interaction. Global reductions in EEG alpha amplitude were apparent with opening of the eyes and caffeine ingestion; again, there was no interaction. Caffeine had a larger effect than opening the eyes on SCL, but their relative effect sizes were reversed in alpha. The two dependent measures showed the predicted negative correlation in both eyes-closed placebo and eyes-open caffeine conditions, with the latter substantially reduced relative to the former. It was concluded that caffeine and opening the eyes have additive effects on two measures of arousal, increasing SCL and reducing global EEG alpha. However, the independent variable effects are not equivalent, suggesting that one or both measures reflect additional non-arousal processes. As caffeine is widely used by both children and adults, knowledge of the additivity of arousal effects of caffeine and opening the eyes is important in controlling participant state in EEG studies [13344].

Moderated effect of anxiety

One experiment examined the effect of a moderate dose of caffeine on perceptions of leg-muscle pain during a bout of high-intensity cycling exercise and the role of anxiety sensitivity in the hypalgesic effect of caffeine on muscle pain during exercise. Sixteen college-age women ingested caffeine (5 mg/kg body weight) or a placebo and 1 hour later completed 30 min of cycling on an ergometer at 80 percent of peak aerobic capacity. The conditions were completed in a counterbalanced order, and perceptions of leg-muscle pain were recorded during the bouts of exercise. Caffeine resulted in a large reduction in leg-muscle pain-intensity ratings compared with placebo, and the reduction in leg-muscle pain-intensity ratings was larger in those with lower anxiety-sensitivity scores than those with higher anxiety-sensitivity scores. The results support that caffeine ingestion has a large effect on reducing leg-muscle pain during high-intensity exercise, but the effect is moderated by anxiety sensitivity [08318].

Psychological effects

Caffeine's metabolic and performance effects have been widely reported. However, caffeine's effects on affective states during prolonged exercise are unknown. Therefore, this was examined in one study. Following an overnight fast and in a randomised, double-blind, counterbalanced design, twelve endurance trained male cyclists performed 90 min of exercise at 70 percent of VO₂max 1h after ingesting 6 mg/kg body weight of caffeine (CAF) or
placebo (PLA). Dimensions of affect and perceived exertion were assessed at regular intervals. During exercise, pleasure ratings were better maintained in the CAF trial compared to the PLA trial with significantly higher ratings at 15, 30 and 75 min. Perceived exertion increased throughout exercise and values, overall, were significantly lower in the CAF trial compared to the PLA trial. Perceived arousal was elevated during exercise but did not differ between trials. Overall, the results suggest that a moderate dose of CAF ingested 1h prior to exercise maintains a more positive subjective experience during prolonged cycling. This observation may partially explain caffeine's ergogenic effects [13346].

**Psychological effects**

One study examined the effect of coffee ingestion on physiological responses and ratings of perceived exertion (RPE) during submaximal endurance exercises by 10 healthy young adults. Participants performed a submaximal endurance cycling exercise corresponding to 60% of maximum oxygen uptake capacity for 60 min. They drank either caffeinated coffee with a caffeine content of 6 mg/kg body-mass of each participant (Caf) or a decaffeinated coffee (Dec) 60 min. before starting exercise. Participants participated in the blind design experiment under both conditions at a one-week interval. Oxygen uptake, respiratory exchange ratio, heart rate, RPE, and plasma lactate concentration were measured during the endurance exercise. The RPE under the Caffeinated coffee condition during the last 60 min. of endurance exercise was significantly lower than that in the Decaffeinated coffee condition. However, no significant differences in any physiological response were observed between conditions. Thus, caffeine ingestion 60 min. before starting exercise had an insignificant effect on the physiological responses, except for RPE during submaximal endurance exercises for 60 min. Caffeine ingestion before endurance exercise of relatively low intensity may have a beneficial effect on psychological responses [07168].

General Factor of Personality (GFP) research is an emergent field in personality research. This paper uses a theoretical mathematical model to predict the short-term effects of a dose of a stimulant drug on GFP and reports the results of an experiment showing how caffeine achieves this. This study considers the General Factor of Personality Questionnaire (GFPQ) a good psychometric approach to assess GFP. The GFP dynamic mechanism of change is based on the Unique Trait Personality Theory (UTPT). This theory proposes the existence of GFP which occupies the apex of the hierarchy of personality, and extends from an impulsiveness-and-aggressiveness pole (approach tendency) to an anxiety-and-introversion pole (avoidance tendency). An experiment with 25 volunteers was performed. All the participants completed the GFPQ and the Sensation-Seeking Scale list of adjectives from the trait version of MAACL-R (Multiple Affect Adjective Checklist Revised) on an empty stomach. The participants in the experimental group (20) received 330 mg of caffeine. All the participants filled in a state version form with the sensation-seeking adjectives every 4.5 minutes. This study considers that the Sensation-Seeking Scale list of adjectives from the MAACL-R, available in both trait and state versions, is a good psychometric approach to assess GFP. The results show that GFP is modified by a single dose of caffeine in the direction predicted by the UTPT [11489].

*Caffeine choices prospectively predicts positive subjective effects d-amphetamine*

Individuals vary in their subjective and behavioral response to psychomotor stimulants and these differences may be associated with the likelihood of developing problematic use of these drugs. The present study sought to determine whether individual differences in caffeine choice prospectively predict subjective response to acute doses of caffeine and d-amphetamine. In phase 1, Choosers and Nonchoosers of caffeine were identified using 10 independent choice trials in which subjects repeatedly chose between caffeine (200 mg/70 kg) and placebo. Choosers were defined as those who chose caffeine over placebo on ≥7 of
the 10 trials; Nonchoosers were those who chose placebo on ≥7 trials. In Phase 2, Choosers and Nonchoosers were compared in their subjective response to caffeine (100, 200, 400 mg/70 kg) and d-amphetamine (5, 10, 20 mg/70 kg). Of the 22 participants completing the study, 11 met criteria for being a caffeine Chooser and 8 were Nonchoosers. In phase 1, Choosers reported higher ratings of positive (i.e., pleasant) and lower ratings of negative (i.e. unpleasant) effects of caffeine during the sampling sessions. In phase 2, caffeine Choosers reported more positive subjective effects and fewer negative effects of caffeine and d-amphetamine, particularly at the highest doses examined. It was concluded that individual differences in caffeine reinforcement predicted subsequent subjective response to both d-amphetamine and caffeine. This observation may have clinical utility for identifying individuals who are vulnerable to the reinforcing effects of abused psychomotor stimulants [11490].

**Caffeinated versus decaffeinated coffee**

One study examined the effect of coffee ingestion on physiological responses and ratings of perceived exertion (RPE) during submaximal endurance exercises by 10 healthy young adults. Participants performed a submaximal endurance cycling exercise corresponding to 60 percent of maximum oxygen uptake capacity for 60 min. They drank either caffeinated coffee with a caffeine content of 6 mg/kg body-mass of each participant or a decaffeinated coffee 60 min before starting exercise. Participants participated in the blind design experiment under both conditions at a one-week interval. Oxygen uptake, respiratory exchange ratio, heart rate, RPE, and plasma lactate concentration were measured during the endurance exercise. The RPE under the caffeinated coffee condition during the last 60 min of endurance exercise was significantly lower than that in the decaffeinated coffee condition. However, no significant differences in any physiological response were observed between conditions. Thus, caffeine ingestion 60 min before starting exercise had an insignificant effect on the physiological responses, except for RPE during submaximal endurance exercises for 60 min. Caffeine ingestion before endurance exercise of relatively low intensity may have a beneficial effect on psychological responses [08319].

Although coffee is largely consumed by adults in Western countries, controversy exists about its impact on the cardiovascular system. It was recently demonstrated that caffeinated and decaffeinated espresso coffee have different acute effects on endothelial function in healthy subjects, measured using flow-mediated dilation of the brachial artery. In one study, it was measured the anti-oxidant capacity of two coffee substances in terms of free stable radical 2,2-diphenyl-1-picryl-hydrazyl 50 percent inhibition (I50 DPPH). The caffeinated coffee had a slightly higher anti-oxidant capacity than decaffeinated espresso coffee. It was suggested that the unfavourable effects observed after caffeinated coffee ingestion are due to caffeine and that the antioxidant activity is responsible for the increased FMD observed after decaffeinated coffee ingestion. Further clinical and epidemiological studies are needed to understand the chronic effects of coffee consumption on health [10499].

**Hematological side effects**

To evaluate the effect of caffeine on white cell distribution and muscle injury markers in professional soccer players during exercise 22 male athletes completed a placebo controlled double blind test protocol to simulate a soccer match, followed by a Yo-Yo intermittent recovery test. Exercise caused an increase in packed cell volume that was enhanced by caffeine. Caffeine and exercise had a synergistic effect on the blood lymphocyte count, which
increased by about 38 percent after exercise, and by an additional 35 percent when combined with caffeine. Caffeine promoted an exercise independent rise in circulating monocytes, and a synergistic action of exercise and caffeine was observed on segmented neutrophils. Caffeine promoted thrombocytosis. Plasma adenosine deaminase, aspartate aminotransferase, and lactate dehydrogenase concentrations were enhanced by exercise, and alanine transaminase concentration was enhanced in both groups, with a synergistic effect of caffeine. It was concluded that the pronounced increase in the white cell count in the group receiving caffeine appeared to be caused by greater muscle stress and consequently more intense endothelial and muscle cell injury. The use of caffeine may augment the risk of muscle damage in athletes [07176].

Experimental

Caffeine and taurine

Caffeine enhances endurance performance; however, its effect on accumulated lactate remains unclear. Conversely, taurine, which also enhances endurance performance, decreases accumulated lactate. In one study, the effect of combination of caffeine and taurine on endurance performance was assessed. Mice ran on a treadmill, and the accumulated lactate was measured. In addition, muscle fibers from the gastrocnemius muscle of the mice were stained with ATPase and analyzed. The use of caffeine and taurine over a 2 week period enhanced endurance performance. Moreover, taurine significantly decreased the accumulated concentration of lactate over long running distances. However, the diameter of the cross-sections and ratios of Types I, IIA, and IIB muscle fibers were not affected [09258].

Interaction with amitriptylin

The interaction of caffeine (1 mg/kg) and amitriptyline (15 mg/kg) on the immobility time during Porsolt's forced swimming test was investigated in female Wistar rats. Vehicle-treated animals had a significant increase of immobility time during the second day of the test. Amitriptyline only prevented the increase of immobility time during the second session. While caffeine alone prevented the increase of immobility time in both groups, the methylxanthine abolished the effect of amitriptyline, leaving the antidepressant action. These results suggest that the anti-immobility effect of amitriptyline is mediated in part by endogenous adenosine [08320].

Muscle metabolism

Caffeine (1,3,7-trimethylxanthine) has been implicated in the regulation of glucose and lipid metabolism including actions such as insulin-independent glucose transport, glucose transporter 4 expression, and fatty acid utilization in skeletal muscle. These effects are similar to the exercise-induced and 5'adenosine monophosphate-activated protein kinase (AMPK)-mediated metabolic changes in skeletal muscle, suggesting that caffeine is involved in the regulation of muscle metabolism through AMPK activation. It was explored whether caffeine acts on skeletal muscle to stimulate AMPK. Incubation of rat epitrochlearis and soleus muscles with Krebs buffer containing caffeine (≥3 mmol/L, ≥15 minutes) increased the phosphorylation of AMPK-alpha Thr(172), an essential step for full kinase activation, and acetyl-coenzyme A carboxylase Ser(79), a downstream target of AMPK, in dose- and time-dependent manners. Analysis of isoform-specific AMPK activity revealed that both AMPKalpha1 and alpha2 activities increased significantly. This enzyme activation was
associated with a reduction in phosphocreatine content and an increased rate of 3-O-methyl-d-glucose transport activity in the absence of insulin. These results suggest that caffeine has similar actions to exercise by acutely stimulating skeletal muscle AMPK activity and insulin-independent glucose transport with a reduction of the intracellular energy status [09259].

Caffeine activates 5'AMP-activated protein kinase (AMPK), a signalling intermediary implicated in the regulation of glucose, lipid and energy metabolism in skeletal muscle. Skeletal muscle expresses two catalytic α subunits of AMPK, alpha1 and alpha2, but the isoform specificity of caffeine-induced AMPK activation is unclear. The aim of this study was to determine which α isoform is preferentially activated by caffeine in vitro and in vivo using rat skeletal muscle. Rat epitrochlearis muscle was isolated and incubated in vitro in the absence or presence of caffeine. In another experiment, the muscle was dissected after intravenous injection of caffeine. Isoform-specific AMPK activity, the phosphorylation status of AMPKalpha Thr(172) and acetyl-CoA carboxylase (ACC) Ser(79), the concentrations of ATP, phosphocreatine (PCr) and glycogen, and 3-O-methyl-d-glucose (3MG) transport activity were estimated.

Incubation of isolated epitrochlearis muscle with 1 mm of caffeine for 15 min increased AMPKα1 activity, but not AMPKα2 activity; concentrations of ATP, PCr and glycogen were not affected. Incubation with 3 mm of caffeine activated AMPKα2 and reduced PCr and glycogen concentrations. Incubation with 1 mm of caffeine increased the phosphorylation of AMPK and ACC and enhanced 3MG transport. Intravenous injection of caffeine (5 mg/kg) predominantly activated AMPKalpha1 and increased 3MG transport without affecting energy status. The results suggest that of the two alpha isoforms of AMPK, AMPKalpha1 is predominantly activated by caffeine via an energy-independent mechanism and that the activation of AMPKalpha1 increases glucose transport and ACC phosphorylation in skeletal muscle [11205].

Effect of fat free weight

The influences of creatine and caffeine supplementation associated with power exercise on lean body mass (LBM) composition are not clear. The purpose of this research was to determine whether supplementation with high doses of creatine and caffeine, either solely or combined, affects the LBM composition of rats submitted to vertical jumping training. Male Wistar rats were randomly divided into 8 groups: sedentary (S) or exercised (E), placebo (Pl), creatine (Cr), caffeine (Caf) or creatine plus caffeine (CrCaf). The supplemented groups received creatine (load: 0.430 g/kg of body weight for 7 days; and maintenance: 0.143 g/kg of body weight for 35 days), caffeine (15 mg/kg of body weight for 42 days) or creatine plus caffeine. The exercised groups underwent a vertical jump training regime (load: 20 to 50 % of body weight, 4 sets of 10 jumps interspersed with 1 min resting intervals), 5 days/wk, for 6 weeks. LBM composition was evaluated by portions of water, protein and fat in the rat carcass. Exercised animals presented a lower carcass weight (11 %), as compared to sedentary animals. However, no effect of supplementation was observed on carcass weight. There were no significant differences among the groups for percentage of water in the carcass. The percentage of fat in the group SCr was higher than in the groups SCaf and ECr. A higher percentage of protein was observed in the groups EPI and ECaf when compared to the groups SPI and SCaf. The percentage of fat in the carcass decreased, while those of water and protein increased in exercised animals, compared to sedentary animals. Caffeine groups presented reduced percentage of fat when compared to creatine supplemented groups. It was concluded that high combined doses of creatine and caffeine does not affect the LBM composition of either sedentary or exercised rats, however, caffeine supplementation alone reduces the percentage of fat. Vertical jumping training increases the percentages of water and protein and reduces the fat percentage in rats [11206].

Practical recommendations
The ergogenic effects of caffeine on athletic performance have been shown in many studies, and its broad range of metabolic, hormonal, and physiologic effects has been recorded, as one review of the literature shows. However, few caffeine studies have been published to include cognitive and physiologic considerations for the athlete. The following practical recommendations consider the global effects of caffeine on the body: lower doses can be as effective as higher doses during exercise performance without any negative coincidence; after a period of cessation, restarting caffeine intake at a low amount before performance can provide the same ergogenic effects as acute intake; caffeine can be taken gradually at low doses to avoid tolerance; and caffeine can improve cognitive aspects of performance, such as concentration, when an athlete has not slept well. Athletes and coaches also must consider how a person's body size, age, gender, previous use, level of tolerance, and the dose itself all influence the ergogenic effects of caffeine on sports performance [08321].

The position of the International Society of Sports Nutrition regarding caffeine supplementation and sport performance is summarized by the following seven points [10176]:

- caffeine is effective for enhancing sport performance in trained athletes when consumed in low-to-moderate dosages (about 3-6 mg/kg) and overall does not result in further enhancement in performance when consumed in higher dosages (≥9 mg/kg)
- caffeine exerts a greater ergogenic effect when consumed in an anhydrous state as compared to coffee
- it has been shown that caffeine can enhance vigilance during bouts of extended exhaustive exercise, as well as periods of sustained sleep deprivation
- caffeine is ergogenic for sustained maximal endurance exercise, and has been shown to be highly effective for time-trial performance
- caffeine supplementation is beneficial for high-intensity exercise, including team sports such as soccer and rugby, both of which are categorized by intermittent activity within a period of prolonged duration
- the literature is equivocal when considering the effects of caffeine supplementation on strength-power performance, and additional research in this area is warranted
- the scientific literature does not support caffeine-induced diuresis during exercise, or any harmful change in fluid balance that would negatively affect performance

Caffeinated “energy shots”

Caffeine (1,3,7-trimethylxanthine) is widely used among athletes, and it was in 2007 found that 73 percent of 140 athletes surveyed at the 2005 Ironman Triathlon World Championships believed caffeine improved performance. Via ingestion of coffee, capsules, or anhydrous powder, caffeine improves performance of moderate to high intensity endurance exercise. However, most studies examining caffeine utilized anhydrous caffeine, which is not readily accessible to coaches and athletes. Additionally, most researchers utilize doses relative to body weight instead of the absolute doses commonly found in commercially available caffeine products. While it could be argued that weighing out a relative dose may be plausible for elite athletes, who have access to dietitians and other trained staff, most track and field/cross country running coaches in the United States have large numbers of athletes to supervise, and this is relatively impractical. Energy drink usage among athletes is also quite widespread, and it was reported 48 percent of 401 collegiate athletes regularly consumed energy drinks. Thus, a coach is far more likely to tell an athlete to “Drink this” prior
to a competition instead of calculating a target caffeine dose for each athlete. Recent research has examined effects of more accessible forms of caffeine, such as energy drinks, on exercise performance in athletes. A, yet to be evaluated, caffeine supplement is the energy "shot" which is smaller in volume (generally 59-88 mL), as it lacks the large amounts of sugars, carbohydrates, and/or carbonated water of energy drinks containing caffeine. This low volume and energy content may make their intake practical for runners, who typically avoid supplements due to the onset of gastrointestinal disturbances, as running has a higher occurrence of GI symptoms than cycling. Despite the large amount of literature examining the ergogenic properties of caffeine, few studies have evaluated the efficacy of caffeine ingestion on running performance using ecologically valid assessments, such as time trials. Energy shots may prove to be a viable pre-competition supplement for runners. Six male runners (mean ± SD age 22.5 ± 1.8 years) completed three trials [placebo (PLA; 0 mg caffeine), Guayaki Yerba Maté Organic Energy Shot™ (YM; 140 mg caffeine), or Red Bull Energy Shot™ (RB; 80 mg caffeine)]. Treatments were ingested following a randomized, placebo-controlled crossover design. Participants ran a five kilometer time trial on a treadmill. No differences in performance were detected with RB or YM ingestion compared to placebo. Thus, ingestion of two commercially available energy shots with differing levels of caffeine does not alter treadmill five kilometer time-trial performance, RPE, or physiological variables in well-trained runners, compared to a placebo. The results must be considered preliminary due to the small sample size, and further studies should examine the effects of energy shot ingestion on performance in a variety of sport and field settings. While some research has been conducted on energy drinks, this is the first study to examine the efficacy of energy shots on exercise performance. Therefore, additional study is needed to examine their long-term effects on body composition and health, in addition to athletic performance, before recommendations about their usage can be provided to coaches and athletes [13438].

Caffeinated chewing gum

One investigation reports the effects of caffeinated chewing gum on fatigue and hormone response during repeated sprint performance with competitive cyclists. Nine male cyclists completed four high-intensity experimental sessions, consisting of four sets of 30 s sprints (5 sprints each set). Caffeine (240 mg) or placebo was administered via chewing gum following the second set of each experimental session. Testosterone and cortisol concentrations were assayed in saliva samples collected at rest and after each set of sprints. Mean power output in the first 10 sprints relative to the last 10 sprints declined by 5.8 ± 4.0 percent in the placebo and 0.4 ± 7.7 percent in the caffeine trials, respectively. The reduced fatigue in the caffeine trials equated to a 5.4 percent performance enhancement in favour of caffeine. Salivary testosterone increased rapidly from rest (53 %) and prior to treatments in all trials. Following caffeine treatment, testosterone increased by a further 12 ± 14 percent relative to the placebo condition. In contrast, cortisol concentrations were not elevated until after the third exercise set; following the caffeine treatment cortisol was reduced by 21 ± 31 percent relative to placebo. The acute ingestion of caffeine via chewing gum attenuated fatigue during repeated, high-intensity sprint exercise in competitive cyclists. Furthermore, the delayed fatigue was associated with substantially elevated testosterone concentrations and decreased cortisol in the caffeine trials [10389].

Low-dose caffeine administered in chewing gum does not enhance cycling to exhaustion. The purpose of the current investigation was to examine the effect of low-dose caffeine (CAF) administered in chewing gum at 3 different time points during submaximal cycling exercise to exhaustion. Eight college-aged (26 ± 4 years), physically active (46 ± 6 mL/kg/min) volunteers participated in 4 experimental trials. Two pieces of caffeinated
chewing gum (100 mg per piece, total quantity of 200 mg) were administered in a double-blind manner at 1 of 3 time points (-35, -5, and +15 minutes) with placebo at the other 2 points and at all 3 points in the control trial. The participants cycled at 85 percent of maximal oxygen consumption until volitional fatigue and time to exhaustion (TTE) were recorded in minutes. Venous blood samples were obtained at -40, -10, and immediately postexercise and analyzed for serum-free fatty acid and plasma catecholamine concentrations. Oxygen consumption, respiratory exchange ratio, heart rate, glucose, lactate, ratings of perceived exertion, and perceived leg pain measures were obtained at baseline and every 10 minutes during cycling. The results showed that there were no significant differences between the trials for any of the parameters measured including TTE. These findings suggest that low-dose CAF administered in chewing gum has no effect on TTE during cycling in recreational athletes and is, therefore, not recommended [12290].

Research has shown that standard chewing gum can affect aspects of both attention and memory. One study examined the effects of Think Gum®, a caffeinated-herbal chewing gum, on both concentration and memory using a series of paper-based and online testing. Compared to standard chewing gum and a no-gum control, chewing caffeinated-herbal gum during testing improved aspects of memory, but did not affect concentration. The findings suggest that caffeinated-herbal chewing gum is an effective memory aid [13350].

The purpose of this study was to determine the most efficacious time to administer caffeine (CAF) in chewing gum to enhance cycling performance. Eight male cyclists participated in 5 separate laboratory sessions. During the first visit, the subjects underwent a graded exercise test to determine maximal oxygen consumption (VO\textsubscript{2max}). During the next 4 visits, 3 pieces of chewing gum were administered at 3 time points (120-minute precycling, 60-minute precycling, and 5-minute precycling). In 3 of the 4 visits, at 1 of the time points mentioned previously, 300 mg of CAF was administered. During the fourth visit, placebo gum was administered at all 3 time points. The experimental trials were defined as follows: trial A (-120), trial B (-60), trial C (-5), and trial D (Placebo). After baseline measurements, time allotted for gum administration, and a standard warm-up, the participants cycled at 75% VO\textsubscript{2max} for 15 minutes then completed a 7kJ/kg cycling time trial. Data were analyzed using a repeated measures analysis of variance. Cycling performance was improved in trial C (-5), but not in trial A (-120) or trial B (-60), relative to trial D (Placebo). CAF administered in chewing gum enhanced cycling performance when administered immediately prior, but not when administered 1 or 2 hours before cycling [13466].

**Caffein gel**

It was investigated the effects of ingesting carbohydrate gels with and without caffeine on a 90 minute, four blocks intermittent sprint test (IST), in 12 recreationally trained male athletes. Using a cross-over design, one 70 ml dose of gel containing either 25 g of carbohydrate with (CHOCAF) or without (CHO) 100 mg of caffeine, or a non-caloric placebo (PL) was ingested on three occasions: one hour before, immediately prior to and during the IST. Blood glucose, rating of perceived exertion (RPE) and fatigue index (FI) were analysed. Glucose showed significantly higher values for both CHOCAF and CHO at the first, second and third blocks when compared with PL, while only CHOCAF was significantly different to PL at the fourth block. CHOCAF showed an improved FI compared with CHO and PL, a significantly lower RPE compared with PL and a trend in respect of CHO after the third block. In conclusion, ingesting CHOCAF one hour before, prior to and during an IST is effective at transiently reducing fatigue and RPE whilst maintaining higher glucose levels at the final stages of the exercise [13455].
Guarana

Guarana (*Paullinia cupana*), a climbing plant in the maple family, native to the Amazon basin and especially common in Brazil, contains a high amount of guaranine, a chemical substance with the same characteristics as caffeine. Guaranine, a synonym for caffeine, is defined only as the caffeine chemical in guarana and is identical to the caffeine chemical derived from other sources (e.g. coffee, tea, maté). Guarana features large leaves, clusters of flowers, and a fruit similar in size to the coffee bean. As a dietary supplement, guarana is a useful caffeine source with guarana seeds containing approximately twice the amount of caffeine (2-4.5 %) compared with 1-2 percent for coffee beans. Guarana, alongside other natural sources of caffeine, also contains varying mixtures of other xanthine alkaloids such as theobromine and theophylline. Guarana is generally recognised as an acceptable ingredient and can be found in drinks, “energy shots”, herbal teas or capsules. Consequently, guarana is best known for its stimulatory properties, providing similar benefits to caffeine, such as reducing fatigue, increasing alertness, and as an ergogenic aid in the athletic arena. The maximal ergogenic benefits of caffeine and guarana can be seen at small to moderate caffeine doses (2-3 mg/kg). Theoretically, it is possible to overdose on caffeine or guarana, with the fatal dose being estimated at a single dose of 10 g pure caffeine/guaranine [11150].
OTHER STIMULANTS

While the extent of explicitly named substances among S.6 of WADA's prohibited list remained nearly unchanged in 2011 compared to the preceding year, the growing number of new designer stimulants in addition to earlier abandoned and now re-discovered agents with stimulating properties (e.g. mephedrone, naphyrone, methylene, methedrone, flephedrone) has required more comprehensive screening efforts and attention by doping control laboratories [12016].

Central nervous system (CNS) stimulants may be used to reduce tiredness and increase alertness, competitiveness, and aggression. Central nervous system (CNS) stimulants were originally used by athletes to improve performance on the day of competition. Although there was evidence that these drugs might be linked with sudden collapse or death, usually from cardiac or respiratory arrest, particularly during competition, the long term side effects of addiction and physiological damage to the body were regarded as minor or were not mentioned. The class of stimulants includes psychomotor stimulants, sympathomimetics, and miscellaneous CNS stimulants – for example, caffeine, amphetamines, ephedrines, and cocaine. These substances are either prohibited or monitored, previously by the International Olympic Committee (IOC) and now by the World Anti-Doping Agency (WADA), and are screened for daily by accredited laboratories. There are several potential dangers involving their misuse in contact sports. One paper reviewed the three main CNS stimulants, ephedrine, amphetamine, and cocaine, in relation to misuse in sport. CNS stimulants have psychotropic effects that may be perceived to be ergogenic. Some are prescription drugs, such as Ephedra alkaloids, and there are issues regarding their appropriate therapeutic use. Recently attention has been given to their widespread use by athletes, despite the lack of evidence regarding any ergogenic or real performance benefit, and their potentially serious side effects. Recreational drugs, some of which are illegal (cocaine, amphetamines), are commonly used by athletes and cause potential ergolytic effects. Overall, these drugs are important for their frequent use and mention in anti-doping laboratories statistics and the media, and their potentially serious adverse effects. Doping with CNS stimulants is a real public health problem and all sports authorities should participate in its prevention. Dissemination of information is essential to prevent doping in sport and to provide alternatives. Adequate training and education in this domain should be introduced [06171].

Stimulants are banned in-competition for all categories of sports by the World Anti-Doping Agency. A simple liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay employing electrospray ionisation in positive mode was developed in that work for the quantification in urine specimens of 4-methyl-2-hexanamine, a primary amine exhibiting sympathomimetic properties. Following a simple pretreatment procedure, the analyte was separated using a gradient mobile phase on reverse phase C8 column. Selected reaction monitoring m/z 116.2-->57.3 was specific for detection of 4-methyl-2-hexanamine and the assay exhibited a linear dynamic range of 50-700 ng/mL. The validated method has been successfully applied to analyze the target compound in food supplements as well as in urine specimens. The administered drug (40 mg) was detected at the level of 350 ng/mL in the urine up to 4 days [09249].

Stimulants have been frequently detected in doping control samples and represent a structurally diverse class of compounds. Comprehensive sports drug-testing procedures have been developed using gas or liquid chromatography combined with mass spectrometric detection, and they have revealed various adverse analytical findings, as demonstrated with 2 examples, 4-methylhexan-2-amine and methoxyphenamine. Moreover, the necessity of
controlling the use or misuse of stimulating agents is outlined by means of pseudoephedrine, a compound that was prohibited in sports until the end of 2003. Since the ban was lifted, monitoring programs proved a significant increase in pseudoephedrine applications as determined from urine samples collected in competition. As a consequence, a reimplementation of this drug in future doping controls was decided [10185].

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The evidence for the ergogenic properties of the most potent stimulants, amphetamines, cocaine and ephedrine, is mostly insubstantial. Low doses of amphetamines may aid performance where effects of fatigue adversely affect higher psychomotor activity. Pseudoephedrine, at high doses, has been suggested to improve high intensity and endurance exercise but phenylpropanolamine has not been proven to be ergogenic. Only caffeine has substantial experimental backing for being ergogenic in exercise. The mode of action of these stimulants centres on their ability to cause persistence of catecholamine neurotransmitters, with the exception of caffeine which is an adenosine receptor antagonist. By these actions, the stimulants are able to influence the activity of neuronal control pathways in the central (and peripheral) nervous system. Rodent models suggest that amphetamines and cocaine interact with different pathways to that affected by caffeine. Caffeine has a variety of pharmacological effects but its affinity for adenosine receptors is comparable with the levels expected to exist in the body after moderate caffeine intake, thus making adenosine receptor blockade the favoured mode of ergogenic action. However, alternative modes of action to account for the ergogenic properties of caffeine have been supported in the literature. Biochemical mechanisms that are consistent with more recent research findings, involving proteins such as DARPP-32 (dopamine and cAMP-regulated phosphoprotein), are helping to rationalize the molecular details of stimulant action in the central nervous system [08292].

One review examined the pharmacology of stimulants prohibited by the World Anti-Doping Agency. Stimulants that increase alertness and reduce fatigue or activate the cardiovascular system can include drugs like ephedrine available in many over-the-counter medicines. Others such as amphetamines, cocaine and hallucinogenic drugs, available on prescription or illegally, can modify mood. A total of 62 stimulants (61 chemical entities) are listed in the WADA List, prohibited in competition. Athletes may have stimulants in their body for one of three main reasons: inadvertent consumption in a propriety medicine; deliberate consumption for misuse as a recreational drug and deliberate consumption to enhance performance. The majority of stimulants on the list act on the monoaminergic systems: adrenergic (sympathetic, transmitter noradrenaline), dopaminergic (transmitter dopamine) and serotonergic (transmitter serotonin, 5-HT). Sympathomimetic describes agents, which mimic sympathetic responses, and dopaminomimetic and serotoninomimetic can be used to describe actions on the dopamine and serotonin systems. However, many agents act to mimic more than one of these monoamines, so that a collective term of monoaminomimetic may be useful. Monoaminomimetic actions of stimulants can include blockade of re-uptake of neurotransmitter, indirect release of neurotransmitter, direct activation of monoaminergic
receptors. Many of the stimulants are amphetamines or amphetamine derivatives, including agents with abuse potential as recreational drugs. A number of agents are metabolized to amphetamine or metamphetamine. In addition to the monoaminomimetic agents, a small number of agents with different modes of action are on the list. A number of commonly used stimulants are not considered as "Prohibited Substances" [08293].

Stimulatory substances applied during competition possess a reasonable potential as performance enhancing agents and their misuse in elite sport has been frequently reported during the last few decades. An analytical method for the qualitative determination of selected stimulants containing a primary or secondary amine moiety in human urine for doping control purposes was developed. A rapid and highly specific procedure based on a sample preparation using weak cation exchange solid phase extraction (SPE-XCW) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a C6-Phenyl analytical column allowed the unambiguous identification of the target analytes down to low concentration levels. Validation provided recovery rates of better than 75 percent, precisions of less than 20 percent and a linear approximation in the required working range (10-750 ng/mL) were obtained for 19 different target compounds. This method provides a rugged and highly specific alternative to the established method utilising gas or liquid chromatography after liquid-liquid extraction [08294].

One study tested the related hypotheses that acute fatigue reduces +Gz tolerance and endurance, and that stimulant can partially reverse this impact. To determine the effect of fatigue on +Gz tolerance and the impact of stimulant use, 10 male centrifuge subjects, mean age 32, were tested in a repeated measures study under five nighttime conditions following an average of 22 h of sustained wakefulness during their circadian nadir. Using a within-subject design, subjects received placebo, dextroamphetamine 10 mg, modafinil 200 mg, methylphenidate 10 mg, and pemoline 37.5 mg at night, and were tested during a daytime control session. No difference in +Gz tolerance or endurance was detected among conditions. The cognitive/performance tests also did not detect any differences. Subject perception that anti-G straining maneuver (AGSM) difficulty was greater during the night placebo condition than during the daytime control, methylphenidate and modafinil night conditions reached statistical significance [08295, 08296].

The objective of one study was to determine the prevalence of asthma-like symptoms and asthma and the use of asthma medication in Danish elite athletes. A cross-sectional questionnaire survey of Danish elite athletes was conducted in 2006. All elite athletes (n=418) financially supported by the national organization of elite athletes comprised the study group; 329 (79 %) completed the questionnaire concerning their sport, asthma-like symptoms, asthma and use of asthma medication. Asthma-like symptoms at rest were reported by 41 percent of respondents; 55 percent reported asthma-like symptoms at rest or at exercise. Physician-diagnosed asthma was present in 16 percent and 14 percent had current asthma. Asthma medication was taken by 7 percent of the athletes, of whom 79 percent used inhaled corticosteroids and 21 percent used inhaled beta2-agonists only. Athletes participating in endurance sports had significantly higher prevalences of current asthma (24 %) and use of asthma medication (15 %) than all other athletes. Athletes participating in endurance sports have a higher prevalence of asthma and use of asthma medication. The frequency of asthma medication is lower than the prevalence of current asthma indicating that there is no overuse of asthma medication among Danish elite athletes [08297].

One paper reviewed the prevalence of legal and illegal stimulants in relation to doping-control analysis. Stimulants are among the oldest classes of doping agents, having been used since ancient times. Despite the ease with which they can be detected and the
availability of sensitive detection methods, stimulants are still popular among athletes. Indeed, they remain one of the top three most popular classes of prohibited substances. Because the list of legal and illegal stimulants is extensive only a selection is discussed in detail. The compounds selected are caffeine, ephedrines, amphetamine and related compounds, methylphenidate, cocaine, strychnine, modafinil, adrafinil, 4-methyl-2-hexaneamine, and sibutramine. These compounds are mainly prevalent in sport or are of therapeutic importance. Because stimulants are the oldest doping class the first detection methods were for this group. Several early detection techniques including GC-NPD, GC-ECD, and TLC are highlighted. The more novel detection techniques GC-MS and LC-MS are also discussed in detail. In particular, the last technique has been shown to enable successful detection of stimulants difficult to detect by GC-MS or for stimulants previously undetectable. Because stimulants are also regularly detected in nutritional (food) supplements a section on this topic is also included [11192].

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Ergonomic effects

The actual ergonomic benefits of amphetamines, cocaine, and ephedrines are unclear, but all are banned or monitored to varying degrees by different sporting federations and associations in relation with their real side effects in sport practice. This discrepancy between actual benefit and banned status highlights the difficulty sport physicians have in advising athletes, parents, coaches, and the sporting community about the proper approach to monitoring, education, and testing for these drugs. Together, they should take action in the areas of education, scientific research, and social and health measures to protect athletes, harmonise standards and coordinate the legislation in relation to doping [06171].

Theobromine

The effects of theobromine in man are underresearched, possibly owing to the assumption that it is behaviourally inert. Toxicology research in animals may appear to provide alarming results, but these cannot be extrapolated to humans for a number of reasons. Domestic animals and animals used for racing competitions need to be guarded from chocolate and cocoa-containing foods, including foods containing cocoa husks. Research ought to include caffeine as a comparative agent, and underlying mechanisms need to be further explored. Of
all constituents proposed to play a role in our liking for chocolate, caffeine is the most convincing, though a role for theobromine cannot be ruled out. Most other substances are unlikely to exude a psychopharmacological effect owing to extremely low concentrations or the inability to reach the blood-brain barrier, whilst chocolate craving and addiction need to be explained by means of a culturally determined ambivalence towards chocolate [11209].

**Theobromine and theophylline**

Theobromine and theophylline are methylxanthine alkaloids originally identified from the chocolate or cocoa plant Theobroma cacao L. (Malvaceae, syn. Sterculiaceae), but subsequently identified in tea, cola, guarana and many other plants. Theobromine and theophylline are isomers of one another and paraxanthine. All three are structurally similar to caffeine and all three are metabolites of caffeine in the human body, although paraxanthine is much more so. Both theobromine and theophylline have been used in energy- or metabolism-enhancing sports supplements for their stimulant effects. Beyond their presence in foods, beverages and sports supplements where they may work as stimulants, pure theophylline is widely prescribed as an antiasthmatic, and is under investigation as a treatment for other cardiorespiratory diseases. Theophylline is an alleged ergogenic aid, though studies both support and refute this capacity. One study found no effect of theophylline (serum concentrations 10–20 mg/L) on VO\textsubscript{2max}, muscle or lung measures, or reaction time in nonasthmatic athletes, concluding it was not ergogenic. In contrast, two other groups dosed athletes with 4.5 mg/kg theophylline/body mass and reported improved time-to-exhaustion when athletes cycled either at 80 percent of their VO\textsubscript{2max} or intermittently (1 min cycling at 120 percent VO\textsubscript{2max} alternating with 3 min rest) and concluded it was ergogenic. Although a more thorough review exists elsewhere, this sample of studies demonstrates why the potential ergogenic effects of theophylline are still being questioned. Similar studies of theobromine could not be found. Both body mass and exercise are known to influence the metabolism of both compounds and, given current reports, it is likely that both may act additively or synergistically with other compounds. For example, theophylline is known to augment the effects of ephedrine, another stimulant discussed previously in this series. Both theobromine and theophylline have proven anti-inflammatory actions at cellular and molecular levels, including abilities to regulate reactive oxygen species (ROS), as well as to modulate the behaviours of inflammatory cells. The past (International Olympic Committee and World Anti-Doping Association) and present (National Collegiate Athletic Association) restrictions on high doses of caffeine intake by athletes in competition has created some discussion on whether theobromine and theophylline should also be regulated. Antidoping agencies have never placed restrictions on the use of these compounds, though one research team argued during the period when urinary caffeine concentrations were restricted to <12 microg/mL, that urinary levels of 5 μg/ml for theophylline and a theophylline/paraxanthine ratio of 0.50 should be used for doping standards. Quantities of theobromine and theophylline in commercially available products are probably too low to elicit any ergogenic benefits when such products are consumed in typical or even tolerable quantities, and pure theophylline is only available by prescription in many countries. Available data suggest that current policies regarding these compounds should be maintained but not cemented [12300].

**Ephedrine**

Echinacea and ephedra are herbs used by athletes worldwide. Echinacea is believed to strengthen the immune system against upper respiratory infections, while ephedra is supposed to promote alertness, endurance and strength. Purported bioactive molecules
include alk(yl)amides, caffeic acid derivatives and polysaccharides from echinacea, and alkaloids (ephrine, pseudoephedrine, phenylpropanolamine) from ephedra. Supplements are made from echinacea roots or flowering tops and ephedra stems. Taxonomically, Echinacea is an American angiosperm genus of nine species, whereas Ephedra is a global gymnosperm genus of about 50 species. Vernacularly, “echinacea” commonly refers to three species (Echinacea angustifolia, Echinacea pallida and Echinacea purpurea), whereas “ephedra” often refers to one Asian species (Ephedra sinica, Ma Huang) or sometimes a supplement containing one isolated alkaloid. Supplements from both plants may be purchased over the counter; additionally, ephedra alkaloids are components in cough syrups, decongestants and diet aids. Ephedra sympathomimetic alkaloids are structurally similar to methamphetamine, releasing catechoalamines and acting on cellular alpha and beta receptors and adrenoreceptors; these actions increase cardiovascular variables. Studies have looked at ergogenic effects of ephedra by dosing athletes with isolated alkaloids (usually pseudoephedrine, usually up to 120 mg) and together have reported non-significant and often heterogeneous results, reviewed elsewhere. One study that supplemented athletes with a whole herb supplement found no effect of 60 mg of ephedra when used in conjunction with 300 mg of caffeine on strength or power. Despite widespread public belief, ephedra does not have ergogenic properties in applicable contexts. However, it does carry serious adverse effect risks. The World Anti-Doping Agency (WADA) prohibits urinary concentrations of ephedrine and methylephedrine of >10 μg/ml and pseudoephedrine >150 μg/ml. Clinical studies of echinacea supplements typically utilise commercial whole herb formulations from above-ground parts and focus on immune enhancing outcomes. Research on athletes given echinacea supplements (doses varied or not given) reported upper respiratory tract infection prophylaxis and good tolerability with few adverse effects. Echinacea is a relatively modern remedy, studied first in the late 19th century as opposed to those herbs studied for centuries. Ephedra has been used in Chinese medicine for thousands of years. It became widely used in the late 1920s when its apparent ergogenic properties were observed. Despite several attributed fatalities and many adverse events, it was only after a notorious sporting fatality in 2003 that ephedra was banned [10391].

In recent years, the use of herbal preparations containing Ephedra has increased and so has the number of reported negative health effects. Ephedra products are used as a slimming aid, (sports) performance enhancer, stimulant during long working hours and as a plant-based ecstasy-surrogate in the party scene. The health effects caused by the use of Ephedra products are mainly restlessness, agitation, tachycardia and palpitations. The most important risk factors are the relatively high amounts of Ephedra alkaloids in the preparations available, the varying concentrations within different batches of the same product, the relatively high risk of voluntary overdosing in order to improve results and the occurrence of interactions with the concurrent use of other stimulants and medicines [10503].

Ephedrine is a stimulant with a chemical structure closely related to amphetamine. It is derived from ephedra herbs also known as ma huang. It possesses alpha and beta adrenergic agonistic effects, enhances the release of norepinephrine, and stimulates the central nervous system. Pseudoephedrine is closely related and also possesses central nervous system stimulant properties in addition to being a popular decongestant. Previously available ephedrine compounds in the US included such brands as Metabolife356 and Ripped Fuel. Before being banned in 2004, the FDA recommended that daily ephedra alkaloid intake remain under 25 mg and not be continued longer than 1 week. Pseudoephedrine in cold remedies is taken in maximal amount at 60 mg. Combining ephedrine with caffeine or its herbal form guarana poses significant risk and was prohibited by the FDA in the 1980s [06003].
The sympathomimetic drug ephedrine is used to treat symptoms of infection with the cold virus. It was originally prescribed as a bronchodilator for asthma, although it is now regarded as less suitable for this use since it has been linked with cardiac arrhythmia. Ephedrine is likely to be misused for its stimulant effect, but can also be ingested inadvertently because of its widespread availability in over-the-counter medicines [06171].

One study examined trends in stimulant use and attitudes toward use among American collegiate hockey athletes. All 139 players in one college hockey conference completed a comprehensive questionnaire. Over half of the athletes (52 %) confirmed stimulant use before a hockey game or practice. About half of the respondents (49 %) reported having used ephedra at least one time to improve athletic performance. Additionally, 17 percent reported using pseudoephedrine to improve performance in the 30 days prior to survey administration. Over half (55 %) were aware of the recent national ban on ephedra. Fifty-nine percent stated the national ban made them less likely to use ephedra products. The majority of athletes began use prior to college. Coaches, athletic trainers, and team physicians should be aware of athletes' patterns of stimulant use. Improved educational efforts directed at younger athletes are necessary to deter abuse of metabolic stimulants [06177].

Ephedra alkaloids, which are popular components of many nutritional supplements, are naturally occurring CNS stimulants obtained from several Ephedra species. Historically, Ephedra alkaloids have been used for both asthma and allergies in China for more than 5000 years. Currently, it is found in various pharmaceuticals, mainly as a decongestant, and in numerous nutritional and dietary supplements as an energy stimulant and anorexic agent. Pseudoephedrine can be found in prescription and over-the-counter preparations for respiratory infections or allergies (mostly for the treatment of congestion). Until its recent voluntary removal from the market because of reports of increased risk for stroke in women, phenylpropanolamine was also used similarly to pseudoephedrine and in over-the-counter diet pills. Purified forms of these substances include ephedrine, pseudoephedrine, norephedrine, methylephedrine, norpseudoephedrine and methylpseudoephedrine. Phenylpropanolamine is a synthetic compound functionally similar to the ephedra alkaloids in effect and use. Ephedrine, which is now also produced by chemical synthesis, is closely related in structure to metamfetamine, although its CNS actions are much less potent but longer acting than those of the amphetamines. Its peripheral stimulant actions are similar to, but less powerful than, those of adrenaline (epinephrine), a hormone produced in the body by the adrenal glands [06171].

**Chinese preparations**

Sho-seiryu-to is one of the most common Traditional Chinese Medicine preparations for the attenuation of colds. Ephedrae Herba is one of the prescriptions of Sho-seiryu-to. The major ingredients of Ephedrae Herba, ephedrines, are banned substances on the WADA list. The purpose of one study was to investigate the elimination of urinary ephedrines after administering Sho-seiryu-to preparation and to determine the possibility of positive ephedrines test results in urine. Six healthy volunteers took a single 2.5-g dose of concentrated Sho-seiryu-to preparation. All urine was collected for 48 h. The concentrations of urinary ephedrines were analyzed by high-performance liquid chromatography and the elimination half-life of the ephedrines was estimated. The results show that ephedrine and cathine (norpseudoephedrine), the prohibited substances of the WADA, were excreted in the urine after taking a single dose of Sho-seiryuto preparation. The peak concentration of ephedrine was 3.9 ± 1.9 mg/mL, which was lower than the WADA permitted value (10 mg/mL). The estimated elimination half-lives of ephedrine, norephedrine, pseudoephedrine, and norpseudoephedrine following administration of this preparation were 5.3 ± 1.2, 4.9 ±
The study concluded that the urine would not violate the antidoping rules after administering a single dose of Sho-seiryu-to preparation. Nevertheless, an applied multiple-dose study upon administering the preparation for three times per day for three days showed a positive urine ephedrine result (13.7 mg/mL). Athletes should be careful when taking more than a single dose of Sho-seiryu-to preparation [09261].

Kakkon-to is one of the most common traditional Chinese medicine preparations for the attenuation of colds. Ephedrae Herba is one of the prescriptions of Kakkon-to. The major ingredients of Ephedrae Herba, ephedrines, are banned substances on the WADA list. The purpose of one study was to investigate the elimination of urinary ephedrines after administering Kakkon-to and to determine the possibility of urinary positive ephedrine test results. Six healthy volunteers took one single dose of 2.5 g Kakkon-to extract granules. The concentrations of urinary ephedrines were analyzed by high-performance liquid chromatography. The result showed that ephedrine and norpseudoephedrine were excreted in the urine after taking one single dose of Kakkon-to. However, the highest amount of ephedrines in urine was ephedrine and the peak concentration was $4.4 \pm 1.8$ microg/mL, which was lower than the WADA permitted value (10 microg/mL). The estimated elimination half-lives of ephedrine, norephedrine, pseudoephedrine, and norpseudoephedrine following administration of this preparation were: $5.2 \pm 1.2$, $4.2 \pm 1.3$, $4.2 \pm 0.9$, and $6.5 \pm 2.8$ h, respectively. The study concluded that the urine would not violate the rule of doping after administering a single dose of Kakkon-to. Nevertheless, a further study on administering the preparation for 3 times per day for 3 days showed a positive ephedrine result. Athletes should be careful when taking more than a single dose of Kakkon-to [08303].

**Use in women**

Ephedra is a dietary supplement used for performance enhancement due to its stimulant effects. Ephedra contains the alkaloids ephedrine and pseudoephedrine. It is a sympathomimetic and has a chemical structure close to amphetamine. Its use as a performance-enhancing supplement was popularized due to reported improvement in weight loss, energy levels, and athletic performance. Ephedra and ephedra-containing products were removed from the market in 2004 in the US; ephedra is the only dietary supplement that has been removed since the creation of the Dietary Supplement Health and Education Act in 1994. Nonetheless, the ban has not stopped its use by athletes 07[086].

**Prevalence**

The NCAA study of substance use habits of college student-athletes found that ephedra use increased in female athletes from 2001 to 2005 despite highly publicized adverse health effects. Prevalence of ephedrine use increased from 3.2 to 11.9 percent in women's ice hockey and 0.3 to 2.7 percent in lacrosse from 2001 to 2005, while prevalence increased or stayed the same in women's basketball, softball, and tennis. It was found that, of 270 high school athletes, 26 percent of girls and 12 percent of boys have tried ephedrine products [07086].

**Legal affairs**

In 2004, ephedrine was the first supplement removed from the US market by the FDA since the 1994 supplement act. However, in April 2005 a US District Court judge ruled to overturn the ban, stating that the FDA had not proved that a 10-mg dose was dangerous. She ruled that “[the prior ban] would be directly contrary to the statutory language placing the burden of proof on the government…. ” It is likely that this legal battle will continue with possible appeals by the FDA, although this ruling may open the door to ephedrine's return to the
supplement marketplace. The International Olympic Committee, NCAA, Major League Baseball, National Basketball Association, and National Football League all have banned the systemic use of ephedrine products [06003].

Physiological effects

Ephedrine traditionally has been used by athletes to provide quick energy and to aid in fat loss, consequently improving speed and appearance. The majority of studies that are available show no change in athletic performance with ephedrine use. A few investigations have reported improvements in quadriceps strength or stationary cycling, but these have involved high doses of ephedra compounds and sometimes were combined with caffeine, which poses significant risk to the athlete [06003].

Ephedrine is the most potent thermogenic of the Ephedra alkaloids. It is a mixed sympathomimetic agent which acts as a CNS stimulant by enhancing the release of noradrenaline from sympathetic neurones and stimulating alpha and beta receptors. Ephedrine not only stimulates the heart rate and thereby increases cardiac output, but also causes peripheral constriction, resulting in an increase in peripheral resistance, which can lead to a sustained rise in blood pressure. Ephedrine has moderately potent bronchial smooth muscle relaxant properties, and is used as a decongestant and for temporary relief of shortness of breath caused by asthma. Ephedrine is excreted in the urine largely unchanged and the usual elimination half-life is three to six hours which can be prolonged with increased urine pH [06171].

Side effects

It has been reported that ephedra accounted for 0.82 percent of herbal product sales but was implicated in 64 percent of adverse herbal reactions in the United States in 2001. Adverse reactions are wide ranging and include hypertension, arrhythmias, anxiety, tremors, insomnia, seizures, paranoid psychoses, cerebral vascular accident, myocardial infarction, and death. In fact, in athletic performance studies, the most consistent finding is increased heart rate. In 2000 critically it was evaluated the 140 adverse events reported to the FDA from 1997 to 1999 and concluded that 62 percent of the adverse events reported were definitely or possibly related to the drug. In that series, 10 users were younger than 18 years, and there were 10 deaths and 13 cases of permanent disability. These included a 22-year-old man who was taking Ripped Force and sustained a cardiac arrest and was left with a permanent neurologic disability and a 15-year-old girl who was taking Ripped Fuel and sustained a fatal arrhythmia [06003].

The common side effects of ephedrine are qualitatively similar to those produced by amfetamines but are generally milder: headache, dizziness, irritability, anxiety, tremor, and psychosis. Higher doses (overdose) can cause restlessness and anxiety, dizziness, insomnia, tremor, rapid pulse, sweating, respiratory difficulties, confusion, hallucinations, delirium, and convulsions. The most dangerous symptoms of overdose are abnormally high blood pressure and rapid, irregular heartbeat. A dose of ephedrine only two or three times the therapeutic maximum can cause a significant increase in blood pressure. Finally, a number of instances of psychosis, clinically similar to amphetamine psychosis, have resulted from chronic high-dose misuse. There are serious doubts concerning the safety of food supplements containing ephedra alkaloids. Because supplements are not considered therapeutic, they are not held to the same level of rigor in claiming efficacy and safety as that required of prescribed and over-the-counter medicines. Since the 1994 deregulation, an increased number of reports of adverse events, including hypertension, arrhythmia,
myocardial infarction, seizure, cerebrovascular accidents and death, has prompted the US Food and Drug Administration to recommend a limit on the use of ephedra alkaloids. Furthermore, joint use of ephedrine and caffeine can augment adverse cardiovascular and CNS effects [06171].

To evaluate ephedra's effects on weight loss, athletic performance, and adverse events, it was conducted a meta-analysis of trials using ephedra and similar compounds. They concluded that, compared with placebo, ephedra had a weight loss of 0.6 kg/months (95% confidence interval 0.2 to 1.0). Seven trials using different exercise type and outcome measures did not show significant improvement on exercise performance. This conclusion was supported by another study where it was concluded that standard ephedra doses did not have a performance-enhancing effect. Safety data from 50 trials produced an estimated risk 2.2 to 3.6 times that of developing psychiatric, autonomic, or gastrointestinal symptoms, and heart palpitations. No clinical trials have been specifically performed in women athletes to evaluate ephedrine's performance-enhancing effect [07086].

Much of the literature has focused on ephedra's adverse effects related to the cardiovascular and central nervous systems. In one study it was examined 140 cases submitted to the US Food and Drug Administration (FDA) between 1997 and 1999. Adverse effects included hypertension, arrhythmia, myocardial infarction, hemorrhagic stroke, seizure, and death. Thirty-one percent of cases were definitely or probably related to ephedra substances, while another 31 percent were possibly related. In many cases, ephedra was combined with caffeine [07086].

Heart
These are usually found through the internet or on the sports black market under names like “ma-huang”, “herbal Ecstasy”, and so on. Very frequent cardiovascular complications have been described as a result of their use, such as arrhythmias, hypertension, acute myocardial infarction, stroke, myocarditis, dilated cardiomyopathy, and sudden death. The pathophysiological mechanism of action is similar to that of the other sympathomimetic amines [12126].

Little has been done by way of measuring both the acute/chronic effects of stimulant use/abuse on a range of CV risk factors. While significant CV events are associated with their use, the effect on long-term atherosclerotic risk even with moderate usage, through negative alterations in known CV risk factors is worthy of further examination. All stimulants structurally related to amphetamine can cause catecholamine-mediated cardiotoxicity. Increased catecholamine levels can lead to vasoconstriction, vasospasm, tachycardia and hypertension and it is as a result of these responses that oxygen supply to the heart is compromised and hypertrophy, fibrosis and necrosis can result. Clearly, such conditions develop over time, as a consequence of chronic exposure and the repercussions may include myocardial infarction, aortic dissection and sudden cardiac death. There have been numerous reported cases of serious adverse cardiovascular (CV) events, including fatalities, linked with the use of ephedrines. The use of ephedrine has also been linked to cardiomyopathy and stroke while pseudoephedrine use has also been linked with stroke and coronary artery spasm with myocardial infarction. Further, there are numerous case reports of significant cardiovascular events following the administration of over-the-counter stimulants. As with AS use, the interpretation of such case reports is difficult, since it may be that serious adverse events are as a consequence of preexisting medical conditions combined with drug use. What case studies do promote is further experimental study. For example, doses equivalent to three to four times greater than the recommended therapeutic dose of pseudoephedrine have raised diastolic blood pressure above 90 mm Hg. These results were in accord with two other studies: it was reported significant increases in heart
rate and systolic blood pressure following relatively high doses of pseudoephedrine (120 and 180 mg), and noted that doses of 120 and 180 mg produced statistically significant increases in both pulse and systolic blood pressure. The clinical relevance of these blood pressure changes is not known. Reports of the CV effects of sympathomimetics in therapeutic doses have been conflicting. It was observed that a single dose of ephedrine (25 mg) significantly elevated both heart rate and systolic blood pressure while a single therapeutic dose of pseudoephedrine (60 mg) significantly elevated only systolic arterial blood pressure. However, it was noted little change in CV function following therapeutic doses. Increased blood pressure has been demonstrated in cases whereby ephedrines have been co-administered with a moderate dose of caffeine. Caffeine is thought to exacerbate the action of ephedrine's since it too may cause vasoconstriction through antagonism of adenosine and release of catecholamines [12114].

**Ephedrine in sport**

With their stimulant properties and sympathomimetic actions, ephedra alkaloids have been perceived as products that can potentially be used to enhance athletic performance and lending unfair advantages to athletes, even if used in supplement forms. Research has shown that the isolated use of ephedrine, pseudoephedrine and phenylpropanolamine alone at usual dosages has an inconsistent, and probably insignificant, ergogenic benefit for power, endurance, strength, or speed. Other studies looking at the use of ephedrine combined with vitamins, minerals, or caffeine have supported potential ergogenic effects. Indeed, many athletes use food supplements containing ephedra alkaloids because of perceived benefits of increased energy, decreased time to exhaustion and potential thermogenic properties with increased metabolism, increased fat loss, and improved muscle strength. In particular, a series of studies evaluated the effects of ephedrine in combination with caffeine showing an increased time to exhaustion and decreased rating of perceived exhaustion on cycle ergometry compared with either drug alone or placebo. The medical use of ephedrine is tolerated by WADA and IOC at therapeutic levels. Nevertheless, urine concentrations of greater than 10 microg/ml are considered positive. Ephedrine is a category S6 prohibited substance [06171].

**Side effects of ephedrine in relation to sport**

The recent highly publicised tragedies have prompted various athletic associations to focus on further evaluations of the use of these substances and on trying to educate athletes about potential health risks associated with their use. Continued evaluation of the use of these substances is necessary, as is continued education of athletes, parents, coaches, and trainers regarding the health risks associated with ephedrine alkaloids and corresponding supplements [06171].

**Fatal effect**

Ephedrine or ephedra herbal products have occasionally been used to enhance sports performance and energy or to aid weight loss. The most serious side effects are those on cardiovascular function, including acute myocardial infarction, severe hypertension, myocarditis and lethal cardiac arrhythmias. A 19-year-old woman was taking ephedrine to enhance her sports performance. After 10 days of this medication she developed hemodynamically unstable ventricular tachycardia resistant to cardioversion and amiodarone treatment. She converted to sinus rhythm 60 hours later, presumably when the plasma ephedrine level had sufficiently decreased. In an electrophysiological study the ventricular tachycardia could be induced and successfully ablated. There were no recurrences during follow-up of more than a year. The use of ephedrine carries a risk of development of life-threatening arrhythmias. Ephedrine alone cannot be considered as the ultimate cause of tachycardia in our patient; however, it is highly probable that ephedrine triggered the
tachycardic attack. The proarrhythmic effect most likely occurred because of underlying idiopathic left ventricular tachycardia. Although the patient could have developed her first attack of ventricular tachycardia at any time in her life, it is highly improbable that the attack following the ephedrine abuse was purely coincidental. The experience with the reported patient shows that ephedrine alone, or in combination with substances that increase its effects on the cardiovascular system, may also trigger paroxysms of non-ischemic ventricular tachycardia. The use of ephedrine carries a risk of development of life-threatening arrhythmias and should be discouraged [06178].

Dietary supplements containing ephedrine and other alkaloids related to ephedrine are largely consumed in various countries, with the purpose of energetic stimulation and weight loss. Despite the fact that it is not approved for marketing in Brazil, these products may be freely purchased over the Internet or at gyms/fitness centers. It was reported a case of a young athlete with no risk factors for cardiovascular disease who experienced a myocardial infarction during the period in which he used an ephedrine-rich supplement [06179].

**Laboratory technique**

The separation and quantification of hydrophilic basic compounds continues to challenge reversed-phase chromatography. Ephedrines are an example where the optimal separation of their isomers and related substances is complicated due to both their hydrophilicity and basic nature. Here we study two potential ultra-high pressure liquid chromatography (UHPLC) methods and present the merits and limitations of a high pH reversed-phase and a hydrophilic interaction liquid chromatography (HILIC) approach for the separation and quantification of ephedrines for doping control analysis. The study compares a hybrid silica material used for the HILIC separations with a C18 reversed-phase material produced from the same hybrid silica. While both analytical approaches provide good retention and resolution, HILIC offers benefits in terms of peak shape, sample loading capacity and enhanced sensitivity with electrospray ionisation-mass spectrometry (ESI-MS). HILIC permits favourable kinetic performance owing to the low viscosity mobile phase and hence better mass transfer characteristics. Common problems associated with HILIC including retention shifts and undesirable peak shapes are investigated and overcome using a suitable re-equilibration time and injection solvent. Matrix effects were shown to have a negligible effect on ionisation variability in each mode, with inter-day retention times also being repeatable (<0.17 % RSD). HILIC gave increased sensitivity with ESI-MS, giving a 6-fold increase in signal over the RPLC approach. In this application, we demonstrate the use of UHPLC technology coupled with a hybrid quadrupole time-of-flight (QToF) mass analyser. This approach provides fast scanning medium-resolution accurate mass detection for reliable identification and quantification purposes [13476].

Among the stimulants prohibited by WADA, four (cathine, ephedrine, pseudoephedrine, and methylephedrine) are not considered as anti-doping rule violation when their urinary concentration remains below their respective threshold level. Hence, quantitation is required, and Gray et al. compared the performance of reversed-phase and hydrophilic interaction LC in combination with a medium-resolution (ca. 10 000 FWHM) TOF MS set-up concerning separation capability and general fitness-for-purpose for doping control analyses. Urine samples were prepared for analysis by a 45-fold dilution in mobile phase followed by another dilution step (1:1, v/v) with a volume containing the isotopically labelled internal standard. Subsequently, the samples were analyzed on either a C-18 (2.1 x 50 mm, particle size 1.7 µm) or a HILIC (2.1 x 100 mm, particle size 1.7 microm) column, connected via ESI to a Q/TOF MS operated in positive ionization mode. Both approaches allowed baseline separation of the target analytes within 5 min and fulfilled the desired criteria in terms of sensitivity, accuracy, precision, and specificity [13009].
A simple method for the determination of ephedrine alkaloids: ephedrine (EF), pseudoephedrine (PE), norpseudoephedrine (NPE), norephedrine (NE) and methylpseudoephedrine (MPE) in dietary supplements by gas chromatography-mass spectrometry is described. After the addition of 3,4-methylenedioxypropylamphetamine as internal standard, a liquid-liquid extraction procedure in alkaline conditions with chloroform/isopropanol (9:1, v/v) was applied to the samples prior to analysis. Chromatography was performed on a fused capillary column and analytes, derivatized with pentafluoropropionic anhydride, were determined in the selected-ion-monitoring (SIM) mode. The method was validated in the range 0.3-10 microg/mg for EP, 0.06-2.5 microg/mg for PE and NPE and 0.04-1 microg/mg NE and MPE. Mean recovery ranged between 66 and 81 percent for the different analytes in dietary supplements. The quantification limits were 0.3 microg/mg for EP, 0.06 microg/mg for PE, 0.04 microg/mg for NE and MPE. The method was applied to analysis of various dietary supplements containing Ma-huang (Ephedra Sinica) and Sida Cordifolia plant extracts promoted for aiding weight control and boosting sports performance and energy [06180].

Ephedrine (EPH), pseudoephedrine (PEPH), phenylpropanolamine (PPA), methylephedrine (MEPH) and cathine are sympathomimetic amines. These drugs are commonly found in over-the-counter (OTC) cold medicines and some dietary supplements. In Taiwan, the misuse of these drugs has resulted in an increase in athletic violations. Excretion studies of the ephedrine-related drugs have been performed to better understand the metabolic yields of ephedrines in urine. After consuming a single clinical dose of each of these drugs, urine samples from volunteers (n=3 for each drug) were subjected to tert-butyl-methyl-ether (TBME) extraction and trifluoroacetic acid (TFAA) derivatization before gas chromatography-mass spectrometry (GC-MS) analysis. Most ephedrines were excreted unchanged in urine, including EPH (41 %), PEPH (72 %), and PPA (59 %). However, only a relatively small amount of MEPH (16 %) was excreted unchanged in urine. In addition, an trace amount of PPA (2 %) and cathine (1 %) was found to be the metabolites of EPH and PEPH, respectively. Urinary EPH, PEPH, and PPA reached peaks at 2-6h and disappeared in urine at approximately 24-48 h post-administration. For MEPH, the peaks of excretion extended from 4 to 12h post-administration and were undetectable at approximately 48 h. A single clinical dose of EPH (25 mg) may exceed threshold level (10 microg/mL) in sport drug testing if the urine samples are tested within approximately 8h post-administration. However, a single dose of MEPH (20 mg) never reached the threshold value (10 microg/mL) [06181].

A rapid and simple liquid chromatography tandem mass spectrometry method was developed and validated for the simultaneous determination of L-ephedrine, pseudosephedrine, and caffeine in male Fisher-344 rat plasma at nanogram-per-milliliter concentrations for use in support of toxicology studies. Only 25 μL of plasma is required, and extraction is performed using a simple, single-step protein precipitation. The method was validated over a range of 2.09 to 5460 ng/mL for L-ephedrine, 2.09 to 5050 ng/mL for pseudoeephydine and 2.03 to 5340 ng/mL for caffeine. The method was evaluated for linearity, recovery, precision, accuracy, and stability, and it was successfully applied in toxicokinetic studies of ephedrine, administered alone, in combination with caffeine, and in the herbal source Ma Huang [11210].

The compound 3,4-dimethyl-5-phenyl-1,3-oxazolidine can appear as an artifact during the gas chromatographic analysis of ephedrines. Its presence is a risk for doping control and forensic analyses. An evaluation about the consequences of its formation showed the possibility of a false positive for ephedrine, a false negative for pseudophedrine and increased uncertainty in the quantitative approach. Misinterpretations can be avoided with the observation of fragments m/z 56 and 71 in the ephedrine mass spectrum during GC-MS.
analysis and also by the formation of N-TFA-O-TBDMS derivatives prior to GC analysis. These N-TFA-O-TBDMS derivatives lead to an increase in the number and mass of diagnostic ions, meet the identification criteria, and provide an improvement in chromatographic resolution, allowing the separation of the ephedrines [11211].

A simple, rapid and sensitive CE-ESI-MS method for the simultaneous analysis of seven stimulants and narcotics (amphetamine, ephedrine, methadone, pethidine, tetracaine, codeine and heroin) was developed. The CE-ESI-MS experimental conditions were optimized as follows: 20 mmol/L ammonium acetate with pH 9.0 as running buffer, the separation voltage of 22 kV and the sheath liquid of isopropanol/water (1:1 v/v) containing 7.5 mmol/L acetic acid with 3.0 μL/min flow rate. Under the optimized conditions, the stimulants and narcotics were well separated within 4.6 min using a 70-cm length fused-silica capillary (50 μm id). The detection limits (S/N=3) of the CE-ESI-MS analysis were in the range of 0.40-1.0 ng/mL. Method repeatability of intra-day and inter-day was satisfactory. The recoveries obtained from the analysis of spiked urine samples were between 84.1 and 108%. The developed method was successfully applied for the simultaneous analysis of methadone, pethidine and codeine and their in vitro metabolites [11212].

A simple and sensitive HPLC technique was developed for the qualitative determination of ephedrine and pseudoephedrine (ephedrines), used as precursors of clandestine d-methamphetamine hydrochloride of high purity. Good separation of ephedrines from bulk d-methamphetamine was achieved, without any extraction or derivatization procedure on a CAPCELLPACK C(18) MGII (250 × 4.6 mm) column. The mobile phase consisted of 50 mM KH₂PO₄-acetonitrile (94:6 v/v %) using an isocratic pump system within 20 min for detecting two analytes. One run took about 50 min as it was necessary to wash out overloaded methamphetamine for column conditioning. The analytes were detected by UV absorbance measurement at 210 nm. A sample (20 mg) was simply dissolved in 1 mL of water, and a 50 μL aliquot of the solution was injected into the HPLC. The detection limits for ephedrine and pseudoephedrine in bulk d-methamphetamine were as low as 3 ppm each. This analytical separation technique made it possible to detect ephedrine and/or pseudoephedrine in seven samples of high-purity d-methamphetamine hydrochloride seized in Japan. The presence of trace ephedrines in illicit methamphetamine may strongly indicate a synthetic route via ephedrine in methamphetamine profiling. This method is simple and sensitive, requiring only commonly available equipment, and should be useful for high-purity methamphetamine profiling [11213].

Solvent systems for use with LC-MS often result in a compromise between chromatographic performance and mass spectrometric detection, exemplified here by a LC-MS/MS method development for the analysis of ephedrines in doping control. Ephedrines, frequently found in therapeutic and nutritional preparations, are among the most commonly administered doping agents in competitive sport. Improved separation of these hydrophilic, basic compounds, some of which are diastereoisomers, is achieved in reversed-phase LC by the use of a high pH mobile phase in order to suppress analyte ionisation, and thus alter their polarity, resulting in reduced peak tailing and enhanced retention. However, when coupled to an ESI-MS detector, this eluent composition generated a non-linear and poorly reproducible signal. APCI yielded greater stability and reproducibility and is here presented as an ion source for the analysis of basic compounds under conditions that suppress their ionisation. Errors as large as 49 percent were observed with ESI, compared with 15 percent generated using APCI, for pseudoephedrine over the calibration range (25-400 μg/mL) in urine with a simple dilution and injection of samples. These data highlight the importance of suitable MS conditions for stable performance, necessary for accurate quantification, without undue compromise to the LC separation [11214].
In one paper, a fast and economical HPLC method (on a Phenomenex Polar-RP column with a solution of phosphoric acid:triethylamine:diisobutylamine:water and methanol) was developed, and applied for the determination of norephedrine, norpseudoephedrine, ephedrine (E), pseudoephedrine (PE) and methylephedrine (ME) in 64 samples of three species from main habitats in China. Quantitation data showed that total alkaloid content in Ephedra equisetina Bge. is higher than that in Ephedra sinica Stapf. and Ephedra intermedia Schrenk et C. A. Mey., but the range of total alkaloid content in each species is so wide that the ranges of the three species greatly overlap. The contents of E, PE and ME are different among the three species. The ratio E/total alkaloid content and ratio E/PE as well as E and ME contents can be used for identification of Ephedra sinica, Ephedra intermedia and Ephedra equisetina from one another [11215].

Ephedrines are sympathomimetic amines which have central nervous system stimulating properties and, for this reason, some of them are forbidden in sport by the World Anti-Doping Agency. The amines are screened and quantitated in urine by several published techniques and confirmed by gas chromatography/mass spectrometry (GC/MS). In one paper, a simple and easy confirmation procedure for norpseudoephedrine, norephedrine, ephedrine and pseudoephedrine in human urine by GC/electron ionization (EI)-MS was described. After the addition of diphenylamine as internal standard, a liquid-liquid extraction procedure under alkaline conditions with tert-butyl methyl ether was applied to the samples. The analytes were derivatized with acetone and pyridine to form the correspondent oxazolidine derivatives (acetonide). The EI mass spectra of all the studied substances have many diagnostic ions with relative abundance in accordance with WADA requirements and show great structural information [08302].

One article described a method for the detection and quantitation of cathine, pseudoephedrine, ephedrine, and methylephedrine in urine, using their deuterated analogues as internal standards and derivatization to form the corresponding trimethylsilyl derivatives after a simple liquid-liquid extraction. The study was designed to evaluate whether the urinary cutoff values set by the World Anti-Doping Agency for the banned ephedrines (cathine >5 microg/mL, ephedrine and methylephedrine >10 microg/mL) can be exceeded after the normal self-administration of common over-the-counter medicaments containing nonbanned ephedrines. The present method, after validation, has been applied on real urine samples obtained from 9 healthy volunteers taking different doses of over-the-counter preparations containing ephedrines. Results obtained from excretion studies show high interindividual differences in the urinary concentrations of both pseudoephedrine and cathine, not dependent on body weight or sex nor, in some instances, on the administered dose. The same typical therapeutic dose of pseudoephedrine (60 mg) produced a urinary concentration of more than 5 microg/mL for cathine and of more than 100 microg/mL for pseudoephedrine in 2 of 9 subjects only. When a dose of 120 mg was administered, cathine concentration exceeded 5 microg/mL in 4 of 7 subject, and also concentrations of pseudoephedrine above 100 microg/mL. After administration of 5 x 120 mg of pseudoephedrine (120 mg administered every 7 days for 5 weeks) to one of the subjects exceeding the urinary threshold values, the urinary concentration of cathine and pseudoephedrine exceeded 5 microg/mL (peak concentration 14.8 microg/mL) and 100 microg/mL (peak concentration 275 microg/mL), respectively. When the same subject took 180 mg of pseudoephedrine, the urinary concentration values were below 5 microg/mL for ephedrine and 100 microg/mL for pseudoephedrine. In the case of ephedrine administration in a sustained-release formulation containing 12 mg of ephedrine, 2 of 3 subjects exceeded the urinary cutoff value of 10 microg/mL. The high interindividual variability is still significant even if the urinary concentration values are adjusted by specific gravity and/or creatinine. These results confirm a high interindividual variability in the urinary concentration of ephedrines after the administration of the same therapeutic dose of a preparation [09260].
A novel capillary electrophoresis (CE) method coupled with monolithic molecular imprinted polymer (MIP) fiber based solid phase microextraction (SPME) was developed for selective and sensitive determination of ephedrine (E) and pseudoephedrine (PE). With in situ polymerization in a silica capillary mold and E as template, the MIP fibers could be produced in batch reproducibly and each fiber was available for 50 extraction cycles without significant decrease in extraction ability. Using the MIP fiber under optimized extraction conditions, CE detection limits of E and PE were greatly lowered from 0.20 to 0.00096 microg/mL and 0.12 to 0.0011 microg/mL, respectively. Analysis of urine and serum samples by the MIP-SPME-CE method was also performed, with results indicating that E and PE could be selectively extracted. The recoveries and relative standard deviations (RSDs) for sample analysis were found in the range of 91-104 percent and 3.8-9.1 percent, respectively [11496].

In one study, hollow fiber liquid phase microextraction (HF-LPME) based on pH gradient and electromembrane extraction (EME) coupled with high-performance liquid chromatography (HPLC) was compared for the extraction of ephedrine from biological samples. The influences of fundamental parameters affecting the extraction efficiency of ephedrine were studied and optimized for both methods. Under the optimized conditions, preconcentration factors of 120 and 35 for urine and 51 and 8 for human plasma were obtained using EME and HF-LPME, respectively. The calibration curves showed good linearity for urine and plasma samples by both methods with the coefficient of estimations higher than 0.98. The limits of detection were obtained 5 and 10 ng/mL using EME and 60 and 200 ng/mL by HF-LPME for urine and plasma samples respectively. The relative standard deviations of the analysis were found in the range of 5.2-8.6 percent (n=3). The results showed that in comparison with HF-LPME based on pH gradient, EME is a much more effective transport process, providing high extraction efficiencies in very short time [11497].

In oral fluid
One study was designed to optimize a method for the identification and quantification of ephedrines in oral fluid (OF) and for its application to subjects taking different doses of pseudoephedrine. Ephedrines use by athletes is banned by World Anti-Doping Agency (WADA), only "in competition" if their concentration in urine exceeds the cutoff limit. The study aimed to establish if there is a correlation in terms of times of elimination and of concentration trends of ephedrine in OF and urine after administration of therapeutic doses of pseudoephedrine to various subjects. Results obtained from excretion studies performed on eight subjects showed reproducible times of disappearance of ephedrines from OF. Pseudoephedrine was generally at low concentrations or undetectable in oral fluid samples 12 h after administration, whereas urine samples collected in the same period of time showed higher ephedrine concentrations and exceeding cutoff values generally between 8 and 24 h after administration of the drug. Within- and between-individual variability was observed in terms of concentrations of pseudoephedrine in OF following the administration of the same dose. Only in the case of sustained-release drugs were constant pseudoephedrine concentrations achieved in OF [10187].

Pseudoephedrine
Since the re-introduction of pseudoephedrine as a banned stimulant in 2010, an adverse analytical finding is to be reported by doping control laboratories when urinary concentrations greater 150 microg/ml are determined. This threshold value was deduced from two studies with healthy subjects receiving a total of 240 mg of pseudoephedrine within 24 or 48 h using different administration regimens and drug formulations. Peak concentrations were found between 100 and 175 microg/mL [13012].
Pseudoephedrine is an over-the-counter drug to relieve nasal and sinus congestion. Although it has been suggested that pseudoephedrine could be a stimulant and ergogenic aid, pseudoephedrine was recently removed from the banned substance list by the International Olympic Committee and placed on the monitoring program (from January 2004). It was felt that evidence was lacking for an ergogenic effect, although few studies have investigated the effects of pseudoephedrine on exercise performance. One study, therefore, aimed to investigate the effects of pseudoephedrine on 1500-m running performance. In a double-blind, randomized crossover design, seven male athletes completed two 1500-m running trials on an outdoor track after having completed a familiarization trial. All trials were 7 d apart. After a 12-h overnight fast, subjects reported to the laboratory and received a standardized breakfast (energy asymptotically equal to 500 kcal 50% CHO). Subjects were given either 2.5 mg/kg bw pseudoephedrine or 2.5 mg/kg bw maltodextrins (placebo) in gelatin capsules 70 min before the start of the warm-up, which started 20 min before they ran 1500 m all-out. Pre- and postexercise blood samples were collected and analyzed for lactate and glucose concentrations, partial pressure of oxygen (PO$_2$) and carbon dioxide (PCO$_2$), and percent oxygen saturation. Pseudoephedrine significantly decreased time to completion of 1500-m time trials in the present study by 2 percent with no reported side effects. No changes in the measured blood parameters were found, suggesting a central effect of pseudoephedrine rather than a metabolic effect. It was concluded that the finding was that 2.5 mg/kg bw pseudoephedrine ingested 90 min preexercise improves 1500-m running performance [06182].

The Drug Enforcement Administration (DEA) is removing the thresholds for importation, exportation, and domestic distributions of the List I chemicals pseudoephedrine and phenylpropanolamine. This rulemaking is being conducted as part of DEA's implementation of the Combat Methamphetamine Epidemic Act of 2005 and is needed to implement the Act's requirements for import and production quotas and to address the potential diversion of these chemicals. DEA is also clarifying that all transactions of drug products containing ephedrine, pseudoephedrine, and phenylpropanolamine, except retail transactions, are considered to be regulated transactions [10392].

The aim of one study was to investigate the effect of 180 mg of pseudoephedrine (PSE) on cycling time-trial (TT) performance. Six well-trained male cyclists and triathletes underwent two performance trials in which they completed a 25-min variable-intensity (50-90 % maximal aerobic power) warm-up, followed by a cycling TT in which they completed a fixed amount of work (7 KJ/kg body mass) in the shortest possible time. Sixty minutes before the start of exercise, they orally ingested 180 mg of PSE or a cornstarch placebo (PLA) in a randomized, crossover, double-blind manner. Venous blood was sampled immediately pre- and postexercise for the analysis of pH plus lactate, glucose, and norepinephrine (NE). PSE improved cycling TT performance by 5 percent (95 % confidence interval 0 to 10 %) compared with PLA. There was a significant Treatment x Time interaction for NE, with NE increasing during the PSE trial only. Similarly, blood glucose also showed a trend for increased levels postexercise in the PSE trial. The ingestion of 180 mg of PSE 60 min before the onset of high-intensity exercise improved cycling TT performance in well-trained athletes. It is possible that changes in metabolism or an increase in central nervous system stimulation is responsible for the observed ergogenic effect of PSE [10188].

Two studies were conducted to characterize multiple-dose pharmacokinetics and potential drug interactions of ibuprofen and pseudoephedrine combined in a suspension and to evaluate safety of this combination in children with common cold, flu, or sinusitis. In the pharmacokinetic study, 24 healthy children aged 4-11 years were administered ibuprofen - pseudoephedrine suspension at 7.5 and 1.125 mg/kg, respectively, every 6 hours for 5 doses. Serial blood samples were drawn over 6 hours after final dose for assessment of
steady-state pharmacokinetics. In the open-label, multicenter safety study, more than 100 children aged 2-11 years experiencing symptomatic rhinitis were enrolled. Ibuprofen-pseudoephedrine suspension was administered as needed at similar mg/kg doses every 6-8 hours for up to 3 days. Subjects enrolled in the pharmacokinetic study showed no accumulation of either drug; their weight-adjusted clearances were independent of age, and results were comparable with those from previous single-ingredient studies. In the safety study, adverse events were reported for 18 percent of subjects; most were mild to moderate intensity. There was little difference in incidence of adverse events among different age and weight groups. In conclusion, administration of combined ibuprofen and pseudoephedrine in children demonstrated similar pharmacokinetics when compared with reports of the pharmacokinetics for the single-ingredient products, consistent with no apparent drug interactions. The combination suspension was generally well tolerated [10393].

Due to its stimulatory effects on the central nervous system, and its structural similarity to banned stimulants such as ephedrine and methamphetamine, pseudoephedrine (PSE) at high doses is considered as an ergogenic aid for boosting athletic performance. However, the status of PSE in the International Standard of the Prohibited List as established under the World Anti-Doping Code has changed over the years, being prohibited until 2003 at a urinary cut-off value of 25 microg/mL, and then subsequently removed from the Prohibited List during the period 2004-2009. The re-consideration of this position by the World Anti-Doping Agency (WADA) List Expert Group has led to the reintroduction of PSE in the Prohibited List in 2010. In this manuscript, we present the results of two WADA-sponsored clinical studies on the urinary excretion of PSE and its metabolite cathine (CATH) following the oral administration of different PSE formulations to healthy individuals at therapeutic regimes. On this basis, the current analytical urinary threshold for the detection of PSE as a doping agent in sport has been conservatively established at 150 microg/mL [11216].

During recent years, a syndrome of hypokinesia, dysarthria, dystonia, and postural impairment, related to intravenous use of a "designer" psychostimulant derived from pseudoephedrine using potassium permanganate as the oxidant, has been observed in drug addicts in several countries in Eastern Europe with some cases also in Western countries. A levodopa unresponsive Parkinsonian syndrome occurs within a few months of abusing the homemade drug mixture containing ephedrine (methcathinone) and manganese. The development of this neurological syndrome has been attributed to toxic effects of manganese, but the role of the psychostimulant ephedrone is unclear. This paper describes the clinical syndrome, results of neuroimaging, and therapeutic attempts [11217].

Since the re-introduction of pseudoephedrine as a banned stimulant in 2010, an adverse analytical finding is to be reported by doping control laboratories when urinary concentrations greater 150 mg/mL are determined. This threshold value was deduced from two studies with healthy subjects receiving a total of 240 mg of pseudoephedrine within 24 or 48 h using different administration regimens and drug formulations. Peak concentrations were found between 100 and 175 mg/mL [12017].

Pseudoephedrine (PSE) is an over-the-counter decongestant that might have ergogenic effects. The World Anti-Doping Agency has prohibited large doses (>150 microg/mL) of PSE, while the National College Athletic Association (NCAA) does not include it on their banned-substance list. One study examined the effect of body-weight dosing of PSE on 800-m-run times of NCAA female runners. Fifteen NCAA female track athletes volunteered to participate in the randomized, double-blind, crossover design. Participants were given 2.5 mg/kg PSE or placebo in trials separated by a week. Ninety minutes postingestion, participants completed an 800-m individual time trial on an indoor track. Finishing time was recorded with an automated video timing device. Heart rate and anxiety state scores were recorded
immediately after each trial. Fourteen runners completed both trials, and 1 was an outlier: n=13. Despite the dose being well above normal therapeutic levels (144 ±1.7 mg), there was no significant difference in 800-m times between PSE and placebo trials, in postexercise heart rate or in anxiety-state levels. A 2.5-mg/kg dose of PSE had no effect on 800-m performance for female NCAA runners [12295].

Due to its stimulatory effects on the central nervous system, and its structural similarity to banned stimulants such as ephedrine and methamphetamine, pseudoephedrine (PSE) at high doses is considered as an ergogenic aid for boosting athletic performance. However, the status of PSE in the International Standard of the Prohibited List as established under the World Anti-Doping Code has changed over the years, being prohibited until 2003 at a urinary cut-off value of 25 microg/mL, and then subsequently removed from the Prohibited List during the period 2004-2009. The re-consideration of this position by the World Anti-Doping Agency (WADA) List Expert Group has led to the reintroduction of PSE in the Prohibited List in 2010. In this manuscript, we present the results of two WADA-sponsored clinical studies on the urinary excretion of PSE and its metabolite cathine (CATH) following the oral administration of different PSE formulations to healthy individuals at therapeutic regimes. On this basis, the current analytical urinary threshold for the detection of PSE as a doping agent in sport has been conservatively established at 150 micro/mL [12296].

Dose-response in cycling

The purpose of one study was to examine a possible dose-response between pre-exercise pseudoephedrine intake and cycling time trial performance. Ten trained male endurance cyclists (26.5 ± 6.2 years, 75.1 ± 5.9 kg, 70.6 ± 6.8 mL/kg/min) undertook three cycling time trials in which a fixed amount of work (7 kJ/kg body mass) was completed in the shortest possible time. Sixty minutes before the start of exercise, subjects orally ingested either 2.3 mg/kg or 2.8 mg/kg body mass of pseudoephedrine or a placebo in a randomised and double-blind manner. Venous blood was sampled at baseline, pre- and post-warm up and post-exercise for the analysis of pH and lactate and glucose concentrations; plasma catecholamine and pseudoephedrine concentrations were measured at all times except post-warm up. Cycling time trial performance (30 min) was not enhanced by pseudoephedrine ingestion. Plasma pseudoephedrine concentration increased from pre-warm up to post-exercise in both treatment conditions, with the 2.8 mg/kg body mass dose producing the highest concentration at both time points (2.8 mg/kg >2.3 mg/kg >placebo). It was concluded that there was large individual variation in plasma pseudoephedrine concentration between subjects following pseudoephedrine administration. A number of factors clearly influence the uptake and appearance of pseudoephedrine in the blood and these are not yet fully understood. Combined with subsequent differences in plasma pseudoephedrine between individuals, this may partially explain the present findings and also the inconsistencies in performance following pseudoephedrine administration in previous studies [13477].

Influence of preexercise food intake

One study examined the influence of preexercise food intake on plasma pseudoephedrine (PSE) concentrations and subsequent high-intensity exercise. In addition, urinary PSE concentrations were measured under the same conditions and compared with the present threshold of the World Anti-Doping Agency (WADA). Ten highly trained male cyclists and triathletes undertook four cycling time trials (TT), each requiring the completion of a set amount of work (7 kJ/kg BM) in the shortest possible time. Participants were randomized into a fed or nonfed condition and orally ingested 2.8 mg·kg BM of PSE or a placebo (PLA) 90 min before exercise; in the fed trials, they consumed a meal providing 1.5 g·kg BM of CHO.
Venous blood was sampled at 30, 50, and 70 min and pre-warm-up and postexercise for the analysis of plasma PSE and catecholamine concentrations, and urine was also collected for the analysis of PSE concentration. Independent of the preexercise meal, 2.8 mg/kg BM of PSE did not significantly improve cycling TT performance. The fed trials resulted in lower plasma PSE concentrations at all time points compared with the nonfed trials. Both plasma epinephrine and blood lactate concentrations were higher in the PSE compared with the PLA trials, and preexercise and postexercise urinary PSE concentrations were significantly higher than the threshold (150 microg/mL) used by WADA to determine illicit PSE use. It was concluded that irrespective of the preexercise meal, cycling TT performance of approximately 30 min was not improved after PSE supplementation. Furthermore, 2.8 mg/kg BM of PSE taken 90 min before exercise, with or without food, resulted in urinary PSE concentrations exceeding the present WADA threshold.

Methylephedrine

A simple and effective method of capillary electrophoresis-amperometric detection (CE-AD) coupled with transient isotachophoresis (tITP) was developed for the trace determination of doping substances. Compared with the conventional capillary electrophoresis method, the maximum enhancement factor in terms of peak heights was up to 5500-fold when the tITP technique was adopted. Under the optimum conditions, the detection limit for methylephedrine (MDP), celiprolol (CEL), sotalol (SOT) and indapamide (IDP) were $4.2 \times 10^{-14}$, $6.3 \times 10^{-13}$, $5.8 \times 10^{-14}$ and $9.5 \times 10^{-13}$ mol/L, respectively. The RSDs of four analytes were 1.0-2.3 percent for migration time and 2.6-3.8 percent for peak current, respectively. The proposed method was successfully applied to determine the contents of SOT and IDP in real urine sample, and the excretion curve of IDP within 48 h was also investigated. The recoveries of the four doping in urine ranged 90-102 percent.

Synephrine

The occurrence of some cases of positive results in anti-doping analysis of octopamine requires clarification as to whether its methylated derivative synephrine could be a metabolic precursor of octopamine itself. Synephrine is a natural phenylethylamine derivative present in some food supplements containing Citrus aurantium, permitted in sport regulations. A simulative laboratory study had been done using a photocatalytic process, to identify all possible main and secondary transformation products, in a clean matrix; these were then sought in biological samples obtained from three human volunteers and four rats treated with synephrine: the parent compound and its new potential metabolic products were investigated in human urine and rat plasma samples. The transformation of synephrine and octopamine and the formation of intermediate products were evaluated, adopting titanium dioxide as photocatalyst. Several products were formed and characterized using the HPLC-HRMS(n) technique. The main intermediates identified in these experimental conditions were compared with the major synephrine metabolites found in in vivo studies on rats and humans. Some more oxidized species, already formed in the photocatalytic process, were also found in urine and plasma samples of treated animals. These new findings could be of interest in further metabolism studies. The main photocatalytic pathway involving synephrine appears to be N-demethylation to give octopamine. On the contrary, it was demonstrate the inconsistency of this reaction in both rat and human in vivo determinations, resulting in forensic importance.
Prenylamine

Prenylamine is a vasodilator of phenylalkylamine structure and was used for the treatment of angina pectoris, until reports of undesirable effects including ventricular tachycardia led to a decreasing use of the drug in the 1980s. Metabolic N-dealkylation of orally ingested prenylamine can liberate amphetamine in humans and cause positive findings for amphetamine in doping and forensic analysis. In 2010, the World Anti-Doping Agency (WADA) classified prenylamine as a non-specified stimulant according to the 2010 Prohibited List, thus banning its use in sports in-competition. Supporting the development of a liquid chromatography-tandem mass spectrometry (LC-MS/MS) based detection method, a post-administration urine sample following a single oral prenylamine ingestion (Segontin® 60 mg) was analyzed for urinary metabolites. The LC-separated analytes were ionized in positive electrospray ionization (ESI) mode and detected as protonated ions using an AB Sciex TripleTOF 5600 quadrupole-time-of-flight hybrid mass spectrometer. Over 40 phase I metabolites were detected, including previously unknown mono- bis-, tris- and tetra-hydroxylated prenylamine, several hydroxylated and methoxylated prenylamine metabolites and (hydroxylated) diphenylpropylamine. Investigation of the collision-induced dissociation behaviours of the metabolites by high resolution/high accuracy mass spectrometry allowed for the assignment of the nature and the site of observed metabolic transformations. The most abundant phase I metabolite was confirmed as p-hydroxy-prenylamine by chemical synthesis and stable isotope labelling of reference material. An existing routine screening assay based on direct injection and LC-MS/MS analysis of urine was modified and validated according to common guidelines, in order to allow for the detection of p-hydroxy-prenylamine in sports drug testing. The assay demonstrated the ability to detect the target metabolite at 0.1 ng/ml at intra- and inter-day imprecisions below 10 percent [12298].

Octopamine

The biogenic amine octopamine [4-(2-amino-1-hydroxyethyl)phenol] is prohibited in sports owing to its stimulating and performance-enhancing properties. Adverse analytical findings in athletes' doping control samples commonly result from surreptitious applications; however, the occurrence of octopamine in nutritional supplements and in selected invertebrates as well as the assumption that its N-methylated analog synephrine [4-(1-hydroxyethyl-2-methylamino)phenol, not banned by anti-doping authorities but currently monitored in prevalence studies) might be converted in-vivo into octopamine have necessitated a study to investigate the elimination of synephrine and octopamine present in over-the-counter products. Urine samples collected after administration of nutritional supplements containing octopamine and/or synephrine as well as urine samples collected after therapeutic application of octopamine- or synephrine-containing drugs were analyzed using a validated solid-phase extraction-based procedure employing a weak cation exchange resin and liquid chromatographic/tandem mass spectrometric detection with electrospray ionization and multiple reaction monitoring. In the case of therapeutic octopamine application, the urinary concentration of the target compound increased from baseline levels below the lower limit of detection to 142 microg/mL, while urine samples collected after synephrine as well as dietary supplement administration did not yield any evidence for elevated renal excretion of octopamine [12299].
Dopamine

In one work, a highly sensitive and selective biomimetic electrochemical sensor for the amperometric detection of trace dopamine (DA) in human serums was achieved by gold nanoparticles (AuNPs) doped molecularly imprinted polymers (MIPs). Functionalized AuNPs (F-AuNPs), a novel functional monomer bearing aniline moieties on the surface of the AuNPs, were prepared via a direct synthesis method and then used to fabricate the conductive MIPs film on the modified electrode by electropolymerization method in the presence of DA and p-aminobenzenethiol (p-ATP). The obtained electrochemical sensor based on the conductive film of AuNPs doped MIPs (AuNPs@MIPs) could effectively minimize the interferences caused by ascorbic acid (AA) and uric acid (UA). The linear range for amperometric detection of DA was from 0.02 micromol/L to 0.54 micromol/L with the detection limit of 7.8 nmol/L. Furthermore, the AuNPs@MIPs modified electrode (AuNPs@MIES) was successfully employed to detect trace DA in different human serums [13481].

Phenylethylamine

The drug scene is constantly changing and evolving. Traditionally, drugs of abuse are associated with popular street drugs such as marijuana, heroin, cocaine, and methamphetamine. In the 1990s, several other drugs were added to this list, including the so-called “club drugs”: ecstasy, ketamine, and gamma hydroxybutyric acid. In more recent years, “designer drugs” have emerged, which are either chemically altered natural substances or completely synthetic molecular structures that have psychotropic effects. Due to the widespread use of the Internet, information regarding synthesis of and access to novel compounds is more accessible than ever. This poses new challenges to the medical community in terms of treatment as well as identification of the abused substance, especially in patients unable to communicate. In recent years, access to information regarding acquisition and synthesis of newer designer drugs has been at an all-time high due largely to the Internet. As these drugs have become more prevalent, laboratory techniques have been developed and refined to identify and screen for this burgeoning population of drugs. This provides a unique opportunity for learning about many of these methods. Laboratory testing techniques and instrumentation are obscure to many health care professionals, yet their results are crucial. Here, it was presented a case of an overdose of an uncommon designer drug (2C-E) and discuss the basics of liquid chromatography and mass spectrometry, two important techniques used in isolating and identifying the drug. Although often overlooked and taken for granted, these techniques can play a pivotal role in the diagnosis and subsequent management of select patients. Here it was presented a case of a fatal overdose of the designer drug known as 2C-E (4-ethyl-2, 5-dimethoxy-b-phenethylamine), a phenethylamine derivative [13538].

Modafinil

Modafinil was originally introduced in the clinical literature as a wake-promoting agent in 1988. Modafinil was first approved by the US Food and Drug Administration (FDA) in 1998 and marketed as the racemic mixture of R- and S-enantiomers and later as a formulation containing only the R-enantiomer, which is pharmacokinetically distinct from the S-enantiomer in humans as described later. It has been viewed throughout its history as a “novel” wake-promoting therapeutic, and apparently is still viewed in the same manner to this day. The fact that this mainstream therapeutic agent is still thought of as novel presumably
stems from some unique pharmacological and clinically relevant properties of modafinil relative to other wake-promoting agents. Modafinil, in its two clinical formulations (Provigil® and Nuvigil®), is a widely prescribed wake-promoting therapeutic agent. It binds competitively to the cell-membrane dopamine (DA) transporter and is dependent on catecholaminergic (dopaminergic and adrenergic) signaling for its wake-promoting effects. The clinical spectrum of effects for modafinil is distinct from the effects seen with other catecholaminergic agents. Relative to other commonly used agents that act through catecholaminergic mechanisms, modafinil has a relatively low abuse potential, produces wakefulness with an attenuated compensatory sleep recovery thereafter, and does not ameliorate cataplexy in narcolepsy. These clinically relevant phenomenological differences between modafinil and agents such as amphetamines and cocaine do not eliminate catecholaminergic effects as a possible mediator of its wake-promoting action; they merely reflect its unique pharmacological profile.

Modafinil is an exceptionally weak, but apparently very selective, DA transporter inhibitor. The pharmacodynamic response to modafinil, as measured by DA levels in brain microdialyzate, is protracted relative to other agents that act via catecholaminergic mechanisms. The conformational constraints on the interaction of modafinil with the DA transporter – and probably, as a consequence, its effects on trace amine receptor signaling in the catecholaminergic cell – are unique among catecholaminergic agents. These unique pharmacological properties of modafinil should be considered both in seeking to thoroughly understand its putatively elusive mechanism of action and in the design of novel therapeutic agents [13479].

Like many compounds, modafinil was found to be clinically useful long before its pharmacological target was known. Still, as with any new wake-promoting agent, a number of potential targets came to mind in the search for its mechanism of action. Among the potential targets for modafinil were the cell-membrane monoamine transporters. These monoamine-selective transporters serve, in a non-selective fashion, to clear the monoamines dopamine (DA), noradrenaline (NE), and serotonin (5-HT) from the extracellular space surrounding the neurons that release them. The transporters are named for the neurochemical identity of the cells that express them at the highest levels – the dopamine transporter (DAT), the noradrenaline transporter (NET), and the serotonin transporter (SERT). Despite this nomenclature, they are not truly selective for their namesake neuromodulators. For instance, the Michaelis constant (K_m) for DA uptake by the NET is lower than the K_m for NE uptake by the NET – at least in genetically engineered cells expressing NET under an exogenous construct – indicating a higher affinity for DA. This fact, and the promiscuity among catecholaminergic (dopaminergic and adrenergic) receptors in responding to both DA and NE, may lead to some confusion with regard to modafinil’s mechanism of action. At the time when modafinil’s wake-promoting effect was discovered, various agents that bind to and inhibit the activity of monoamine transporters, such as cocaine and amphetamines, were known to also promote wakefulness. Thus, it was reasonable to hypothesize that modafinil might act through monoamine transporter inhibition to produce wakefulness. The first indication that modafinil binds competitively to a monoamine transporter, specifically DAT, came in 1994. In that study, modafinil competitively displaced the binding of radiolabeled (−)-2-beta-carbomethoxy-3-beta-(4-fluorophenyl) tropane (abbreviated beta-CFT, also known as WIN 35,428), a known DAT ligand, in extracts of a tissue enriched for DAT, the striatum, with a concentration that inhibits 50 percent (IC_{50}) value of 3.19 μM. The ability of modafinil to displace SERT and NET ligands was investigated in the same study and no effect was detected [13479].

Psychostimulants have been used to treat many symptoms associated with advanced cancer. The primary role of psychostimulants in such cases is the treatment of symptoms such as cancer-related fatigue, opioid-induced sedation, depression, and cognitive dysfunction associated with malignancies. These uses for psychostimulants came after approval for treatment of disorders such as attention deficit disorder. Modafinil, a new
psychostimulant, is following a similar path after its approval for use in attention deficit disorder in 1998. Modafinil has been used to treat fatigue associated with neurodegenerative disorders such as multiple sclerosis and amyotrophic lateral sclerosis. It is now being increasingly used for cancer-related symptoms targeted by psychostimulants. Preliminary evidence from literature review suggests that modafinil is efficacious in improving opioid-induced sedation, cancer-related fatigue, and depression. There is no evidence to support its use in the treatment of cognitive dysfunction related to cancer or to support its having analgesic properties. Well-designed, randomized, controlled clinical trials are still needed to further elucidate the precise role of this drug in the care of patients with cancer. Specifically, large placebo-controlled trials with modafinil must be conducted in patients with cancer, with specific attention paid to pain control, depression, cognitive function, and adverse effects.

Modafinil is a novel wake-promoting agent that has US Food and Drug Administration approval for narcolepsy and shift work sleep disorder and as adjunctive treatment of obstructive sleep apnea/hypopnea syndrome. Modafinil has a novel mechanism and is theorized to work in a localized manner, utilizing hypocretin, histamine, epinephrine, gamma-aminobutyric acid, and glutamate. It is a well-tolerated medication with low propensity for abuse and is frequently used for off-label indications. The objective of this study was to systematically review the available evidence supporting the clinical use of modafinil. The search term modafinil OR Provigil was searched on PubMed. Selected articles were mined for further potential sources of data. Abstracts from major scientific conferences were reviewed. Lastly, the manufacturer of modafinil in the United States was asked to provide all publications, abstracts, and unpublished data regarding studies of modafinil. There have been 33 double-blind, placebo-controlled trials of modafinil. Additionally, numerous smaller studies have been performed, and case reports of modafinil's use abound in the literature. It was concluded that modafinil is a promising drug with a large potential for many uses in psychiatry and general medicine. Treating daytime sleepiness is complex, and determining the precise nature of the sleep disorder is vital. Modafinil may be an effective agent in many sleep conditions. To date, the strongest evidence among off-label uses exists for the use of modafinil in attention-deficit disorder, postanesthetic sedation, and cocaine dependence and withdrawal and as an adjunct to antidepressants for depression.

Modafinil (Provigil@) is an example of a drug operating on state variables that is widely discussed with regard to cognitive enhancement. The compound was first marketed nearly 20 years ago in Europe as an agent to offset excessive sleepiness associated with narcolepsy; it was approved by the Food and Drug Administration for this use and for the treatment of certain sleep disorders. While the effects of modafinil on sleep and its EEG correlates are well-documented only a few studies have examined its effects on locomotor activity. This is surprising given that the drug is often referred to as a psychostimulant. In any event, modafinil causes a marked increase in activity in mice but only modest effects in rats or on daytime locomotion by monkeys. The reasons for these discrepancies are an important topic for future work, but the results as they stand indicate that the psychological state variables affected by modafinil are quite different from those targeted by Ritalin@. There is a large and often conflicting literature on the effects of modafinil on components of cognition. Some studies obtained a clear improvement in sustained attention in healthy human subjects but others failed to find such effects. Similar discrepancies occur in the literature on animals.

The FDA has approved modafinil to improve wakefulness in adult patients with excessive sleepiness associated with sleep apnea, shift work disorder, and narcolepsy. Despite seeming acceptance of modafinil for PESA purposes, the FDA has not approved modafinil for additional indications, so physicians prescribing modafinil to patients for other conditions...
occurs off-label. Studies have shown that modafinil serves as a countermeasure for sleep loss and sustains continued wakefulness to improve attention, alertness, spatial planning, and visual pattern recognition memory. A recent, multi-factorial analysis provided convincing evidence that moderate doses of modafinil improve attention in healthy middle-aged rats without affecting motivation or locomotor activity. Importantly, these effects became evident only as attentional demands were increased. In all, it seems reasonable at this point to conclude that modafinil's effects on basic psychological state variables – wakefulness – can translate into selective improvements in attention. There is also a sizable literature suggesting that the above conclusion can be extended to memory encoding. An intriguing aspect of these studies in rodents and humans is that they generally point to a drug influence on working memory as opposed to the encoding of long-term memory for specific information. For example, the above noted work on middle-aged rats found no evidence for accelerated acquisition of a visual discrimination problem, with minimal demands on working memory, despite clear improvements in attention. There are, however, studies showing that modafinil accelerates the acquisition of simple rules (“win-stay”), a spatial learning protocol, and a non-match to position problem in rodents. It is tempting to speculate that we are here seeing hierarchical effects of modafinil such that enhanced wakefulness produces greater attention that in turn improves both working memory and simple rule learning. By far the greater part of the human studies with modafinil involves subjects with impairments to performance (sleep deprivation) or psychiatric disorders. A retrospective analysis of several studies led to the conclusion that modafinil does not produce a "global" enhancement of cognition. Uncertainties about the cellular effects of modafinil limit the utility of the neurobiological dimension in evaluating the drug's likely effects on cognition. Recent work has reinforced earlier arguments that modafinil binds to forebrain dopamine transporters in rats, monkeys, and humans, and that this is accompanied by the expected increases in extracellular dopamine concentrations. There is also evidence for binding to norepinephrine transporters in human thalamus. These effects would be expected to promote wakefulness via mechanisms engaged by classical stimulants. But then the question arises as to why modafinil's behavioral profile (e.g. locomotor activity) differs so clearly from those for amphetamine, methylphenidate, and cocaine. One possibility is that the dopamine effects are less potent than seen with the stimulants but are supplemented by other, more unusual actions. Studies have established that modafinil activates the perifornical region and the tuberomammillary nucleus of the hypothalamus, two areas that are of primary importance to sleep-wakefulness. The first of these contains orexin synthesizing neurons that have been directly implicated in narcolepsy while the latter region is the exclusive location of wake-promoting histaminergic neurons. Modafinil still causes wakefulness as well as its other behavioral, EEG, and Fos expression effects in orexin null mice and so is not likely to produce its major effects via the sleep suppressing peptide. It does however increase extracellular histamine levels in the posterior hypothalamus and there is considerable evidence that histamine plays an important role in maintaining the vigilant, wakeful state. It seems reasonable then to assume that modafinil, at moderate doses, exerts its effects via a combined activation of histaminergic and catecholaminergic projections into telencephalon. With regard to cortical networks, modafinil is a compound that enhances inputs that are diffusely distributed and whose terminal populations are vastly outnumbered by the glutamatergic endings generated by the cortical neurons themselves. These arrangements are suggestive of a modulatory role wherein the activated inputs change global parameters of networks as opposed to discrete changes in communication patterns within and between networks, or in the various synaptic plasticity effects used to store information. It is also highly likely that the pertinent lower brain areas focus some component of their projections on subcortical areas that exert strong effects on particular cortical areas. For example, the histaminergic cells in posterior hypothalamus give rise to projections to the relatively small medial septum; it can be imagined that this results in a strong histaminergic influence over
cholinergic and GABAergic septal projection neurons that regulate hippocampal rhythms [11434].

One paper examined the social construction of the new wakefulness-promoting drug Modafinil (brand name Provigil) in the British press. Key themes in this newspaper coverage include the potential "uses" and "abuses" of this drug in relation to: (i) medical conditions; (ii) lifestyle choices; (iii) military operations; and (iv) sporting competition. The British press played a dual role in reporting on these trends and developments: on the one hand constructing this as something of a "wonder drug" in relation to the treatment of a number of medical complaints or conditions, on the other hand articulating and amplifying a range of cultural concerns and anxieties about the non-medical "uses" and "abuses" of this drug, both now and in the future. These issues, it is argued, are best interpreted in terms of media concerns over the pharmaceuticalisation rather than the medicalisation of everyday/night life. The paper concludes with some further thoughts and reflections on these issues, including the potential reworking of notions of "pharmaceutical Calvinism" and the spirit of (bio)capitalism [08298].

Previous research has demonstrated cognitive-enhancing effects of modafinil in humans and generated evidence for its therapeutic potential in psychiatric disorders. The neurochemical basis of these effects remains unresolved although a role for alpha1-adrenoceptors has been hypothesised. In one within-subject, double-blind, placebo-controlled study, 12 healthy male adults received modafinil (300 mg), the alpha1-adrenoceptor antagonist prazosin (3 mg), both together and placebo on separate occasions at least 5 days apart. Cognitive effects were assessed using a well-validated testing battery focusing on executive and working memory functions. Blood pressure, heart rate and salivary alpha-amylase were measured at hourly intervals. Cognitive effects of modafinil and prazosin were identified at the difficult levels of the One-Touch Stockings of Cambridge (OTSOC) planning task. Prazosin antagonized the error-reducing effect of modafinil when the agents were given together. In contrast, the combined agents acted synergistically to increase time taken to complete OTSOC problems compared with placebo. The tachycardic and amylase-elevating effects of prazosin were also potentiated by concurrent modafinil administration. The current data suggest that the cognitive effects of modafinil on performance accuracy and latency are dissociable in terms of their neurochemical mechanisms. The findings support the hypothesised involvement of alpha1-adrenoceptors in some of the cognitive-enhancing effects of modafinil [09263].

Modafinil is a non-amphetamine wakefulness-promoting agent used for the treatment of various sleep disorders characterized by excessive daytime sleepiness. There is little information in the medical literature with respect to supratherapeutic doses of this medication. It was performed a retrospective review of the California Poison Control System database for all cases of single-substance ingestion of modafinil with follow-up to a known outcome for the time period 1998-2008. Data collected included age, gender, dose ingested, clinical effects, and medical outcome. There were a total of 87 patients, 53 (61 %) of which were female. Patient ages ranged from 1 to 72 years with a mean of 30 years; 17 (20 %) patients were aged 6 years or less. Thirty-three (38 %) were intentional overdoses. Most commonly reported effects were tachycardia (n=23), agitation (n=14), anxiety (n=11), headache (n=8), hypertension (n=6), dystonia/tremor (n=6), and dizziness (n=5). Forty-nine patients (56 %) were managed at home, and 38 (44 %) were managed in a healthcare setting. Therapies administered included activated charcoal (n=8), benzodiazepines (n=7), antihistamines (n=2), intravenous fluids (n=2), haloperidol (n=2), and beta-blockers (n=1). Effects were classified as none (n=22), minor (n=54), and moderate (n=11). No major effects and no deaths occurred. Effects of modafinil overdose appear to be mild in most cases, with
tachycardia and CNS symptoms predominating. Clinically significant effects requiring treatment occurred in a small number of patients [10381].

Modafinil, a wake-promoting drug used to treat narcolepsy, is increasingly being used as a cognitive enhancer. Although initially launched as distinct from stimulants that increase extracellular dopamine by targeting dopamine transporters, recent preclinical studies suggest otherwise. To measure the acute effects of modafinil at doses used therapeutically (200 mg and 400 mg given orally) on extracellular dopamine and on dopamine transporters in the male human brain. In this pilot study, modafinil blocked dopamine transporters and increased dopamine in the human brain (including the nucleus accumbens). Because drugs that increase dopamine in the nucleus accumbens have the potential for abuse, and considering the increasing use of modafinil, these results highlight the need for heightened awareness for potential abuse of and dependence on modafinil in vulnerable populations [09264].

Adrafinil and modafinil have received wide publicity and have become controversial in the sporting world when several athletes were discovered allegedly using these drugs as doping agents. By acknowledging the facts, the World Anti-Doping Agency (WADA) banned these drugs in sports since 2004. One study explored the possibility of differentiating adrafinil and modafinil and their major metabolites under electron impact ionization in gas chromatograph-mass spectrometer (GC-MSD) and electrospray ionization in liquid chromatograph-mass spectrometer (LC-MS/MS) by studying the fragmentation pattern of these drugs. Adrafinil, modafinil and their major metabolite, modafinilic acid were analyzed on EI-GC-MSD and ESI-LC-MS/MS using various individual parameters on both the instruments. The results show that adrafinil, modafinil and their major metabolite modafinilic acid could be detected as a single artifact without differentiation under EI-GC-MSD analysis. However, all drugs could be detected and differentiated under ESI-LCMS/MS analysis without any artifaction. The GC-MSD analysis gives a single artifact for both the drugs without differentiation and thus can be used as a marker for screening purposes. Further, the Multiple Reaction Monitoring (MRM) method developed under LC-MS/MS is fit for the purpose for confirmation of suspicious samples in routine sports testing and in forensic and clinical analysis [09265].

Stimulant medications appear effective at restoring simple alertness and psychomotor vigilance in sleep deprived individuals, but it is not clear whether these medications are effective at restoring higher order complex cognitive capacities such as planning, sequencing, and decision making. After 44 hours awake, participants received a double-blind dose of one of 3 stimulant medications or placebo. After 45-50 hours awake, participants were tested. Participants were randomly assigned to 1 of 3 stimulant medication groups, including caffeine, 600 mg (n=12), modafinil, 400 mg (n=12), dextroamphetamine, 20 mg (n=16), or placebo (n=14). At the doses tested, modafinil and dextroamphetamine groups completed the task in significantly fewer moves than the placebo group, and the modafinil group demonstrated greater deliberation before making moves. In contrast, subjects receiving caffeine completed the task in fewer moves than all 3 of the other groups, although speed of completion was not influenced by the stimulants. Finally, the modafinil group outperformed all other groups on indices of perseverative responding and perseverative errors. Although comparisons across tasks cannot be made due to the different times of administration, within-task comparisons suggest that, at the doses tested here, each stimulant may produce differential advantages depending on the cognitive demands of the task [09266].

A new and reliable two-step liquid chromatography/tandem mass spectrometry (LC/MS/MS) method in combination with gas chromatography/mass spectrometry (GC/MS) for the screening and confirmation of adrafinil and its major metabolites, modafinil and modafinil acid, in human urine has been developed and validated. The method involved reversed-
phase C18 solid-phase extraction cartridge extraction and MS analysis by means of LC/MS/MS and GC/MS. The study illustrated that the ESI capillary temperature played a key role in the formation of the protonated molecule. The limits of detection (LODs) of the developed method for the three compounds were lower than the minimum required performance limit (MRPL) of the World Anti-Doping Agency (WADA). The human urine samples obtained after the oral administration of modafinil and from the Beijing 2008 Olympic Games were analyzed by using the described method, which has also been successfully applied to routine analyses and the WADA Proficiency Test [09267].

Sports and military environments have many common features – intense physical activity, rigorous physical environment (heat, cold, high or low pressure, hypoxia, acceleration...), specific psychosocial atmosphere, team spirit. If combined with jet lag syndrome, these specific conditions can favor altered physical and mental performance. There is always the temptation to use drugs as a simple way to reduce the penalizing effects. The available compounds known to affect sleep and wakefulness include hypnotics, benzodiazepines and non benzodiazepines such as temazepam, zolopidem, and zopiclone, stimulants such as amphetamine and amphetamine-like agents, adrafinil, modafinil, caffeine and chronobiotics substances such as melatonin and, more recently, slow release caffeine. In the sports area, all of these substances except caffeine are on the list of forbidden products, although special authorizations linked to known disease conditions are allowed. In the military setting, the environment may be similar, but the context of use is very different. In the context of a rescue mission, the current practice in the French military organization is to place modafinil pills in the ejection seat of fight planes and in rescue boats. A second context is the use of anti-sleep agents under orders; the debate continues on this and the appropriate recommendations in this context. Self-medication is a third condition, in which case no rules have been defined [07178].

The neuro-enhancement Modafinil promises to dramatically increase users' waking hours without much sacrifice to clarity of thought and without serious side effects (inducing addiction). For Modafinil to be advantageous, its usage must enable access to goods that themselves improve the quality of one's life. I draw attention to a variety of conditions that must be met for an experience, activity or object to improve the quality of one's life, such as positional, relational, and saturation conditions, as well as it's being good for its own sake. I discuss and describe the contexts in which widespread usage (legal or not) of Modafinil would undermine these conditions being met, and thus users would fail to significantly improve the quality of their lives and would in fact potentially make both themselves and nonusers worse off in important respects thus far overlooked by critics. In the right contexts, where free time is protected and prolonged, Modafinil does have a variety of potential benefits including, most interestingly, a distinctive form of agency possible only in free time. The potential disadvantages and advantages highlighted in this article are relevant not only to public institutions deciding whether to legalize Modafinil's use as an enhancement but also to individuals deciding whether to use it illegally, as well as to the questions of how and whether to alter key features of one's context (e.g. regulating work hours or extending social services) rather than, or in addition, to regulating the use of enhancement drugs such as Modafinil [12301].

Combined with cocaine

Modafinil is a wake-promoting drug effective at enhancing alertness and attention with a variety of approved and off-label applications. The mechanism of modafinil is not well understood but initial studies indicated a limited abuse potential. A number of recent publications, however, have shown that modafinil can be rewarding under certain conditions. The present study assessed the reinforcing properties of modafinil using conditioned place
preference and locomotor sensitization in mice. Experiment 1 examined a high dose of modafinil (75 mg/kg) as well as its interactions with cocaine (15 mg/kg). Cocaine alone and modafinil co-administered with cocaine induced sensitization of locomotor activity; modafinil alone showed little or no locomotor sensitization. Animals given modafinil alone, cocaine alone, and modafinil plus cocaine exhibited a strong and roughly equivalent place preference. When tested for sensitization using a low challenge dose of modafinil, cross-sensitization was observed in all cocaine-pretreated mice. Experiment 2 examined a low dose of modafinil that is similar to the dose administered to humans and has been shown to produce cognitive enhancements in mice. Low dose modafinil (0.75 mg/kg) did not produce conditioned place preference or locomotor sensitization. Together, these results suggest that modafinil has the potential to produce reward, particularly in cocaine addicts, and should be used with caution. However, the typical low dose administered likely moderates these effects and may account for lack of addiction seen in humans [12302].

Combined with cannabis

Marijuana (cannabis) is the most widely used illicit substance globally, and cannabis use is associated with a range of adverse consequences. Currently, no medications have been proven to be effective for the treatment of cannabis addiction. The goals of this study were to examine the safety and efficacy of a potential treatment medication, modafinil, in combination with oral delta9-tetrahydrocannabinol (THC). Twelve male and female occasional cannabis users participated in an outpatient double-blind, placebo-controlled, crossover study. Across four sessions, participants were randomly assigned to a sequence of four oral treatments: (1) 400 mg modafinil+placebo, (2) 15 mg THC+placebo, (3) 400 mg modafinil+15 mg THC, or (4) placebo+placebo. Outcome measures included heart rate, blood pressure, performance on the Rapid Visual Information Processing (RVIP), and the Hopkins Verbal Learning Test (HVLT), and subjective measures. Oral THC increased heart rate, and produced increased subjective ratings of feeling "high" and "sedated," as well as increased ratings of euphoria. Modafinil alone increased the Profiles of Mood States (POMS) subscales of vigor and tension. These findings support the safety of modafinil in combination with THC. The effects of modafinil in combination with a range of doses of THC need to be determined in future studies [11196].

Adrafinil

A new and reliable two-step liquid chromatography/tandem mass spectrometry (LC/MS/MS) method in combination with gas chromatography/mass spectrometry (GC/MS) for the screening and confirmation of adrafinil and its major metabolites, modafinil and modafinil acid, in human urine has been developed and validated. The method involved reversed-phase C18 solid-phase extraction (SPE) cartridge extraction and MS analysis by means of LC/MS/MS and GC/MS. The study illustrated that the ESI capillary temperature played a key role in the formation of the protonated molecule. The limits of detection (LODs) of the developed method for the three compounds were lower than the minimum required performance limit (MRPL) of the World Anti-Doping Agency (WADA). The human urine samples obtained after the oral administration of modafinil and from the Beijing 2008 Olympic Games were analyzed by using the described method, which has also been successfully applied to routine analyses and the WADA Proficiency Test [09268].

Armodafinil

Armodafinil, a non-amphetamine, wakefulness-promoting medication, is the R- and longer-lasting isomer of racemic modafinil. Armodafinil has been shown to improve wakefulness in
patients with excessive sleepiness associated with treated obstructive sleep apnoea, shift work disorder or narcolepsy. In comparison with modafinil, armodafinil maintains higher plasma concentrations later in the day in healthy subjects. The objective of this analysis was to characterize the pharmacokinetic parameters related to those higher concentrations. Data from three randomized studies in healthy adult subjects receiving single doses of either armodafinil (50, 100, 200, 250, 300 or 400 mg) or modafinil (400 mg) were pooled, and subsequently dose-normalized to a 200 mg dose for each drug. Non-compartmental pharmacokinetic parameters were assessed. Armodafinil and modafinil both had a mean single-dose terminal elimination half-life of approximately 13 hours, with similar mean maximum plasma drug concentration ($C_{\text{max}}$) and median time to $C_{\text{max}}$ values. After reaching $C_{\text{max}}$, plasma concentrations appeared to decline in a monophasic manner with armodafinil, but in a biphasic manner with modafinil due to the initial rapid elimination of its S-isomer. As a result, mean area under the plasma drug concentration versus time curve (AUC) from time zero to the time of the last measurable concentration and AUC from time zero to infinity values were 33 percent and 40 percent higher, respectively, with armodafinil compared with modafinil on a milligram-to-milligram basis. Despite similar half-lives, plasma concentrations following armodafinil administration are higher late in the day than those following modafinil administration on a milligram-to-milligram basis [09269].

Armodafinil is the R-enantiomer of modafinil, a wake-promoting agent, that primarily affects areas of the brain involved in controlling wakefulness. Once-daily armodafinil was effective in improving wakefulness in adult patients with excessive sleepiness associated with obstructive sleep apnoea/hypopnoea syndrome (despite treatment of the underlying condition), narcolepsy or shift work sleep disorder in four large (n >195), double-blind, multinational trials of 12 weeks' duration. Compared with placebo, mean sleep latency (coprimary endpoint) was significantly improved with armodafinil 150 or 250 mg once daily in patients with obstructive sleep apnoea/hypopnoea syndrome (or narcolepsy, and with armodafinil 150 mg once daily in patients with shift work sleep disorder, as assessed by the Multiple Sleep Latency Test or the Maintenance of Wakefulness Test. Furthermore, a significantly higher proportion of armodafinil than placebo recipients achieved a response (at least a minimal improvement) on the Clinical Global Impressions of Change scale at study end in these four trials (coprimary endpoint). Once-daily armodafinil was generally well tolerated in adult patients with excessive sleepiness associated with obstructive sleep apnoea/hypopnoea syndrome (despite treatment of the underlying condition), narcolepsy or shift work sleep disorder [09270].

Armodafinil (R-modafinil) is the R- and longer-lasting isomer of the racemic compound modafinil, a wakefulness-promoting medication. Armodafinil is eliminated approximately three times more slowly than the S-isomer of racemic modafinil. Published studies have demonstrated the efficacy of armodafinil for treating excessive sleepiness associated with obstructive sleep apnoea, shift work disorder and narcolepsy. The objectives of one study were to describe the pharmacokinetic profile, tolerability and safety of armodafinil in healthy subjects. Pooled pharmacokinetic data from three separate randomized studies in 119 healthy subjects who received single or multiple (once daily for up to 14 days) oral doses of armodafinil ranging between 50 and 400 mg were analysed. The impact of food on the single-dose pharmacokinetic profile of armodafinil was also assessed in subjects following an overnight fast and after the consumption of a standard fatty meal. Armodafinil was readily absorbed and exhibited linear pharmacokinetics over the 50-400 mg dose range. Peak plasma concentrations were reached around 2 hours after administration in the fasted state. Food had no effect on the overall bioavailability of armodafinil; however, the peak concentration was delayed by approximately 2-4 hours. In the multiple-dose study, dose proportionality was confirmed by linear regression analyses of the log-transformed area under the plasma concentration versus time curve and maximum plasma concentration.
values as a function of dose. After reaching the peak, plasma concentrations of armodafinil declined in a monophasic manner, with a mean elimination half-life of approximately 15 hours. Steady state appeared to be reached within 7 days. At steady state, the systemic exposure to armodafinil was 1.8 times that observed after single-dose administration. Armodafinil was generally well tolerated, the most frequent adverse events being headache, dizziness and nausea. While food affected the rate but not the extent of absorption, peak plasma concentrations were reached in approximately 2 hours when the drug was taken on an empty stomach. With once-daily dosing, steady state appeared to be reached within 7 days. After reaching peak plasma levels, concentrations of armodafinil declined monophasically, with a mean elimination half-life of around 15 hours. Armodafinil was generally well tolerated [09271].

Experimental

Modafinil has been shown to promote wakefulness and some studies suggest the drug can improve cognitive function. Because of many similarities, the mechanism of action may be comparable to classical psychostimulants, although the exact mechanisms of modafinil's actions in wakefulness and cognitive enhancement are unknown. One study aimed to further examine the effects of modafinil as a cognitive enhancer on hippocampus-dependent memory in mice. A high dose of modafinil (75 mg/kg ip) given before training improved acquisition on a Morris water maze. When given only before testing, modafinil did not affect water maze performance. It was also examined modafinil (0.075 to 75 mg/kg) on Pavlovian fear conditioning. A low dose of pretraining modafinil (0.75 mg/kg) enhanced memory of contextual fear conditioning (tested off-drug 1 week later) whereas a high dose (75 mg/kg) disrupted memory. Pretraining modafinil did not affect cued conditioning at any dose tested, and immediate posttraining modafinil had no effect on either cued or contextual fear. These results suggest that modafinil's effects of memory are more selective than amphetamine or cocaine and specific to hippocampus-dependent memory [09272].

Methylphenidate

Methylphenidate (Ritalin®), a much-discussed compound with regard to the usage of enhancers by healthy individuals, is a good example of a drug whose actions can be classified with regard to psychological variables. Its effects on arousal are extensively documented beginning with a marked, amphetamine-like increase in rat exploratory behavior. Differences between Ritalin's influence on behavior relative to classical stimulants are subtle at best. That a drug whose primary effect is to increase arousal would produce positive effects on components of cognition, including attention and memory encoding, would not be surprising given the long history of work pointing to an inverted U-curve relationship between arousal levels and performance on complex problems. But whether Ritalin does in fact produce such effects in the absence of disturbances to other aspects of cognition, the latter a possibility inherent in the inverted U, is debatable. In large measure this reflects the fact that the great majority of animal and human studies on Ritalin are concerned with its effects on attention deficit hyperactivity disorder and other psychiatric problems; a much smaller number of experiments deal with control animals or healthy humans. Surveys of the older literature indicate that Ritalin and other stimulants increase attention or vigilance, although a recent study did not obtain this result in a test in which human subjects were required to discriminate significant stimuli from distracters. It seems likely that Ritalin's positive effects on attention are most apparent in relatively simple tasks requiring sustained engagement and less evident for more difficult problems requiring selective attention. There is also evidence that Ritalin can improve spatial working memory in healthy humans under
some but not all conditions. Specifically, performance was improved on novel spatial problems but impaired in repeat tests. Other studies confirmed the improvements in spatial working memory but found them to be evident only in subjects with low baseline scores the latter point, as it happens, also arises in discussions of modafinil’s effects on cognitive operations. Ritalin is also reported to improve digit span test score in healthy individuals, so its influence on working memory is not restricted to spatial problems. There is, however, little evidence for a positive effect on the encoding of long-term episodic or data memory. An early paper found that low to moderate doses have no effect on retention of various types of information on tests carried out 24 h post-learning, while the highest concentration tested produced an impairment. Others provided evidence for improved scores shortly after learning but not at later time points. In all, a limited set of results suggest that Ritalin's pronounced effects on arousal can facilitate the attentional and working memory components of cognition but that these effects are situation- and perhaps subject-dependent. Processes are generally negative. An interesting study using a complex video game that requires the evolution of strategies for optimizing performance found that Ritalin disrupted normally occurring improvements in scores. Ritalin appears to have little effect on verbal fluency, set shifting decision making or selecting shapes to fit within a matrix (Raven's test). Perhaps a fair, though necessarily tentative, conclusion for this collection of findings is that enhancing arousal and some components of cognition do not produce positive outcomes on integrated cognitive operations in fully engaged subjects. Ritalin also provides an illustrative case with regard to neurobiological mechanism of action. It increases the concentration of dopamine and noradrenaline at synapses by blocking their reuptake an action that fits well with its generalized effects on arousal. The drug thus belongs to a sizable group of therapeutically useful compounds acting on diffuse, ascending biogenic amine systems (ACh, 5-HT, NE, and DA). As implied by the name, these projections originate from a relatively small number of cells in the lower brain that generate widely branching projections to broad areas of the forebrain. Such anatomical arrangements constitute a convergent/divergent system in which multiple inputs innervate relatively small targets (the biogenic amine nuclei) that disperse their outputs across much larger target regions. However, dopaminergic projections may exert one type of effect in cortical, particularly frontal, areas where they are comparatively sparse and another in the striatum where they are present in high numbers [11434].

Attention can be readily measured in experimental animal models. Animal models of attention have been used to better understand the neural systems involved in attention, how attention is impaired, and how therapeutic treatments can ameliorate attentional deficits. One review focused on the ways in which animal models are used to better understand the neuronal mechanism of attention and how to develop new therapeutic treatments for attentional impairment. Several behavioral test methods have been developed for experimental animal studies of attention, including a 5-choice serial reaction time task (5-CSRTT), a signal detection task (SDT), and a novel object recognition (NOR) test. These tasks can be used together with genetic, lesion, pharmacological and behavioral models of attentional impairment to test the efficacy of novel therapeutic treatments. The most prominent genetic model is the spontaneously hypertensive rat (SHR). Well-characterized lesion models include frontal cortical or hippocampal lesions. Pharmacological models include challenge with the NMDA glutamate antagonist dizocilpine (MK-801), the nicotinic cholinergic antagonist mecamylamine and the muscarinic cholinergic antagonist scopolamine. Behavioral models include distracting stimuli and attenuated target stimuli. Important validation of these behavioral tests and models of attentional impairments for developing effective treatments for attentional dysfunction is the fact that stimulant treatments effective for attention deficit hyperactivity disorder (ADHD), such as methylphenidate (Ritalin®), are effective in the experimental animal models. Newer lines of treatment including nicotinic agonists, alpha4beta2 nicotinic receptor desensitizers, and histamine H₃ antagonists, have also been found to be effective in improving attention in
these animal models. Good carryover has also been seen for the attentional improvement caused by nicotine in experimental animal models and in human populations. Animal models of attention can be effectively used for the development of new treatments of attentional impairment in ADHD and other syndromes in which have attentional impairments occur, such as Alzheimer's disease and schizophrenia [11355].

The primary purpose of one study was to examine the extent to which two of the three sources of risk-taking – dispositional and ecological – in adolescence and demographic variables were related to Ritalin®, tranquilizer and narcotics misuse. The secondary aim of the study was to distinguish subgroups of Ritalin®, tranquilizer, and narcotics misusers using dispositional, ecological and demographic variables. An archival dataset containing 1672 participants (11-18 years old) was used. Ritalin®, tranquilizer, and narcotics misuse were dichotomized and hierarchical logistic regressions were computed for dispositional and ecological sources of risk-taking and demographics. To distinguish subgroups of misusers, hierarchical multinomial regressions were computed. Dispositional, ecological, and demographic variables were related to Ritalin®, tranquilizer, and narcotics misuse and distinguished among non-users, experimenters/occasional misusers, and frequent misusers. Prescription drug prevention programs should incorporate demographic, dispositional, and ecological variables and should parallel the guidelines currently used for developing effective substance abuse prevention programs [11356].

Acute bupropion (dopamine/noradrenaline reuptake inhibitor) administration significantly improved time trial performance and increased core temperature in the heat (30 degrees C). One study was performed to examine the effect of a dopaminergic reuptake inhibitor on exercise capacity and thermoregulation during prolonged exercise in temperate and warm conditions. Eight healthy well-trained male cyclists participated in this study. Subjects ingested either placebo (20 mg) or methylphenidate (Ritalin 20 mg) 1 h before the start of exercise in temperate (18 degrees C) or warm (30 degrees C) conditions and cycled for 60 min at 55 percent Wmax, immediately followed by a time trial to measure exercise performance. It was shown that Ritalin has a clear ergogenic effect that was not apparent in 18 degrees C. The combination of a dopamine reuptake inhibitor and exercise in the heat clearly improved performance and caused hyperthermia without any change in the perception of effort or thermal stress compared with the placebo trial. This response may potentially increase the risk of developing heat illness during exercise in individuals taking drugs of this nature [08299].

Psychostimulants are effective treatments for attention-deficit/hyperactivity disorder (ADHD) but may be associated with euphoric effects, misuse/diversion, and adverse effects. These risks are perceived by some clinicians to be greater in substance-abusing adolescents relative to non-substance-abusing adults. The present study evaluates the subjective effects, misuse/diversion, and adverse effects associated with the use of osmotic-release oral system methylphenidate (OROS-MPH), relative to placebo, for treating ADHD in adolescents with a substance use disorder (SUD) as a function of substance use severity and compared these risks with those associated with the treatment of ADHD in adults without a non-nicotine SUD. Datasets from two randomized placebo-controlled trials of OROS-MPH for treating ADHD, one conducted with 303 adolescents (13-18) with at least one non-nicotine SUD and one with 255 adult smokers (18-55), were analyzed. Outcome measures included the Massachusetts General Hospital Liking Scale, self-reported medication compliance, pill counts, and adverse events. Euphoric effects and misuse/diversion of OROS-MPH were not significantly affected by substance use severity. The euphoric effects of OROS-MPH did not significantly differ between the adolescent and adult samples. Adults rated OROS-MPH as more effective in treating ADHD, whereas adolescents reported feeling more depressed when taking OROS-MPH. The adolescents lost more pills relative to the adults regardless of
treatment condition, which suggests the importance of careful medication monitoring. Higher baseline use of alcohol and cannabis was associated with an increased risk of experiencing treatment-related adverse events in OROS-MPH, but baseline use did not increase the risk of serious AEs or of any particular category of AE and the adolescents did not experience more treatment-related AEs relative to the adults [11503].

Methylphenidate (MPH) is an amphetamine-like psychostimulant drug approved for the treatment of attention-deficit-hyperactivity-disorder (ADHD) and narcolepsy. An increase in MPH abuse has been observed in the United States in recent years. MPH is abused for recreational purposes with the aim of inducing feelings of euphoria. However, the use of MPH as so-called cognitive enhancer to boost performance has become a focus of concern. Data from poison centres suggest only mild or moderate toxicity in most cases of MPH abuse. Non-medical use of methylphenidate is increasing. Little is known about potential acute medical complications associated with recreational use of methylphenidate. Retrospective case series of methylphenidate abuse cases presenting to an inner city emergency department. It was identified 14 cases of methylphenidate abuse between 2003 and 2010. Ten of these patients abused methylphenidate alone while four co-ingested other drugs, mainly alcohol. The route of ingestion was oral in nine patients, nasal in one and intravascular in four. Severe toxicity was exclusively observed in users who injected the drug. Two cases involved accidental intra-arterial injection and resulted in tissue necrosis leading to the amputation of a forearm and of fingertips, respectively. Clinical findings in the non-serious cases included mild to moderate symptoms and signs of sympathetic nervous stimulation such as agitation, tachycardia, hypertension, anxiety, hallucination, headache, tremor and dizziness. Nine of the fourteen patients were taking methylphenidate as a prescribed drug. Eight patients were former or current multiple substance abusers. It was concluded that methylphenidate misuse is not a significant burden for emergency departments in Switzerland. Oral and nasal administration of methylphenidate did not result in severe toxicity. However, injection of crushed methylphenidate pills lead to serious local toxicity. Most patients with methylphenidate abuse had a prescription for the drug indicating deviation from medical use. A history of multiple substance use may be a risk factor for non-medical use of methylphenidate [11504].

Transdermal

An 8-week, open-label trial assessed the efficacy of methylphenidate transdermal system in 14 adult individuals diagnosed with ADHD and with a history of stimulant misuse, abuse, or dependence. The primary efficacy endpoint was the Wender-Reimherr Adult ADHD Scale, and secondary efficacy endpoints included the Clinical Global Impression ratings and substance abuse as quantified by urine drug screens and self-reported use. Significant improvements from baseline were found on both the measurements. No abuse of the study medication was observed. The findings suggested that methylphenidate transdermal system may improve ADHD symptoms in adults with a history of stimulant misuse; however, there were limitations. The study data showed the need for subsequent randomized studies that further explore findings made in this study [11505].

Laboratory technique

A novel electrophoric derivatisation procedure using o-(pentafluorobenzylxyloxy carbonyl)-2,3,4,5-tetrafluorobenzoyl chloride for the quantitative determination of methylphenidate in human plasma is described. The drug can be quantitatively measured down to 0.006 pg/mL plasma due to the extraordinary sensitivity of the derivatives under negative ion chemical ionisation mass spectrometry. Plasma samples were made alkaline with carbonate buffer
and treated with extraction solvent (n-hexane) and reagent solution for 15 min, which, after concentration was measured by GC-NICI-MS. The method is rapid as extraction and derivatisation occur in one single step. A stable isotope labelled internal standard was used. Validation data are given to demonstrate the usefulness of the assay, including selectivity, linearity, accuracy and precision, autosampler stability, aliquot analysis, robustness, and prospective analytical batch size accuracy. The method has been successfully applied to pharmacokinetic profiling of the drug after oral administration [11357].

A validated, accurate and sensitive LC-MS/MS method for determination of racemic methylphenidate and its metabolite ritalinic acid has been developed. The analytes were quantified by tandem mass spectrometry operating in positive electrospray ionization mode with multiple reaction monitoring. Blood, plasma and oral fluid samples of 100μL were prepared by simple precipitation with 200μL of an aqueous solution of zinc sulphate in methanol. Corresponding deuterated internal standards were used for quantification. Calibrations for methylphenidate and ritalinic acid were linear within the selected range of 0.2-30 ng/mL and 10-1500 ng/mL in blood or plasma and in the range of 1-500 ng/mL and 0.25-125 ng/mL in oral fluid, respectively. The method was successfully applied for the analysis of samples from patients treated with methylphenidate in the dose range of 36-72 mg/day and some representative ante mortem and post mortem samples from clinical and forensic toxicological investigations. A three to fourfold higher concentration of methylphenidate was found in oral fluid compared with blood while for ritalinic acid the concentrations were about 25-fold lower in oral fluid [11358].

**Amphetamine**

Methamphetamine- or amphetamine-type stimulants are the second most frequently used illicit drug worldwide, second only to cannabis. Behavioural treatments are efficacious, but their impact is limited underscoring the need for other treatment options, notably, pharmacotherapy. A review of randomised controlled trials of pharmacotherapies for methamphetamine- or amphetamine-type stimulants was performed using PubMed and Google Scholar databases. Evidence for efficacy of medications is reported. Clinical trials have yielded no broadly effective pharmacotherapy. Promising signals have been observed for methylphenidate, naltrexone, bupropion and mirtazapine in subgroups of patients in reducing stimulant use (e.g. patients with less severe dependence at baseline and men who have sex with men), though none has produced an unambiguous, replicable signal of efficacy. Problems in phase II trials, including high dropout rates, missing data and a lack of agreement on outcomes, complicate efforts to find a broadly effective pharmacotherapy for amphetamine-type stimulant disorders. Efforts to address these problems include calls for better validation of pharmacological target exposure, receptor binding and functional modulation. As well, there is a need for agreement in using findings from preclinical and early phases of the medication development process for selecting better pharmacotherapy candidates. After over 20 years of efforts worldwide to develop a broadly effective medication for dependence on methamphetamine- or amphetamine-type stimulants, no candidate has emerged. This highlights the need for new compounds, consistent and stringent research methods, better integration between preclinical and clinical stages of medication development, and improved collaboration between government, industry and researchers [13482].

Amphetamine (AP) and amphetamine-type stimulants, methamphetamine (MA) and N,N-dimethylamphetamine (DMA), are known as central nervous system stimulants, and their abuse throughout the world has recently increased. Since it is difficult to physically
distinguish among AP, MA and DMA, analysts may not be aware of what abusers have administered. In one study, following the detection of specific metabolites of AP, MA and DMA as biomarkers in abuser urines, a rapid and sensitive method was developed for the identification and classification of AP-type stimulants abusers. After the simple filtration of the urine samples, the samples were directly analyzed using a liquid chromatography/tandem mass spectrometry system with selected reaction monitoring (SRM)-triggered quantitation-enhanced data-dependent MS/MS (QED-MS/MS) for the simultaneous qualitative and quantitative analysis of p-hydroxy AP, p-hydroxy MA, p-hydroxy DMA, AP, MA, DMA and DMA N-oxide. The determination of p-hydroxy AP, p-hydroxy MA, AP, MA, DMA and DMA N-oxide was accurate and reproducible, with the limits of quantitation of 5 ng/mL in urine. When applied to the urine samples of suspected AP-type stimulants abusers, the abused drugs were precisely identified between MA and DMA abusers [10488].

Epidemiological studies on all types of illicit drug use among athletes are essential for both the sport community and drug control achievements. Here, it was investigated the prevalence and associated factors of amphetamine use in body builders in Tehran, Iran, 2007. One study is a secondary analysis of a substance use survey done in 103 randomly selected gymnasia in Tehran (capital city of Iran). The survey was conducted from 2007 to 2008 and included 843 randomly selected bodybuilders (aged 40 years or less). By interviews via questionnaires the following data were obtained: age, job, marital status, education level, housing status, average monthly family income, number of family members, gymnasion area (m²), number of trainers, number of gymnasion members, initiation time (months), weekly duration of the sporting activity (h), monthly cost of the sporting activity, purpose of participating in sporting activity, and history of anabolic steroid and amphetamine use. One hundred twenty (13 %) body builders reported a history of amphetamine use. According to the results of regression analysis, being married and participating in body building to enhance self-esteem or to enhance sport performance had protective effects on amphetamine use. However, having university qualifications using anabolic steroids and participating in sport to maintain fitness were linked to increased risk of amphetamine use. I was concluded that well-educated bodybuilders were more likely to use amphetamines, and why this is so needs to be discovered. If further studies show that they are not aware of the dangers associated with amphetamine use, providing them with information should be considered [12294].

Amphetamines are controlled substances under general drugs legislation, although they have been prescribed as appetite suppressants and for the treatment of narcolepsy. They are known to produce dependence, often in increasing doses. Athletes are likely to use amphetamines to sharpen reflexes and reduce tiredness. However, athletes have died as a result of amphetamine misuse, since the increase in blood pressure combined with increased physical activity and peripheral vasoconstriction makes it difficult for the body to cool down. If the body overheats, it dehydrates and blood circulation decreases, and the heart and other organs are unable to work normally [06171].

Amphetamine was synthesised in 1920 and was used to reduce fatigue and increase alertness during the second world war. Since then, many derivatives have been elaborated – for example, methamphetamine, dimethylamphetamine, methylendioxyamphetamine (MDA), methylendioxydimethylamphetamine (MDMA, “ecstasy”), and selegiline—and they are all forbidden in the practice of sport. Amphetamine was prescribed unsuccessfully as a nasal decongestant, antidepressant, and appetite suppressant, but soon appeared to be a powerful CNS stimulant. It acts primarily by enhancing the brain activity of noradrenaline and dopamine, intensifying psychological sensations of alertness, concentration, and self-confidence. Amphetamine is readily absorbed, mainly from the small intestine, and the plasma concentration peaks one to two hours following administration. Absorption is usually
complete in two and a half to four hours and is accelerated by food intake. The metabolism of amfetamine has been difficult to investigate because of the wide variation among species with regard to its metabolic effects. The principal amfetamine metabolites are p-hydroxy ephedrine and p-hydroxy amfetamine. Amfetamine is lost from the body by renal filtration. For detection of amfetamine use in sport, urine is analysed for the parent compound amfetamine. After a single dose of amfetamine, it has been shown that it can be detected in urine in the first urine void for at least 48 hours after the intake of the drug. The peak concentration in urine is strongly dependent on the individual, but occurs between 3 hours and 12 hours after the intake of the drug. Amfetamine excretion is enhanced by an acidic urine, and treatments that increase the acidity of urine enhance amfetamine loss – a reaction that is useful in the treatment of amfetamine overdose [06171].

**Physiologic effects of amphetamine**

The positive effects of amphetamine include an increase in physical energy, mental aptitude, talkativeness, restlessness, excitement, and good humour. Subjects taking amfetamine also report that they feel confident, efficient, ambitious, and that their food intake is reduced. Some negative effects of amphetamine (that can be dose dependent) are anxiety, indifference, slowness in reasoning, irresponsible behaviour, irritability, dry mouth, tremors, insomnia, and, following withdrawal, depression. Tolerance develops rapidly to many of the effects of the amfetamines. Tolerance is said to be present when, over a period of time, increasing doses of a drug are required to maintain the same response. There is much evidence to show that amfetamines induce drug dependence and the amfetamine dependent person may become psychotic, aggressive, and antisocial. Withdrawal of amfetamines is associated with mental and physical depression [06171].

**Side effects**

The major side effects of amfetamine use include confusion, delirium, sweating, palpitations, dilation of the pupil, and rapid breathing, as well as hypertension, tachycardia, tremors, and muscle and joint pain. Long term administration of amfetamine is associated with myocardial pathology and with growth retardation in adolescents. In most cases, the personality changes induced by chronic low doses of amfetamine are reversed gradually after the drug is stopped. High chronic doses may lead to a variety of persistent personality changes, paranoid delusions, and tactile hallucinations called “amphetamine psychosis” [06171].

**Heart**

Amphetamine is one of the oldest substances used in doping. Many cardiovascular disorders have been described, even SCD, from both their acute and chronic use. Specifically, they can cause coronary artery spasm, resulting in acute myocardial infarction, and or diffuse vasospasm. Their long-term use leads to arterial and pulmonary hypertension, stroke, and cardiomyopathy. Also common is the appearance of rhythm disturbances. Amphetamines are believed to increase levels of intracellular cyclic adenosine monophosphate and sympathetic nervous system tone. Thus, they favour the development of arrhythmias, especially in athletes with myocardial hypertrophy and heterogeneity of ventricular repolarisation [12126].

Atrial fibrillation (AF) is the most frequent cause of prolonged palpitations in young competitive athletes, even including those performing elite sport activity. This arrhythmia may occasionally affect impair athletes’ ability to compete thus leading to non-eligibility at prequalification screening. Competitive sport has a significant impact on the autonomous nervous system. In fact, long-term regular intense physical training determines an increase in
vagal tone leading to resting bradycardia. During physical activity, particularly in the setting of competition, a marked release of catecholamines occurs as a result of both the intense physical effort and emotional stress. Both of these adaptive phenomena may precipitate AF. Furthermore, in several athletes with AF an association with sick sinus syndrome has been found, even though the pathophysiological basis of this finding is not clear. This picture is further complicated by the increasingly intake of illicit substances, whose arrhythmogenic effect has been shown both at the ventricular and atrial levels. Moreover, the use of recreational drugs, such as amphetamines, ecstasy, alcohol, cannabinoids, cocaine and so called new drugs in clubs has dramatically increased, with several cases of drug-induced arrhythmic events. These effects are often exacerbated by the combined use of different drugs, especially in situations such as sports competitions, in which the adrenergic system is already hyperactivated. No data have been published on the efficacy of antiarrhythmic therapy in athletes with AF, but it has been reported that athletes are more predisposed to the development of pro-arrhythmic effects induced by antiarrhythmic drugs when compared to general population. Most recently, radiofrequency catheter ablation involving electrical disconnection of the pulmonary veins in athletes with AF limiting their normal training activity and participation in sports competitions has proven highly effective to restore stable sinus rhythm and enable subsequent re-eligibility [10489].

Amfetamine in sport

The action of amfetamine on sporting performance was first investigated in 1959. It has since been concluded that amfetamines enhanced anaerobic performance while having little or no effect on aerobic performance. Amfetamines may enhance sports performance from a supplemental mental stimulant effect as well as the effects on physical power derived from all three human energy systems – the ATP-CP, lactic acid, and oxygen energy systems. Depending on the type of effect or effort the athlete has to do, the dosage might be important for the user. Aggressiveness seems to increase with high dosage, whereas alertness is stimulated by lower doses. To summarise, amfetamines may:

- improve reaction time when fatigued
- increase muscular strength and endurance
- increase acceleration
- raise lactic acid levels at maximal exercise
- increase aerobic endurance capacity
- stimulate metabolism by inducing a loss of body fat

All amfetamines are banned by the WADA and IOC codes. Laboratory analysis is qualitative only, verifying presence of metabolites in urine. This is sufficient to demonstrate the presence of the substance in the urine and declare the case as an analytical adverse finding. The presence of amfetamine in urine can be described as a severe doping offence because amfetamines are no longer used therapeutically. Many countries prohibit its use because of the adverse effects. Amfetamines are part of the category S6 of the prohibited substances in competition [06171].

Side effects of amfetamine in relation to sport

Side effects of amfetamine, besides headaches, sleeplessness and anxiety, are particularly important to athletes. Indeed, amfetamine use may carry significant health risks for the sportsperson as evidenced by several amfetamine-linked deaths in sport. Two of the major risks are amfetamine induced heatstroke and cardiac arrest, which have resulted in several fatalities among cyclists during arduous effort. Amfetamines obscure pain from injuries and have enabled athletes in some sports to continue to compete and thus exacerbated their injuries. The side effects of amfetamine with regard to behaviour also are important in sport.
The euphoriant effects of amphetamine – taken to promote aggression and lower fatigue – has led in misjudgments and major fouls on the pitch [06171].

In hair

In the preparation of a reference material (RM) for quality assurance, both homogeneity and stability studies are integral parts. In the present study, both homogeneity and stability of a candidate RM for the determination of methamphetamine and amphetamine in hair were examined by an isotope dilution gas chromatography/mass spectrometry (GC/MS) method, which is not only one of the analytical methods validated in our previous study but also one of the primary methods for the preparation of a certified reference material (CRM). Additionally, homogeneity was monitored using a different method: micropulverized extraction followed by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), which was fully validated in the previous study. In order to demonstrate the suitability of the method as an isotope dilution with mass spectrometry (IDMS), the extraction efficiency was also determined according to time. The results showed that the current method, i.e., agitating hair with isotope internal standards in the extraction solvent for 20 h followed by GC-MS, was accepted as an IDMS. No significant difference was observed between bottles of the candidate CRM. The statistical results also showed no significant trends in stability for 92 days at room temperature and 4 degrees C. An inter-laboratory quality assurance program was also performed successfully using this material. The candidate CRM developed in the present study demonstrated its suitability for quality assurance in hair drug analysis. Even though a RM is necessity as a quality control tool, it is not always easy to have an authentic RM containing target drugs and metabolites. Even when an in-house quality control material is used, both homogeneity and stability should be investigated [10490].

Famprofazone

During a sport competition event in Taiwan, one urine specimen was found positive for both methamphetamine (2688 ng/mL) and amphetamine (462 ng/mL). The specimen donor claimed that she had taken Gewolen (a nonprescription drug manufactured in Taiwan) for treating abdominal pain and the medication was presented. Laboratory investigation confirmed that Gewolen contains famprofazone, which is known to metabolize to methamphetamine and amphetamine and is included in the prohibited list of the World Anti-Doping Agency. Study on the excretion profiles of three volunteers ingesting 50 mg famprofazone produced the following patterns similar to that observed in the case specimen: the ratio of methamphetamine to amphetamine was approximately 6 to 1, and d- and l-enantiomers of both methamphetamine and amphetamine were present, while the amount of l-methamphetamine was 3-4-fold greater than its counterpart. The data suggested that famprofazone (as the ingredient of Gewolen) was likely the source of the prohibited drugs found in the case specimen [07179].

Laboratory techniques

A selective and sensitive method for the qualitative screening of urine samples for 27 amphetamine and amphetamine-type drugs in the field of doping analysis is described. The method consists of a liquid-liquid extraction with diethyl ether at pH 14 and analysis of the extracts with a LCQ-Deca mass spectrometer equipped with an atmospheric pressure chemical ionisation interface, operated in positive ionisation mode. The total run time was 15 min. All compounds were analysed in MS2 or MS3. The detection limit for all compounds was lower than 25 ng/mL except for chlorphentermine (detection limit: 250 ng/mL) [06174].
In Japan, a wide variety of designer drugs became popular among juveniles because of their availability via the Internet and mobile phones. Hence, it is necessary to develop simple and rapid screening methods for these drugs. A rapid screening method for 30 abused drugs, including amphetamines, amphetamine-, piperazine-, tryptamine-, and phenethylamine-derived designer drugs and opiates in human urine was devised. The urine sample was digested with urease, and the drugs were analyzed by gas chromatography-mass spectrometry in the scan mode after solid-phase extraction with a Focus column and acetylation. The retention time obtained with the use of a retention time locking technique and three qualifier ions were used to obtain positive results. As the Focus column requires only simple extraction steps and can retain various drugs of a wide range of polarity, screening of 30 abused drugs was feasible within 3 h. The calibration curves were linear in the concentration range of 100-5,000 ng/mL in most drugs with correlation coefficients exceeding 0.99. The absolute recoveries for all drugs in urine samples were 6.9-125.4 percent at the concentration 1,000 ng/mL. This method will be most useful to confirm the presence of many abused drugs in urine in clinical and forensic cases [06175].

The purpose of one study was to evaluate the gas chromatographic-mass spectrometric method (GC-MS) for assay of stimulants of amphetamine type: amphetamine (AMP), methamphetamine (MAMP), ephedrine (EPH), norephedrine (NOREPH), and the amphetamine-derived designer drugs 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA, ecstasy), 3,4-methylenedioxy-N-ethylamphetamine (MDEA) and N-methyl-benzodioxolylbutanamine (MBDB) in urine. These drugs are often encountered in forensic and clinical toxicological analysis. GC-MS method after mixed-mode solid-phase extraction (SPE) and derivatization with heptafluorobutyric anhydride (HFBA) is presented for quick and reliable screening as well as identification and quantification of amphetamines and amphetamine derived designer drugs in urine. The measurement of calibration dependence allowed to determine the extent of linearity in the concentration range from 10 to 2,000 ng/mL for all amphetamines except AMP and for all amphetamine-derived designer drugs with correlation coefficients exceeding 0.98. Detection limits were established at 5 ng/ml and quantification limits at 10 ng/mL for all analytes, except AMP. LOD for AMP was established at 20 ng/ml and LOQ at 50 ng/mL. Extraction efficiency for all analytes at concentration levels 50 and 500 ng/ml (n=6) was established in the range 60-99 percent. Repeatability was measured at concentration levels 50 and 500 ng/mL (n=6), relative standard deviations (RSDs) were under 10% for all analytes. It was concluded that the GC-MS method after mix-mode solid-phase extraction and derivatization with heptafluorobutyric anhydride is presented for screening followed by identification and quantification of AMP, MAMP, EPH, NOREPH, MDA, MDMA, MDEA, and MBDB. The applicability of the GC-MS method was proven by analyzing authentic urine samples [06176].

A fast and selective ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS) method for the determination of amphetamines (amphetamine, methamphetamine, methylenedioxymethamphetamine, methylenedioxymethamphetamine, methylenedioxyethyamphetamine, ephedrine, and p-methoxyamphetamine) in plasma has been developed and validated. Sample preparation was performed by liquid-liquid extraction using ethyl acetate. For optimized chromatographic performance with repeatable retention times, narrow and symmetrical peaks, and focusing all analytes at the column inlet, a gradient start, with acid mobile phase consisting of 0.1 percent formic acid and methanol was chosen. Positive electrospray ionization MS-MS detection was performed with two multiple reaction monitoring transitions for each analyte. Deuteriumlabeled internal standards were used for five of the analytes. The limit of detection was in the range 0.25-1.25 ng/mL, and the limit of quantification was fixed at the lowest calibrator of 2.5 ng/mL for all of the compounds.
The RSD values of the intra- and interassay precision and accuracy were lower than 11% at four concentration levels, including two external quality controls. No or only minor matrix effects were observed, and the extraction method presented recoveries higher than 93 percent for all the compounds. Total run time, including equilibration, was 12 min. The method is routinely used at the National Institute of Criminalistics and Criminology for quantitative determination of the main amphetamines in plasma from forensic and driving under the influence cases [11498].

Capillary electrophoresis, CE, methods with capacitively coupled contactless conductivity detection (C(4)D) were developed for the enantiomeric separation of the following stimulants: amphetamine (AP), methamphetamine (MA), ephedrine (EP), pseudoephedrine (PE), norephedrine (NE) and norpseudoephedrine (NPE). Acetic acid (pH 2.5 and 2.8) was found to be the optimal background electrolyte for the CE-C(4)D system. The chiral selectors, carboxymethyl-beta-cyclodextrin (CMBCD), heptakis(2,6-di-O-methyl)-beta-cyclodextrin (DMBCD) and chiral crown ether (+)-[(18-crown-6)-2,3,11,12-tetra-carboxylic acid (18C6H(4))], were investigated for their enantioseparation properties in the BGE. The use of either a single or a combination of two chiral selectors was chosen to obtain optimal condition of enantiomeric selectivity. Enantiomeric separation of AP and MA was achieved using the single chiral selector CMBCD and (hydroxypropyl)methyl cellulose (HPMC) as the modifier. A combination of the two chiral selectors, CMBCD and DMBCD and HPMC as the modifier, was required for enantiomeric separation of EP and PE. In addition, a combination of DMBCD and 18C6H₄ was successfully applied for the enantiomeric separation of NE and NPE. The detection limits of the enantiomers were found to be in the range of 2.3-5.7 micromol/L. Good precisions of migration time and peak area were obtained. The developed CE-C(4)D method was successfully applied to urine samples of athletes for the identification of enantiomers of the detected stimulants [11499].

The detection of 11 sympathomimetic alkylamines in urine was presented with a focus on human doping control is proposed using liquid chromatography tandem mass spectrometry (LC-QqQ) and high resolution mass spectrometry (LC-HRMS) as a screening tool after a dilute-and-shoot (DS) approach. For the LC-HRMS analyses, several compounds exhibited better limits of detection (LOD) than the LC-QqQ. However, due to their small differences in structure, co-elution among the alkylamines was observed. Therefore, the chemical conversion of the alkylamines into an appropriate derivative for the confirmation analyses using gas chromatography-mass spectrometry (GC-MS) was evaluated. Five derivatization approaches were evaluated in an attempt to increase the analytical response and the confidence of the identification. The choice of the appropriated derivative for each alkylamine makes their spectra more easily interpretable, fulfills the WADA’s rather strict identification criteria and enables the unequivocal identification of alkylamines in urine [13480].

A microwave-assisted fluorescence labeling method for use in CE-LIF (capillary electrophoresis-laser induced fluorescence) is described. Six amphetamine-like designer drugs, namely, α-, m-, p-chloro- and o-, m-, p-fluoro-amphetamine derivatives, were synthesized and used as model compounds. FITC (fluorescein isothiocyanate isomer I) and a blue-laser were used as the fluorescent labeling reagent and excitation source, respectively. When a microwave oven was used, the reaction was complete within 5 min, while the classical method required at least 20 h (usually, an overnight reaction). A mimic oral fluid sample was obtained by spiking oral fluid from a volunteer with the six standards, and after liquid-liquid extraction and microwave-derivatization, it was possible to process the analytes by CE-LIF within a period of 10 min; the wavelength of the blue-laser used was 473 nm. For comparison, data obtained using classical methods, including CZE-UV (capillary zone electrophoresis-UV absorbance detection), sweeping-MEKC-UV (micellar electrokinetic chromatography-UV absorbance detection) and LC-Q-TOFMS (liquid chromatography/
Electrospray ionization quadrupole time-of-flight mass spectrometry) are also reported [13483].

In the context of driving ability diagnostics in Germany, administrative cutoffs for various drugs and pharmaceuticals in urine have been established. Two liquid chromatography-tandem mass spectrometry methods for simultaneous detection and quantification of amphetamines, designer amphetamines, benzylecgonine, benzodiazepines, opiates, and opioids in urine were developed and validated. A 500-microL aliquot of urine was diluted and fortified with an internal standard solution. After enzymatic cleavage, online extraction was performed by an ion-exchange/reversed-phase turbulent flow column. Separation was achieved by using a reversed-phase column and gradient elution. For detection, a Thermo Fisher TSQ Quantum Ultra Accurate Mass tandem mass spectrometer with positive electrospray ionization was used, and the analytes were measured in multiple-reaction monitoring mode detecting two transitions per precursor ion. The total run time for both methods was about 15 min. Validation was performed according to the guidelines of the Society of Toxicological and Forensic Chemistry. The results of matrix effect determination were between 78 and 116 percent. The limits of detection and quantification for all drugs, except zopiclone, were less than 10 ng/mL and less than 25 ng/mL, respectively. Calibration curves ranged from 25 to 200 ng/mL for amphetamines, designer amphetamines, and benzylecgonine, from 25 to 250 ng/mL for benzodiazepines, from 12.5 to 100 ng/mL for morphine, codeine, and dihydrocodeine, and from 5 to 50 ng/mL for buprenorphine and norbuprenorphine. Intraday and interday precision values were lower than 15 percent, and bias values within ± 15 percent were achieved. Turbulent flow chromatography needs no laborious sample preparation, so the workup is less time-consuming compared with gas chromatography-mass spectrometry methods. The methods are suitable for quantification of multiple analytes at the cutoff concentrations required for driving ability diagnostics in Germany [13484].

**Methamphetamine**

Methamphetamine (MA) use is a worldwide problem. Abusers can have cognitive deficits, monoamine reductions, and altered magnetic resonance spectroscopy findings. Animal models have been used to investigate some of these effects, however many of these experiments have not examined the impact of MA on the stress response. For example, numerous studies have demonstrated (+)-MA-induced neurotoxicity and monoamine reductions, however the effects of MA on other markers that may play a role in neurotoxicity or cell energetics such as glucose, corticosterone, and/or creatine have received less attention. In this experiment, the effects of a neurotoxic regimen of (+)-MA (4 doses at 2 h intervals) on brain monoamines, neostriatal GFAP, plasma corticosterone, creatinine, and glucose, and brain and muscle creatine were evaluated 1, 7, 24, and 72 h after the first dose. In order to compare MA’s effects with stress, animals were subjected to a forced swim test in a temporal pattern similar to MA administration (i.e. 30 min/session 4 times at 2 h intervals). Methamphetamine increased corticosterone from 1-72 h with a peak 1 h after the first treatment, whereas glucose was only increased 1 h post-treatment. Neostriatal and hippocampal monoamines were decreased at 7, 24, and 72 h, with a concurrent increase in GFAP at 72 h. There was no effect of MA on regional brain creatine, however plasma creatinine was increased during the first 24 h and decreased by 72 h. As with MA treatment, forced swim increased corticosterone more than MA initially. Unlike MA, forced swim reduced creatine in the cerebellum with no change in other brain regions while plasma creatinine was decreased at 1 and 7 h. Glucose in plasma was decreased at 7 h. It was concluded that methamphetamine and forced swim increase demand on energy substrates.
Methamphetamine (MA) has been implicated in cognitive deficits in humans after chronic use. Animal models of neurotoxic MA exposure reveal persistent damage to monoaminergic systems but few associated cognitive effects. Since questions have been raised about the typical neurotoxic dosing regimen used in animals and whether it adequately models human cumulative drug exposure, these experiments examined two different dosing regimens. Rats were treated with one of the two regimens: one based on the typical neurotoxic regimen (4 x 10 mg/kg every 2 h) and one based on pharmacokinetic modeling designed to better represent accumulating plasma concentrations of MA as seen in human users. On markers of neurotoxicity, methamphetamine showed decreased dopamine (DA) and 5-HT, increased glial fibrillary acidic protein, and increased corticosterone levels regardless of dosing regimen 3 days post-treatment. Behaviorally, methamphetamine-treated groups, regardless of dosing regimen, showed hypoactivity, increased initial hyperactivity to a subsequent MA challenge, impaired novel object recognition, impaired learning in a multiple water maze test of path integration, and no differences on spatial navigation or reference memory in the Morris water maze. After behavioral testing, reductions of DA and 5-HT remained. It was concluded that methamphetamine treatment induces an effect on path integration learning not previously reported. Dosing regimen had no differential effects on behavior or neurotoxicity.

Recently "detox" agents have been popularly used as forms of diets or nutritional supplements. Especially, several cases have been reported that these detox agents have been used to mask drug tests among drug abusers. In the present study, capsule and drink types of detox agents were evaluated for their ability to alter the elimination of methamphetamine in rats. For this study, methamphetamine and its major metabolite, amphetamine in urine samples were determined using LC-tandem mass spectrometry after administration of the detox agents to methamphetamine-treated rats. As a result, significant differences were not shown between control and detox-dosed groups in the amounts of methamphetamine and amphetamine excreted into urine as well as the volume of excreted urine. This result suggests that the detox agents tested may not affect the metabolism or elimination of methamphetamine and further might have minimal effect on narcotics detection in the urine samples of drug abusers.

One of the most notable trends in illegal substance use among Americans over the past decade is the dramatic growth and spread of methamphetamine use. In response to the dramatic rise in methamphetamine use and its associated burden, a broad range of legislations has been passed to combat the problem. In one paper, it was assessed the impact of retail-level laws intended to restrict chemicals used to manufacture methamphetamine (methamphetamine precursor laws) in reducing indicators of domestic production, methamphetamine availability, and the consequences of methamphetamine use. Specifically, we examine trends in these indicators of methamphetamine supply and use over a period spanning the implementation of the US federal Methamphetamine Anti-Proliferation Act (MAPA) (October 2000) and a more stringent state-level restriction enacted in California (January 2000). The results are mixed in terms of the effectiveness of legislative efforts to control methamphetamine production and use, depending on the strength of the legislation (California Uniform Controlled Substances Act versus federal MAPA), the specification of the comparison group, and the particular outcome of interest. Some evidence suggests that domestic production was impacted by these legislative efforts, but there is also evidence that prices fell, purities rose, and treatment episodes increased.

Methamphetamine is a highly addictive stimulant and long-term exposure leads to reductions in dopamine. One therapeutic strategy is to develop and test compounds that normalize...
dopamine. The primary aim of this study was to determine the safety of modafinil treatment during methamphetamine exposure in a controlled clinical setting. Methamphetamine-dependent volunteers (n=13), who were not seeking treatment, were randomized to receive either modafinil (200 mg) or matching placebo over three days (days 1-3 or days 8-10). On day 1, subjects were randomized to modafinil or placebo in the morning, and then 3 and 6 hours later received infusions of methamphetamine (0 and 30 mg, intravenously), after which cardiovascular and subjective effects were assessed. On day 3, participants completed intravenously self-administration sessions during which they made 10 choices for low doses of methamphetamine (3 mg) or saline. Days 4-7 were used as a washout period. On day 8 participants were assigned to the alternate study medication (placebo or modafinil), and the same testing procedures were repeated through day 10. The data reveal that modafinil treatment was well-tolerated and not associated with increased incidence of adverse events. In general, modafinil reduced by approximately 25 percent ratings of methamphetamine-induced “Any Drug Effect”, “High”, and “Want Methamphetamine”, and reduced total number of choices for methamphetamine and monetary value of methamphetamine, though none of these measures reached statistical significance. Given these encouraging, though non-significant trends, the primary conclusion is that it appears safe to proceed with modafinil in further clinical evaluations of therapeutic efficacy [09274].

Following more than two decades of generally increasing trends in the use and abuse of methamphetamine in certain parts of the country, prevalence indicators for the drug began to decrease in the mid-2000’s-but was this decrease signaling the end of the “meth problem”? one paper has compiled historical and recent data from supply and demand indicators to provide a broader context within which to consider the changes in trends over the past half decade. Data suggest supply-side accommodation to changes in precursor chemical restrictions, with prevalence indicators beginning to attenuate in the mid-2000’s and then increasing again by 2009-2010. Results support the need for continuing attention to control and interdiction efforts appropriate to the changing supply context and to continuing prevention efforts and increased number of treatment programs [11500].

To assess the effectiveness of methamphetamine precursor regulations in reducing illicit methamphetamine supply and use a systematic review of 12 databases was used to identify studies that had evaluated the impact of methamphetamine precursor regulations on methamphetamine supply and/or use. The guidelines of the Effective Practice and Organization of Care Group of The Cochrane Collaboration were used to determine which study designs were included and assess their quality. Ten studies met the inclusion criteria. These studies evaluated 15 interventions (13 regulations and two related interdiction efforts), all of which were located in North America. Interventions had consistent impacts across various indicators of methamphetamine supply and use. Seven of the 15 interventions produced reductions in methamphetamine indicators (ranging from 12 % to 77 %). Two of the largest impacts were seen following interdiction efforts, involving the closure of rogue pharmaceutical companies. There was no evidence of a shift into other types of drug use, or injecting use, although the impact on the synthetic drug market was not examined. Null effects were related largely to the existence of alternative sources of precursor chemicals or the availability of imported methamphetamine. It was concluded that methamphetamine precursor regulations can reduce indicators of methamphetamine supply and use. Further research is needed to determine whether regulations can be effective outside North America, particularly in developing countries, and what impact they have on the broader synthetic drug market. Improved data on precursor diversion are needed to facilitate the evaluation of precursor regulations [11501].

Heart rate variability (HRV) reflects a healthy autonomic nervous system and is increased with physical training. Methamphetamine dependence (MD) causes autonomic dysfunction
and diminished HRV. It was compared recently abstinent MD participants with age-matched, drug free controls (DF) and also investigated whether HRV can be improved with exercise training in the MD participants. In 50 participants (MD=28; DF=22) resting heart rate (R-R intervals) was recorded over 5 min while seated using a monitor affixed to a chest strap. Previously reported time-domain (SDNN, RMSSD, pNN50) and frequency-domain (LFnu, HFnu, LF/HF) parameters of HRV were calculated with customized software. MD were randomized to thrice weekly exercise training (ME=14) or equal attention without training (MC=14) over 8 weeks. Groups were compared using paired and unpaired t-tests. Participant characteristics were matched between groups: age 33 ± 6 years; body mass 83 ± 12 kg, BMI 27 ± 4 kg/min. Compared with DF, the MD group had significantly higher resting heart rate, LFnu, and LF/HF as well as lower SDNN, RMSSD, pNN50 and HFnu. At randomization, HRV indices were similar between ME and MC groups. However, after training, the ME group significantly increased SDNN (+15 ± 2 ms, +34 %), RMSSD (+20 ± 4 ms, +63 %), pNN50 (+23 ± 3 %, +173 %), HFnu (+14 ± 2, +60 %) and decreased HR (-5 ± 1 beats/min, -7 %), LFnu (-10 ± 2, -16 %) and LF/HF (-0.7 ± 0.3, -19 %). These measures did not change from baseline in the MC group. It was concluded that HRV, based on several conventional indices, was diminished in recently abstinent, methamphetamine dependent individuals. Moreover, physical training yielded a marked increase of HRV representing increased vagal modulation or improved autonomic balance [13485].

**Treatment of dependence**

Individuals who are methamphetamine dependent exhibit higher rates of cognitive dysfunction than healthy people who do not use methamphetamine, and this dysfunction may have a negative effect on the success of behavioral treatments for the disorder. Therefore, a medication that improves cognition, such as modafinil (Provigil), may serve as a useful adjunct to behavioral treatments for methamphetamine dependence. Although cognitive-enhancing effects of modafinil have been reported in several populations, little is known about the effects of modafinil in methamphetamine-dependent individuals. It was thus sought to evaluate the effects of modafinil on the cognitive performance of methamphetamine-dependent and healthy individuals. Seventeen healthy subjects and 24 methamphetamine-dependent subjects participated in this randomized, double-blind, placebo-controlled, crossover study. Effects of modafinil (200 mg, single oral dose) were assessed on participants’ performance on tests of inhibitory control, working memory, and processing speed/attention. Across subjects, modafinil improved performance on a test of sustained attention, with no significant improvement on any other cognitive tests. However, within the methamphetamine-dependent group only, participants with a high baseline frequency of methamphetamine use demonstrated a greater effect of modafinil on tests of inhibitory control and processing speed than those participants with low baseline use of methamphetamine. It was concluded that although modafinil produced limited effects across all participants, methamphetamine-dependent participants with a high baseline use of methamphetamine demonstrated significant cognitive improvement on modafinil relative to those with low baseline methamphetamine use. These results add to the findings from a clinical trial that suggested that modafinil may be particularly useful in methamphetamine-dependent subjects who use the drug frequently [11502].

**L-methamphetamine**

L-methamphetamine (the non-abused isomer of methamphetamine) is banned in athletic competition because it may improve athletic performance, but there are no studies assessing its effects on performance. In the United States L-methamphetamine is formulated in the
non-prescription Vick’s Vapor Inhaler nasal decongestant. Vick’s Vapor sold elsewhere contain similar inactive ingredients (menthol, camphor and Siberian pine oil) but no L-methamphetamine. One study tested the effects of inhaled L-methamphetamine delivered from a widely available non-prescription product on athletic performance. In a 2-session double-blind placebo-controlled study 12 participants (ages 14-17) were dosed with 4 (session 1) and 12 (session 2) inhalations from Vick’s Vapor with (USA) or without (UK) L-methamphetamine and then performed two 20 minute rides on a stationary bike with rides separated by a 30 minute rest. After about 16 μg L-methamphetamine distance travelled was 5.26 miles versus 5.30 with placebo. After about 48 μg L-methamphetamine distance travelled was not either significantly different. Modest doses of inhaled L-methamphetamine probably do not improve athletic performance but do minimally raise diastolic blood pressure [09275].

Methylamphetamine

The illicit drug methylamphetamine is often prepared from the precursor ephedrine or pseudoephedrine, which in turn are obtained by three processes: extraction from the Ephedra plant (“natural”), via fermentation of sugars (“semi-synthetic”), and by a “fully synthetic” route from propiophenone. We report the first method to differentiate between the three industrial routes used to produce the precursors ephedrine and pseudoephedrine by measurement of stable isotope ratios of nitrogen (delta^{15}N), hydrogen (delta^{2}H), and carbon (delta^{13}C). Analysis of 782 samples of seized methylamphetamine allowed classification into three groups using k-means clustering or the expectation-maximization algorithm applied to a Gaussian mixture model. By preparation of 30 samples of ephedrine by the “fully synthetic” industrial process and measuring their delta^{15}N, delta^{2}H, and delta^{13}C values, it was observed that ^{15}N becomes significantly depleted compared to the methylamine starting material. Conversion of ten ephedrine samples to methylamphetamine showed that this depletion is maintained in the final drug product, of which the delta^{15}N, delta^{2}H, and delta^{13}C values were distinct from those of ephedrine and methylamphetamine samples of a semi-synthetic (fermentation pathway) origin. Combining modeling analysis with the new experiments and published information on the values of delta^{2}H gave a definitive assignment of the three model groups, and equations to obtain probabilities for the precursor origin of any new sample. A simple rule of thumb is also presented. Making an assignment using delta values is particularly useful when no other chemical profiling information is available [13486].

Mephentermine

The urine specimens of numerous athletes were found to be positive for mephentermine both in-competition and out-of-competition in Taiwan. The donor of one specimen claimed she had only taken Mucaine (contains oxethazaine) for relieving symptomatic peptic ulcer and gastritis. Oxethazaine is not included in the prohibited list of the World Anti-Doping Agency; however, its metabolized compounds, mephentermine and phentermine, are included in that list. This study applied LC-MS-MS to analyze the excretions of three volunteers who ingested oxethazaine and presented positive results for mephentermine and/or phentermine. Thus, oxethazaine is the source of mephentermine and phentermine. Moreover, the results showed that 48 brands of gastric medicines containing oxethazaine were legally imported or locally manufactured in Taiwan, information which could be useful for limiting the misuse of oxethazaine by athletes. The data suggested that the sports associations should warn athletes about the risks of taking oxethazaine [09276].
Mephentermine and phentermine, substances prohibited in sports by the World Anti-Doping Agency, were found for the first time in urine specimens following the administration of a therapeutic medication, oxethazaine. In a recent sporting event, a urine specimen donor who tested positive for mephentermine and phentermine claimed consumption of Mucaine® for treating stomach pain was the reason for testing positive. Five volunteers were administrated oxethazaine (a topical anesthetic found in the multi-ingredient medication Mucaine and its generic equivalent, both of which are available in Taiwan), mephentermine, and phentermine. Excretion profiles of mephentermine and phentermine following the administration of these drugs were found to be similar. However, the mephentermine/phentermine ratios found in urine specimens collected at different time points following the administration of oxethazaine and mephentermine were found to be characteristically different [10190].

Metamfepramone

The sympathomimetic agent metamfepramone (2-dimethylamino-1-phenylpropan-1-one, dimethylpropion) (Dimethycathinone, Dimethylpropion) is a stimulant drug related to cathinone and methcathinone and used for the treatment of the common cold or hypotonic conditions, although first evaluated as an appetite suppressant. Due to its stimulating properties and its rapid metabolism resulting in major degradation products such as methylpseudoephedrine and methcathinone, it has been considered relevant for doping controls by the World Anti-Doping Agency (WADA). The rapid degradation of the active drug complicates the detection of metamfepramone itself but the metabolites methylpseudoephedrine and methcathinone can be monitored, and the finding of the latter in particular allows the inference of a metamfepramone administration. In order to improve sports drug testing procedures, metamfepramone, methylpseudoephedrine and methcathinone were characterized using electrospray ionization-high resolution/high accuracy mass spectrometry, and a method employing liquid chromatography/tandem mass spectrometry was established that allowed the analysis of these three analytes by direct injection of 2 microL of urine specimens. The assay was validated with regard to specificity, lower limits of detection (2-10 ng/mL), intraday and interday precision (3-17 %) and ion suppression/enhancement effects. The developed procedure has been used to verify or falsify suspicious signals observed in routine screening procedures based on gas chromatography/mass spectrometry and yielded an adverse analytical finding concerning a metamfepramone administration in an authentic doping control sample. Although the active drug was not detected, the indicative metabolites methylpseudoephedrine and methcathinone were considered sufficient to infer the application of the prohibited drug [09406].

The sympathomimetic agent metamfepramone (2-dimethylamino-1-phenylpropan-1-one, dimethylpropion) is widely used for the treatment of the common cold or hypotonic conditions. Due to its stimulating properties and its rapid metabolism resulting in major degradation products such as methylpseudoephedrine and methcathinone, it has been considered relevant for doping controls by the World Anti-Doping Agency (WADA). The rapid degradation of the active drug complicates the detection of metamfepramone itself but the metabolites methylpseudoephedrine and methcathinone can be monitored, and the finding of the latter in particular allows the inference of a metamfepramone administration. In order to improve sports drug testing procedures, metamfepramone, methylpseudoephedrine and methcathinone were characterized using electrospray ionization-high resolution/high accuracy mass spectrometry, and a method employing liquid chromatography/tandem mass spectrometry was established that allowed the analysis of these three analytes by direct
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Cathinones

A new group of recreational drugs, popularly known as "bath salts", "plant feeders" or "plant food", has recently emerged in numerous countries. Although various products are labeled with warnings "not for human consumption" or "not tested for hazards or toxicity", they are intended to produce a high similar to that obtained with illegal stimulants, such as MDMA, methamphetamine or cocaine. The active compounds in "bath salts" are cathinone derivatives continuously developed and modified by drug designers to avoid detection or legal scrutiny. Around 2010 the most prevalent were mephedrone (4-methylmethcathinone) and MDPV (3,4-methylenedioxypyrovalerone). One review surveyed the current state of knowledge regarding the pharmacotoxicological properties of synthetic cathinones, the prevalence and pattern of their use. Special emphasis is given to the negative consequences of using these products including, among others, cardiovascular, psychiatric and neurologic symptoms, dehydration, rhambdomyolysis, renal and liver failure. Case reports on synthetic cathinones-related fatalities are also presented [13501].

The abuse of synthetic cathinones, widely known as bath salts, has been increasing since the mid-2000s. These substances are derivatives of the naturally occurring compound cathinone, which is the primary psychoactive component of khat. The toxicity of synthetic cathinones includes significant sympathomimetic effects, as well as psychosis, agitation, aggression, and sometimes violent and bizarre behavior. Mephedrone and methylenedioxypyrovalerone are currently the predominantly abused synthetic cathinones [13502].

Designer beta-keto-amphetamines (e.g. cathinones, “bath salts” and “research chemicals”) have become popular recreational drugs, but their pharmacology is poorly characterized. It was determined the potencies of cathinones to inhibit DA, NA and 5-HT transport into transporter-transfected HEK 293 cells, DA and 5-HT efflux from monoamine-preloaded cells, and monoamine receptor binding affinity. Mephedrone, methylone, ethylone, butylone and naphyrone acted as non-selective monoamine uptake inhibitors, similar to cocaine. Mephedrone, methylone, ethylone and butylone also induced the release of 5-HT, similar to 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) and other entactogens. Cathinone, methcathinone and flephedrone, similar to amphetamine and methamphetamine, acted as preferential DA and NA uptake inhibitors and induced the release of DA. Pyrovalerone and 3,4-methylenedioxypyrovalerone (MDPV) were highly potent and selective DA and NA transporter inhibitors but unlike amphetamines did not evoke the release of monoamines. The non-β-keto amphetamines are trace amine-associated receptor 1 ligands, whereas the cathinones are not. All the cathinones showed high blood-brain barrier permeability in an in vitro model; mephedrone and MDPV exhibited particularly high permeability. It was concluded that cathinones have considerable pharmacological differences that form the basis of their suggested classification into three groups. The predominant action of all
cathinones on the DA transporter is probably associated with a considerable risk of addiction [13503].

A rapid, reproducible and sensitive reversed phase liquid chromatography-mass spectrometry method was developed and validated for the identification and semi-quantitative confirmation of stimulants in urine. The method is capable of separating compounds such as cocaine and metabolites, amphetamines, substituted cathinones and other designer drugs, with a total run time of 11 min. The method was subsequently used to confirm the presence of these stimulants in the urine of patients attending the Drug Treatment Centre Board Ireland over the period in which legislation banning some named cathinones was introduced in Ireland. Substituted cathinones were the predominant drug of choice, outside of cocaine use. Mephedrone was the most widely detected cathinone in 2010, whereas 3,4-methylenedioxypyrovalerone featured more prevalently in screenings in 2011. The appearance of adverse effects increases during multi-stimulant use related to synergistic pharmacological combinations, and this method has benefits in identifying multi-drug use between next generation designer drugs and commonly used stimulants [13504].

Laboratory techniques

Recently, clandestine drug lab operators have attempted to bypass controlled substances laws and regulations with "designer" compounds chemically and pharmacologically similar to controlled substances. For example, "bath salts" have erupted onto the scene as "legal highs" containing cathinone analogs that have produced severe side effects in users worldwide. These products have sparked concern among law enforcement agencies, and emergency bans have been placed on the sale of such items. Despite the increasing number of designer drugs available, there are few comprehensive screening techniques for their detection and quantification in biological specimens. The liquid chromatography triple quadrupole tandem mass spectrometry (LC-QQq-MS/MS) method presented here encompasses over thirty important compounds within the phenethylamine, tryptamine, and piperazine designer drug classes. Analytes were determined by LC-QQq-MS/MS in the multiple-reaction monitoring mode after mixed-mode solid-phase extraction. The bioanalytical method was fully validated according to recommended international guidelines. The assay was selective for all analytes with acceptable accuracy and precision. Limits of quantification were in the range of 1-10 ng/mL for each compound with limits of detection near 10 pg/mL. In order to evaluate its applicability in a forensic toxicological setting, the validated method was used to analyze post-mortem specimens from two cases that were suspected of containing designer drugs. The method was able to identify and quantify seven of these compounds at concentrations as low as 11 ng/mL. The method should have wide applicability for rapid screening of important new drugs of abuse at high sensitivity in both post- and ante-mortem forensic analysis [13513].

Methylenedioxypyrovalerone (MDPV)

3,4-Methylenedioxypyrovalerone (MDPV) is a psychoactive, synthetic analog of the central nervous system stimulant cathinone. Its recent popularity as a recreational drug in the United States has led to numerous reports to poison control centers across the country. As with other synthetic cathinones, the recreational use of MDPV has resulted in death. MDPV is thought to exert its pharmacologic effects by inhibiting the reuptake of dopamine and norepinephrine. This report describes the case of an exposure of a 39-year-old male to MDPV, which resulted in his death. Postmortem concentrations of MDPV in various tissues
were measured. The detection of MDPV in tissues and fluids was accomplished using gas chromatography-mass spectrometry analysis after solid-phase extraction. Blood analysis also demonstrated therapeutic levels of lamotrigine, fluoxetine, risperidone, benztropine, pseudoephedrine and ibuprofen. The detection of cathinones in hair was conducted using high-performance liquid chromatography-tandem mass spectrometry after solid-phase extraction. MDPV was uniformly distributed among multiple tissues (blood, brain, muscle, cerebrospinal fluid and lung) at concentrations of approximately 0.4 to 0.6 microg/mL. Tissue and fluids responsible for detoxification/excretion had higher concentrations of MDPV (kidney, liver and bile > 0.8 microg/mL). A blood concentration ≥ 0.4 microg/mL was judged sufficient to cause death. The cause of death was ruled MDPV intoxication resulting in cardiac arrhythmia [13509].

It was identified six synthetic cathinones, commonly found in bath salt products, in 43 cases. Thirty-two cases will be reviewed here, including all of the postmortem cases, all of the human performance cases that had blood specimens submitted, and one urine-only human performance case. The following compounds have been confirmed: 3,4-methylenedioxy-pyrovalerone (MDPV), 3,4-methylenedioxymethcathinone (methylene), pyrovalerone, pentylone, alpha-pyrolidinopentiophenone (alpha-PVP) and methedrone. The method also screens for mephedrone, butylone and 3-fluoromethcathinone. Case demographics show 42 white males and females ranging in age from 19 to 53 years. The remaining case was that of a 34-year-old Hispanic male. The 43 cases represent 17 driving under the influence, two domestic violence, four suicides, 12 overdoses, six accidents, one drug-facilitated assault and one homicide. Data will be presented on the distribution of some of these cathinones in various matrices. After review, blood concentration does not appear to predict outcome regarding fatalities or impairment. The highest MDPV concentration occurred in a suicide by hanging and the highest methylene concentration was in a driver. The confirmation method is a liquid-liquid extraction with detection by liquid chromatography triple quadrupole mass spectrometry using electrospray ionization in multiple reaction monitoring mode [13510].

The analysis of designer drugs, including those in the synthetic cathinone and piperazine classes, may be complicated by the poor stability of these compounds in biological specimens. The stability of four of these compounds was investigated: 3,4-methylenedioxy-pyrovalerone, 4-methyl-N-methylcathinone (mephedrone), N-benzylpiperazine and 1-[3-(trifluoromethyl)phenyl]piperazine. Compound stability was monitored in three different biological matrices when each matrix was stored under three different conditions. These matrices and conditions included human whole blood, human serum and human urine, each stored at -20, 4 and 22°C for a period of 14 days in the dark in a sealed glass container. Analysis by liquid chromatography-tandem mass spectrometry was performed on Day 1 to establish the initial concentration for each drug in each specimen type, and then the samples were divided into three parts for storage under the various conditions. Analysis was performed in triplicate on Days 2, 4, 7 and 14 for each specimen type under each storage condition and the results were compared to those obtained on Day 1. Following analysis of the data, it became clear that mephedrone was not stable, and that care must be taken following specimen receipt to ensure minimal degradation [13511].

**Sudden death**

The rise in popularity of "bath salts" as safe alternatives to MDMA (3,4-methylenedioxy-methamphetamine), methamphetamine, and other illicit substances has resulted in increased scrutiny of the contents and toxicology associated with these products. It was reported a case of sudden death related to the synthetic cathinone methylene (3,4-methylenedioxy-N-methylcathinonomethyl) in a previously healthy 19-year-old man. Although several fatal case reports have been published involving methylene and other synthetic cathinones, this is
the first reported case of sudden cardiac death associated with methylone use. Although lack of published data prevented a comparison of blood methylone concentrations between the case and existing reports, the amount of methylone that was detected postmortem (0.07 mg/dL) is below those reported in MDMA-related fatalities. The report suggests that methylone toxicity has been greatly underestimated by users of this synthetic cathinone [13512].

Mephedrone

New natural and synthetic compounds are continuously introduced into the illicit drug market. Their origin, composition, main and side-effects are often not exactly known by the users themselves. Thus, the control of these substances is extremely difficult. In year 2008, a new synthetic drug called mephedrone (2-metilamino-1-(4-metilfenil) propan-1-on) appeared in Hungary (also known as: Mct, 4-MMC, MM-Cat, Meow, 4-methylmethcathinone. It was summarized its frequency in biological samples investigated for illicit drugs, and experiences of the medical examination of mephedrone-users. Toxicological analyses of biological samples (urine and/or blood) were carried out by GC-MS. Altogether 5386 samples were analyzed in 2010 (4922 in Budapest and 464 in Szeged), and mephedrone was identified in 363 cases (7 %). At present it is not sufficient experience with its long-term effects, tolerance, addiction, withdrawal symptoms or toxic dose. Thus, it is difficult to establish whether addiction and/or mental disorder occurred [11367].

To assess the patterns of use, subjective effect profile and dependence liability of mephedrone, supported by corroborative urine toxicology a cross-sectional structured telephone interview of UK-based drug users associated with the dance music scene was performed. A total of 100 mephedrone users were recruited through their involvement with the dance music scene. Assessment of pattern of use, acute and after effects, DSM dependence used criteria and gas chromatography-mass spectrometry urinalysis. Mephedrone consumption results in typical stimulant-related subjective effects: euphoria, increased concentration, talkativeness, urge to move, empathy, jaw clenching, reduced appetite and insomnia. Thirty per cent of the sample potentially met criteria for DSM-IV dependence and there was evidence of a strong compulsion to use the drug (47 % had used the drug for 2 or more consecutive days). Self-reported recent consumption of mephedrone was confirmed by toxicological analysis in all of the 14 participants who submitted a urine sample. Mephedrone has a high abuse and health risk liability, with increased tolerance, impaired control and a compulsion to use, the predominant reported dependence symptoms [11506].

One study sought to collect information on the former legal-high “mephedrone” using a web-based survey targeted at mephedrone users. The survey was advertised on websites frequented by drug users. Individuals were invited to complete the survey if they had taken mephedrone on at least one occasion in the past. One thousand and six completed forms were received from declared users, making this the largest survey on mephedrone to date. Results showed that mephedrone users consider its effects to compare best with those of MDMA, and while MDMA was considered marginally safer and its effects more pleasurable, mephedrone's appeal lay in its availability, low price and reliable purity [11507].

Mephedrone (4-methylmethcathinone) is the beta-keto analogue of 4-methylmethamphetamine. Before its control in April 2010, it became popular as a legal high in the United Kingdom, displacing methylenedioxyamphetamine as the stimulant drug of choice. The drug has stimulant and psychoactive properties, and therefore has forensic significance
in criminal and morbid toxicology. The purpose of one study was to survey casework involving the drug (impaired driving and sudden death). The cases were received in the laboratory for analysis between late 2009 and the end of 2010. Analysis of blood samples for mephedrone was conducted by liquid chromatography-mass spectrometry (LC-MS). Routine screening for alcohol and a range of other pharmaceuticals and drugs of abuse was conducted using a combination of enzyme-linked immunoassay, gas chromatography (GC) headspace, GC-MS and high-performance liquid chromatography with diode array detection. Mephedrone was detected in a total of 12 fatal cases. Most of these cases involved death by mechanical means; in two cases, death was attributed directly to mephedrone intoxication (blood concentrations of 2.1 and 1.94 mg/L). Mephedrone was detected in a total of 32 impaired driving cases. Blood concentrations ranged up to 0.74 mg/L (mean 0.21, median 0.10). The casework evidence in this study indicated that recreational use of the drug can produce blood levels as high as 0.74 mg/L, although the most common value encountered is likely to lie between 0.2 and 0.3 mg/L [13505].

“Bath salts” are stimulants with high abuse potential that are known to contain agents such as 3,4-methylenedioxypyrovalerone and 4-methylmethcathinone (mephedrone). They are marketed locally and through online retailers as legitimate products in order to evade legal control and facilitate widespread distribution. They have been present in Europe since 2007 but are now becoming a burgeoning presence in American hospitals. Though preliminary efforts are underway in the United States to restrict their usage and distribution, there remains a general unawareness on the part of physicians regarding the drugs' physiological effects. While they mimic the effects of other known stimulants, they are not detected on standard urine screens. It was presented a clinical case that illustrates a typical pattern of usage along with a description of their basic chemistry, appearance, methods of delivery, withdrawal and intoxication characteristics, treatment recommendations, and areas for further research [13506].

**Long-term effects**

The use of cathinone-derivative designer drugs methylene and mephedrone has increased rapidly in recent years. The aim of one study was to investigate the possible long-term effects of these drugs on a range of behavioral tests in mice. Further, it was investigated the long-term effects of these drugs on brain neurochemistry in both rats and mice. It was treated animals with a binge-like regimen of methylene or mephedrone (30 mg/kg, twice daily for 4 days) and, starting 2 weeks later, we performed behavioral tests of memory, anxiety and depression and measured brain levels of dopamine (DA), serotonin (5-HT), their metabolites and norepinephrine (NE). 5-HT and DA transporter (5-HTT and DAT) levels were also measured in rats by 3H-paroxetine and 3H-mazindol binding. Mephedrone reduced working memory performance in the T-maze spontaneous alternation task but did not affect neurotransmitter levels aside from a 22 percent decrease in striatal homovanillic acid (HVA) levels in mice. Methylene had little effect on behavior or neurotransmitter levels in mice but produced a widespread depletion of 5-HT and 5-HTT levels in rats. It was concluded that both methylene and mephedrone appeared to have a long-term effect on either behavioral or biochemical gauges of neurotoxicity in rodents [13507].

**A fatal case**

A death caused by a new designer drug, 4-methylmethcathinone (mephedrone), is reported. Eight small plastic bags containing white powder were found in the jacket of a young dead male. Spot tests conducted by the police officer indicated the presence of 4-bromo-2,5-dimethoxyphenethylamine (2C-B) in the powders. Laboratory routine screening analyses of blood and vitreous humor did not reveal any positive results; therefore, 2C-B was excluded.
Analysis of powders was conducted using gas chromatography-mass spectrometry and high-pressure liquid chromatography with diode array detection. The purity of mephedrone found in all powder samples was in the range of 80-87 percent. In connection with these findings, blood and vitreous humor samples were analyzed for mephedrone. Analyses were conducted using liquid chromatography-tandem mass spectrometry. Mephedrone was found in blood and vitreous humor at the concentrations of 5.5 and 7.1 µg/mL, respectively, revealing that this was a fatal mephedrone intoxication [13508].

Tuaminoheptane

Since January 2007, the list of prohibited substances established by the World Anti-Doping Agency includes the sympathomimetic compound tuaminoheptane (1-methyl-hexylamine, 2-heptylamine). Primarily used as nasal decongestant drug it has been considered relevant for sports drug testing due to its stimulating properties. A confirmatory gas chromatographic-mass spectrometric procedure was developed including liquid-liquid extraction and imine formation of tuaminoheptane employing various aldehydes and ketones such as formaldehyde, acetaldehyde, benzaldehyde and acetone. Extraction and derivatisation conditions were optimised for utmost efficiency, and characteristic fragment ions obtained after electron ionisation allowed for a sensitive and selective analytical assay, which was validated with regard to recovery (50 %), lower limit of detection (20 ng/mL) as well as interday- and intraday precision (<15 %). The applicability to authentic urine samples was demonstrated using administration study specimens obtained from two male persons using Rhinofluimucil (tuaminoheptane hemisulfate) for intranasal application. The administered drug was detected up to 46 h after repeated topical instillation of a total of approximately 3 mg [07180].

Mesocarb

The synthesis and method of analysis of hydroxylated mesocarb metabolites were described. Six potential hydroxylated mesocarb metabolites were prepared, characterized, and compared with the mesocarb metabolites synthesized enzymatically in vitro using human liver proteins and also compared with metabolites extracted from human urine after oral administration of mesocarb. p-Hydroxymesocarb was the most prevalent metabolite (conjugated and non-conjugated) observed. With respect to doping analysis, synthesis of p-hydroxymesocarb, the main urinary metabolite of mesocarb, and its availability as a reference material is important [09262].

The objective of one study was to investigate mesocarb metabolism in humans. Samples obtained after administration of mesocarb to healthy volunteers were studied. The samples were extracted at alkaline pH using ethyl acetate and salting-out effect to recover metabolites excreted free and conjugated with sulfate. A complementary procedure was applied to recover conjugates with glucuronic acid or with sulfate consisting of the extraction of the urines with XAD-2 columns previously conditioned with methanol and deionized water; the columns were then washed with water and finally eluted with methanol. In both cases, the dried extracts were reconstituted and analyzed by ultra-performance liquid chromatography-tandem mass spectrometry. Chromatographic separation was carried out using a C18 column (100 mm x 2.1 mm i.d., 1.7 microm particle size) and a mobile phase consisting of water and acetonitrile with 0.01% formic acid with gradient elution. The chromatographic system was coupled to a mass spectrometer with an electrospray ionization source working in positive mode. Metabolic experiments were performed in multiple-reaction monitoring mode by
monitoring one transition for each potential mesocarb metabolite. Mesocarb and 19 metabolites were identified in human urine, including mono-, di-, and trihydroxylated metabolites excreted free as well as conjugated with sulfate or glucuronic acid. All metabolites were detected up to 48 h after administration. The structures of most metabolites were proposed based on data from reference standards available and molecular mass and product ion mass spectra of the peaks detected. The direct detection of mesocarb metabolites conjugated with sulfate and glucuronic acid without previous hydrolysis has been described for the first time. Finally, a screening method to detect the administration of mesocarb in routine antidoping control analyses was proposed and validated based on the detection of the main mesocarb metabolites in human urine (p-hydroxymesocarb and p-hydroxymesocarb sulfate). After analysis of several blank urines, the method demonstrated to be specific. Extraction recoveries of 100 ± 1 and 106 ± 11 (n=4), and limits of detection of 0.5 and 0.1 ng/mL were obtained for p-hydroxymesocarb sulfate and p-hydroxymesocarb [10382]

**Sydnocarb**

Sydnocarb is a psychomotor stimulant structurally similar to d-amphetamine (D-AMPH) and is used in Russia for the treatment of a variety of neuropsychiatric comorbidities. The nature of sydnocarb-induced facilitation of dopamine (DA) neurotransmission (DA release versus DA transporter, DAT, inhibition) is not clear. One study characterized the pharmacological actions and behavioral effects of intraperitoneal sydnocarb in male Sprague-Dawley rats. Where relevant, comparisons were made with intraperitoneal D-AMPH. Unlike D-AMPH, which causes release of DA from rat synaptosomes, sydnocarb (up to 100 microM) did not. Sydnocarb potently blocked recombinant human DAT expressed in Chinese hamster ovary-K1 cells and less potently blocked the norepinephrine transporter. Sydnocarb at 10 microM did not bind to 64 other targets. In rats, 10 and 30 mg/kg sydnocarb showed a 2-fold longer half-life in plasma and brain and a 5-fold lower brain-to-plasma ratio compared with 0.3 and 1 mg/kg D-AMPH. In the Irwin assay, sydnocarb was well tolerated up to 30 mg/kg; D-AMPH-like stereotypic behaviors were evident at 100 mg/kg. Behavioral effects of 30 mg/kg sydnocarb and 0.3 mg/kg D-AMPH were comparable. In a sleep/wake assay, 10 mg/kg sydnocarb and 1 mg/kg D-AMPH increased wakefulness comparably; however, sydnocarb (up to 30 mg/kg) did not induce D-AMPH-like rebound hypersomnolence (RHS). Like D-AMPH, sydnocarb enhanced theta power, an electrophysiological measure of cognitive function. In conclusion, sydnocarb is a selective and potent DAT inhibitor that produces robust increases in the wake state without RHS, and with potential cognitive-enhancing properties [11219].

**Strychnine**

A simple, rapid, sensitive and low-cost method using capillary electrophoresis (CE) coupled with field-amplified sample stacking (FASS) has been developed and validated for the simultaneous determination of strychnine and brucine residues in human urine. Before sample loading, a water plug (3.5 kPa, 3 s) was injected to contain sample cations and to permit FASS. Electrokinetic injection at a voltage (20 kV, 25 s) was then used to introduce cations. Separation was performed using 20 mM acetate buffer (pH 3.8) with an applied voltage of 20 kV. The calibration curves were linear over a range of 8.00 x 10^2 ng/mL for strychnine and 10.0-3.20 x 10^2 ng/mL for brucine. Extraction recoveries in urine were greater than 80 and 83 percent for strychnine and brucine, respectively, with an RSD of less than 4.9 percent. The detection limits (signal-to-noise ratio 3) for strychnine and brucine
were 2.00 and 2.50 ng/mL, respectively. A urine sample from one healthy female volunteer (26 years old, 50 kg) was pretreated and analyzed. Strychnine and brucine levels in urine could be detected 24 h after administration. On these grounds, this method was feasible for application to preliminary screening of trace levels of abused drugs for both doping control and forensic analysis [09280].

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Benfluorex

Benfluorex [1-(m-trifluoromethylphenyl)-2-(β-benzoyloxyethyl)aminopropane] has been widely used for the treatment of atherogenic metabolic disorders and impaired carbohydrate metabolism (particularly in obese type-II diabetic patients) as well as an anorectic drug. Due to its potentially performance-enhancing properties, benfluorex has been added to the list of prohibited compounds and methods of doping by the World Anti-Doping Agency (WADA) in 2010, necessitating the implementation of the drug as well as its major metabolites into routine doping control procedures. In one study, human urinary metabolites of benfluorex were characterized by gas chromatography-electron ionization-mass spectrometry (GC-EI-MS) as well as liquid chromatography-electrospray ionization-high resolution/high accuracy tandem mass spectrometry (LC-ESI-MS/MS). Commonly employed sports drug testing approaches consisting of liquid-liquid extraction followed by GC-MS or urine dilution and immediate LC-MS/MS analysis were expanded and validated with regard to specificity, recovery (48-54 %, GC-MS only), intra- and interday precision (<25 %), limits of detection (5-8 ng/mL for LC-MS/MS and 80 ng/mL for GC-MS), and ion suppression (for LC-ESI-MS/MS only) to allow the detection of benfluorex metabolites 1-(m-trifluoromethylphenyl)-2-(2-hydroxyethyl)aminopropane (M1), 1-(m-trifluoromethylphenyl)-2-(2-carboxymethyl)aminopropane (M2), and 1-(m-trifluoromethylphenyl)-2-aminopropane (M3) as well as the glucuronic acid conjugate of M1 [11435].

Laboratory techniques

A liquid-chromatography-tandem-mass-spectrometry method using pneumatically assisted electrospray ionisation (LC-ESI-MS/MS) was developed for the simultaneous determination of cathinone, methcathinone, ethcathinone, amfepramone, mephedrone, flephedrone, methedrone, methylene, butylone, cathine, norephedrine, ephedrine, pseudoephedrine, methylephedrine and methylpseudoephedrine in human live and post-mortem whole blood. The blood proteins were precipitated by the addition of methanol, and the extract was
purified by ultrafiltration. The separation of diastereomeric ephedrines was achieved on an ethyl-linked phenyl column. Matrix-matched calibrants combined with the isotope dilution of selected substances were used for quantitative analysis. The relative intra-laboratory reproducibility standard deviations were generally better than 7 percent at concentrations of 20 microg/L, and the mean true recoveries were 87-106 percent in the concentration range of 10-250 microg/L. The detection limits were in the range of 0.5-3 microg/L. The cathinones were unstable in whole blood and sample extracts under neutral conditions, but the stability could be improved by the acidification of the sample matrix [11194].

A novel method for the determination of propranolol hydrochlorid (PRO) in human urine was developed using capillary electrophoresis coupled with electrochemiluminescence detection (CE-ECL). The parameters that affected the separation and detection were optimized. Under the optimal conditions, the linear range for PRO was from 0.003 to 2 µg/mL and the detection limit was 1.3 ng/mL (S/N=3). The method was successfully applied to the study of the pharmacokinetics of PRO in human urine. The relative standard deviations of ECL intensity and migration time were 2.6 and 2.1 percent, respectively (1.0 µg/mL PRO, n=6). The recovery was between 97 and 97 percent. The peak excretion rate in urine was observed during the 0.5-1 h after oral administration of a 10-mg PRO tablet and the urinary excretion ratio of PRO was 14 percent within 12 h. The method was simple, rapid, economical and sensitive, and may improve the detection of PRO as a doping agent in sports [11195].

A method for the simultaneous screening and confirmation of the presence of fourteen tertiary amine stimulants in human urine by gas chromatography-mass spectrometry (GC-MS) in combination with liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been developed and validated. Solid phase extraction (SPE) and liquid-liquid extraction (LLE) approaches were utilized for the pre-treatment of the urine samples. The study indicated that the capillary temperature played a significant role in the signal abundances of the protonated molecules of cropropamide and crotethamide under positive ion electrospray ionization (ESI) conditions. In addition, comparison studies of two different pre-treatment approaches as well as the two ionization modes were conducted. The LODs of the developed method for all the analytes were lower than the minimum required performance limit (MRPL) as set forth in the World Anti-Doping Agency (WADA) technical document for laboratories. The human urine sample obtained after oral administration of prolintane. HCl was successfully analyzed by the developed method, which demonstrated the applicability and reliability of
SIBUTRAMINE

Sibutramine (trade name Meridia in the US and Canada, Ectiva in South Africa, Reductil in Europe and most other countries), usually as sibutramine hydrochloride monohydrate, is an orally administered agent for the treatment of obesity, as an appetite suppressant. It is a centrally-acting serotonin-norepinephrine reuptake inhibitor structurally related to amphetamines, although its mechanism of action is distinct A method for identifying the metabolites of sibutramine 1-(4(chlorophenyl)-N,N-dimethyl-alpha-(2-methylpropyl)) cyclobutanemethanamine) in urine, utilizing a double derivatization strategy, with N-methyl-N-(trimethylsilyl)-trifluoroacetamide and N-methyl-bis-(trifluoroacetamide), in gas chromatography/mass spectrometry was proposed. This methodology results in mass spectra with at least three fragments in abundance superior to 20 percent, attending the World Anti-Doping Agency identification criteria for qualitative assays. Sibutramine was administered to five volunteers and the excretion profile followed for 92 h. Routine analytical, hydroxy-cyclobutane-bis-nor-sibutramine which becomes the more abundant metabolite in the first 10 h and hydroxy-isopropyl-bis-nor-sibutramine which becomes the most abundant after 40 h, were proposed for doping monitoring [09278].

A method for identifying the metabolites of sibutramine (Reductil®, Meridia® and Sibutrex®) 1-(4(chlorophenyl)-N,N-dimethyl-alpha-(2-methylpropyl))cyclobutanemethanamine) in urine, utilizing a double derivatization strategy, with N-methyl-N-(trimethylsilyl)-trifluoroacetamide and N-methyl-bis-(trifluoroacetamide), in gas chromatography/mass spectrometry was proposed. This methodology results in mass spectra with at least three fragments in abundance superior to 20 percent, attending the World Anti-Doping Agency identification criteria for qualitative assays. The characterization of the derivatives was obtained through two ionization modes: Chemical Ionization and Electron Impact ionization, both in full scan mode. Sibutramine was administered to 5 (five) volunteers and the excretion profile followed for 92 h. Routine analytical, hydroxy-cyclobutane-bis-nor-sibutramine which becomes the more abundant metabolite in the first 10h and hydroxy-isopropyl-bis-nor-sibutramine which becomes the most abundant after 40h, were proposed for doping monitoring [09279].

A gas chromatographic/mass spectrometric (GC/MS) study aimed at identifying the metabolites of sibutramine (1-(4-chlorophenyl)-N,N-dimethyl-alpha-(2-methylpropyl)cyclobutanemethanamine) in urine is described. Urinary excretion of sibutramine metabolites following the oral administration of a single dose of sibutramine was followed by GC/MS analysis. After identification of the chromatographic signals corresponding to the six main urinary metabolites, the fragmentation pattern was studied in electron ionization (EI) mode after derivatization to the corresponding methyl and trimethylsilyl derivatives. Urine samples were pretreated according to a reference procedure (liquid/liquid separation, enzymatic hydrolysis, pre-concentration under a stream of nitrogen and derivatization, either under thermal incubation or by microwave irradiation). All sibutramine metabolites were excreted as glucuroconjugates, and retain the chiral carbon present in the sibutramine skeleton. The metabolites identified included mono-desmethyIsibutramine (nor-sibutramine), bi-desmethyIsibutramine (nor-nor-sibutramine), and the corresponding hydroxylated compounds, the hydroxylation taking place either on the cyclobutane or on the isopropyl chain. The excretion profiles of the different metabolites were also evaluated. From an analytical point of view, the method can be applied to different fields of forensic analytical toxicology, including anti-doping analysis. Although the lack of certified reference materials does not allow a precise determination of the limits of detection (LODs) of all the sibutramine metabolites, an estimation taking into account the response factor of similar compounds ensures that all metabolites are still clearly detectable in a range of concentrations between
10 and 50 ng/mL, thus satisfying the minimum required performance limits (MRPLs) of the World Anti-Doping Agency (WADA) [07200].

Since January 2006, the list of prohibited substances established by the World Anti-Doping Agency includes the antidepressant and anti-obesity drug Sibutramine. Due to its rapid degradation to its active metabolites N-desmethyl and N-bisdesmethyl Sibutramine, reference compounds were synthesized and included into an existing screening assay to allow the unambiguous determination of these metabolic products in human urine using liquid-liquid extraction followed by liquid chromatography/tandem mass spectrometry. Characteristic product ions, obtained after electrospray ionization and collision-induced dissociation, were elucidated using high resolution/high accuracy mass measurements with a hybrid linear ion trap/orbitrap mass analyzer. Based on diagnostic product ions, the extended screening procedure was validated for both Sibutramine metabolites using a triple quadrupole mass spectrometer. Items such as lower limits of detection (6-40 ng/mL), recoveries (39-42 %), intraday precision (low: 5.5-10.6%, medium: 4.9-5.9%), high: 12.8-16.4%) and interday precision (low: 15.0-22.8 %, medium: 17.7-18.6 %), high: 16.5-25.6 %) were evaluated, and a clinical spot urine sample was analyzed to demonstrate the applicability of the developed assay in sports drug testing [06219].

Adulteration of botanical food supplements with undeclared synthetic drugs is a common problem. One of the most affected product groups are the slimming agents. There are no analytical protocols for the detection of synthetic adulterants from these products. The present study aimed at the development of a multistep analytical method for the quick and reliable determination of sibutramine, one of the most common adulterants among botanical food supplements. The extract of a sibutramine-containing slimming formula was analysed by colour tests, TLC, HPLC-DAD, MS and NMR. The multistep method proposed by the authors allows the quick identification of sibutramine in counterfeit samples in laboratories with different instrumentation [13563].

A suspected sibutramine analogue was detected in a slimming functional food by an ultra performance liquid chromatography-electrospray ionisation-time of flight mass spectrometry (UPLC-ESI-TOF/MS) method. The ultraviolet (UV) spectrum of this suspected compound showed close similarity to that of sibutramine. The sample was extracted with 70 percent MeOH and isolated by semi-preparative column chromatography. The structure of this compound was identified by spectroscopic analyses (nuclear magnetic resonance [NMR] technique, mass and tandem mass etc.). The structure of the unknown compound was demonstrated to be [([+]-dimethyl-1-[1-(3,4-dichlorophenyl)cyclobutyl]-N,N,3-trimethylbutan-1-amine (molecular formula C17H25NCl2) and named as chloro-sibutramine. Compared with sibutramine, it has one more chlorine atom than the 3-chlorophenyl group so was switched to 3,4-dichlorophenyl. Until now, chloro-sibutramine was isolated for the first time from the undeclared ingredient included in dietary supplements. Although the safety of chloro-sibutramine is unknown, there is a potential health risk to consumers because of a similar skeleton to sibutramine. For public health, this sibutramine analogue has been included in the inspection list of illegal adulterants in Korea [13564].
Asthma, a chronic inflammatory airway disorder, is one of the most frequently occurring chronic diseases. Its prevalence in adults is about 5 percent. Among athletes, however, it is assumed to be 10-20 percent. Inhaled beta-2 agonists are the drug of choice for treatment of asthma. However, beta-2 agonists are prohibited for non-asthmatic athletes according to the most recent list of prohibited substances released by the World Anti-Doping Agency (WADA). The main reason for prohibition in non-asthmatic athletes is its claimed ergogenic potential. Beta-2 agonists have received recent attention from the International Olympic Committee (IOC) and the World Anti-Doping Agency. At the 1992 Barcelona Olympic Games, two US athletes tested positive for the beta-2 agonist clenbuterol and were subsequently disqualified. Controversy at the Olympics about clenbuterol raised questions as to whether beta-2 agonists could affect muscle mass and function. Numerous studies have demonstrated that administration of some beta-2 agonists such as clenbuterol to a variety of species caused muscle growth and alteration in body composition. However, the anabolic effects of salbutamol remain equivocal. In animal studies, the anabolic effect of salbutamol on skeletal muscle was only found after intravenous administration with implanted minipumps, but never after oral administration. Oral administration of slow-release preparation of salbutamol (Volmax, Glaxo, Greenford, UK), caused increases in quadricep and hamstring muscle strength, but did not affect lean body mass in healthy men [07185].

The potential ergogenic effects of asthma medication in athletes have been controversially discussed for decades. The prevalence of asthma is higher in elite athletes than in the general population. The highest risk for developing asthmatic symptoms is found in endurance athletes and swimmers. In addition, asthma seems to be more common in winter-sport athletes. Asthmatic athletes commonly use inhaled beta2-agonists to prevent and treat asthmatic symptoms. However, beta2-agonists are prohibited according to the "Prohibited List of the World Anti-Doping Agency" (WADA). Until the end of 2009 an exception was only allowed for the substances formoterol, salbutamol, salmeterol, and terbutaline by inhalation, as long as a so-called therapeutic use exemption has been applied for and was granted by the relevant anti-doping authorities. From 2010 salbutamol and salmeterol are allowed by inhalation requiring a so called declaration of use [10166].

Respiratory symptoms in relationship to exercise, bronchial hyperresponsiveness (BHR), and exercise-induced asthma (EIA) are very common in elite winter athletes. Symptom-based screening for BHR would facilitate selection of athletes with possible EIA. The aim of one study was to evaluate the diagnostic accuracy of self-reported symptoms as predictors of BHR in an unselected population of adult elite cross-country skiers. Forty-six Swedish adult skiers competing at national or international level were included. They had a mean (SD)
training volume in the past 12 months of 593 (122) hours. Twenty-four subjects had previous physician-diagnosed asthma. The European Community Respiratory Health Survey questionnaire was used to evaluate the presence of respiratory symptoms. BHR was defined as bronchoconstriction to either eucapnic voluntary hyperventilation, dry powder mannitol or methacholine provocation. The “classical” EIA symptom of shortness of breath post-exercise was reported by 17 percent of all skiers. Eight subjects (17 %) had BHR. None of the self-reported respiratory symptoms had high positive predictive values. However, symptoms caused by grass or pollen had high negative predictive values. It was concluded that EIA in elite winter athletes cannot accurately be based only on self-reported symptoms but requires verification with objective testing of BHR. Bronchoprovocation of elite winter athletes reporting respiratory symptoms in rest or because of exercise will probably reveal a high proportion of athletes without BHR [10372].

One study was aimed at defining the prevalence of airway hyperresponsiveness (AHR) and exercise-induced bronchoconstriction (EIB) in swimmers and winter sport athletes according to the previously recommended regulatory sport agencies criteria, the relationship between respiratory symptoms and AHR/EIB, the impact of the chosen cutoff value for AHR on its prevalence, and the effect on the prevalence of the positive eucapnic voluntary hyperpnea (EVH) test of using the highest vs the lowest spirometric post-EVH values to calculate the magnitude of the airway response. It was compared the prevalence of respiratory symptoms with responses to methacholine challenge and EVH in 45 swimmers, 45 winter sport athletes, and 30 controls. Two methacholine challenge cutoffs for AHR were analyzed: < 4 mg/mL (the sport agencies' criteria for AHR) and < 16 mg/mL. Sixty percent of swimmers, 29 percent of winter sport athletes, and 17 percent of controls had evidence of EIB or AHR (with the < 4 mg/mL criteria). Among athletes with a methacholine provocative concentration inducing a 20% decrease in the FEV₁ between 4 and 16 mg/mL, 43 percent of swimmers and 100 percent of winter sport athletes were symptomatic. Prevalence of positive EVH tests were 39 percent in swimmers, 24 percent in winter sport athletes, and 13 percent in controls when the highest FEV₁ value measured at each time point post-EVH was used to identify maximal response for calculation of airway response, although these prevalences were higher if we used the lowest value. This study suggests that AHR/EIB is frequent in swimmers, whereas the frequently reported respiratory symptoms in winter sport athletes are often not related to AHR/EIB. Furthermore, the choice of methods for assessing methacholine challenge and EVH responses influences the prevalences of airway hyperresponsiveness and exercise-induced bronchoconstriction [10373].

The International Olympic Committee-Medical Commission (IOC-MC) accepts a number of bronchial provocation tests for the diagnosis of exercise-induced bronchoconstriction (EIB) in elite athletes, none of which have been studied in elite swimmers. With the suggestion of a different pathogenesis involved in the development of EIB in swimmers, there is a possibility that the recommended test for EIB in elite athletes, the eucapnic voluntary hyperpnoea (EVH) challenge, may be missing the diagnosis in elite swimmers. The aim of one study was to assess the effectiveness of the EVH challenge, the field swim challenge and the laboratory cycle challenge in the diagnosis of EIB in elite swimmers. Thirty-three elite swimmers were evaluated on separate days for the presence of EIB using three different bronchial provocation challenge tests: an 8 minute field swim challenge, a 6 minute laboratory EVH challenge, and an 8 minute laboratory cycle challenge. It was decided that a fall in FEV₁ from baseline of ≥ 10 percent post challenge was diagnostic of EIB. Only 1 of the 33 subjects (3 %) had a positive field swim challenge with a fall in FEV₁ of 16 percent from baseline. Eighteen of the 33 subjects (55 %) had a positive EVH challenge, with a mean fall in FEV₁ of 20 (SD 12) percent from baseline. Four of the subjects (12 %) had a positive laboratory cycle challenge, with a mean fall in FEV₁ of 15 ± 5 percent from baseline. Only 1 of the 33 subjects was positive to all 3 challenges. These results suggest that the EVH challenge is a highly
sensitive challenge for identifying EIB in elite swimmers, in contrast to the laboratory and field-based exercise challenge tests, which significantly underdiagnose the condition. The EVH challenge, a well-established and standardised test for EIB in elite winter and summer land-based athletes, should thus be used for the diagnosis of EIB in elite swimmers, as recommended by the IOC-MC [10374].

Two groups of substances which stimulate the adrenergic system are listed as prohibited by the World Anti-Doping Agency. Stimulants are prohibited in-competition only and beta2-agonists are prohibited in- and out-of-competition. While beta2-agonists act directly on the target receptors, sympathomimetic amines can exert their action directly and indirectly. Due to differences in pharmacology but mainly due to differences in administered dose, differences in detection methods between both groups of substances exist although preparation is similar and consists of an extraction at basic pH. Gas chromatography coupled to mass spectrometry has been the detection methodology of choice for several decades. However, the importance of liquid chromatography-mass spectrometry as a preferred detection methodology is rapidly increasing, especially for the detection of beta2-agonists and new stimulants [10168].

Beta-2 Agonists bronchodilators (salbutamol, salmeterol, formoterol and terbutaline), as well as inhaled corticoids, are prohibited in sports unless the participant follows the procedure known as Therapeutic Use Exemption (TUE). An abbreviated form (ATUE) was accepted until December 2008, but a revised version of International Standard for TUE (approved by the World Anti-Doping Agency (WADA) Executive Committee on 10 May 2008) came into effect on 1 January 2009. In this version, the concept of an ATUE has been abolished. With the abbreviated form, the athlete could start (or continue) the treatment while the formal process was in progress (taking into account that this process used to be very long), while with the standard form, treatment can only start after receiving the authorisation notice. It is the International Olympic Committee Medical Commission who established the criteria for accepting the use of inhaled beta-2 agonists late in 2001 for the Salt Lake City Winter Olympic Games. Due to the success of its application, these criteria were renewed in January 2004 for the Athens Summer Olympic Games and assumed by WADA. These criteria require an athlete to prove the existence of bronchial hyper-responsiveness and the measures of forced expiratory volume in 1 s (FEV1) at rest, as well as changes in FEV1 in response to either an inhaled bronchodilator or to a bronchial provocation test. These are the essential criteria that must be completed on the TUE form for β-2 agonists. To review the methacholine tests performed in our laboratory up until 2008, to compare them with previously reported data in 2006, and to examine if the anti-doping rules are meeting the needs of asthmatic athletes who really need bronchodilator treatment 89 high-level athletes were examined in laboratory in order to obtain an abbreviated therapeutic use exemption for beta-2 agonists between 2004 and 2008. Of these, 50 men (23 years) and 23 women (21 years) performed a methacholine inhalation test with increasing concentrations of methacholine (0.025, 0.25, 2.5, 5, 10 and 25 mg/ml) until a fall of 20 percent in forced expiratory volume in 1 s (FEV1) was achieved. Thirty-one candidates (43 %) had a provocative concentration causing a 20 percent fall in FEV1 (PC20) <2 mg/ml; 12 (16 %) were between 2 and 4 mg/ml; 8 (11 %) between 4.1 and 8 mg/ml and 22 candidates (30 %) had a PC20 >8 mg/ml. Seven of the 73 candidates had an obstructive pattern in the spirometry at rest, demonstrated by a FEV1% <70 % but with a FEV1 >70 % of the reference value. It was concluded that the anti-doping regulations with respect to beta-2 agonists need to be reviewed, and measures should be adopted to include a fall of 70 percent in FEV1% as an obstruction criterion to indicate a bronchodilation test and to extend the criterion for a positive methacholine test to a PC20 of 8 mg/ml [11361].

Asthma, a chronic inflammatory disorder of the airways is associated with variable
obstruction to the airways and is provoked by many triggers including exercise. The management of asthma is primarily pharmacological, but exercise, despite causing bronchoconstriction in almost all asthmatics, is an important adjunct to treatment. With adequate control of the hyperresponsive airways obtained with inhaled corticosteroids (ICS) and inhaled beta 2 agonists (IBA), used as both a pre-exercise preventive agent and a reliever if necessary, all asthmatics should benefit from an exercise program. Some have realised this benefit with such success as to become Olympic and world champions in many sports. Exercise programs should be individually tailored, follow established guidelines and result in similar benefits to those obtained by non-asthmatics. However asthmatics must try to avoid or minimise triggers whenever possible. A specific benefit of a physical training program is that it allows asthmatics to exercise with less bronchoconstriction at the same exercise stress, although it does not abolish or reduce airway hyper responsiveness (AHR) [11362].

In COPD, improvements in lung mechanics following bronchodilator, measured using the forced oscillation technique (FOT), are more sensitive than spirometry at detecting improvement in lung function following bronchodilator. The relationship between these improvements in lung mechanics and improvements in functional outcomes, such as exertional dyspnoea, following bronchodilator, in COPD is unknown. Seventeen COPD subjects were recruited into a double blind placebo controlled randomised cross over study. Dyspnoea was induced using a standardised six-minute walk test (6 MWT), and measured by Borg score throughout the test. Measurement of respiratory system conductance, respiratory system reactance, inspiratory capacity and spirometry were made at baseline and 1 h after a single dose of either 18 microg of tiotropium bromide plus 200 microg salbutamol, or placebo. Subjects had a mean baseline FEV1 of 46 ± 11 percent predicted. The bronchodilator induced reduction in exertional dyspnoea correlated significantly with the increase in respiratory system conductance and approached significance with FEV1 but not with FVC, respiratory system reactance or inspiratory capacity. Increase in respiratory system conductance was the best and sole predictor of reduction in exertional dyspnoea, explaining 41 percent of the variance. There was no additional contribution to the model from the increase in FEV1 or inspiratory capacity. It was concluded that bronchodilator induced improvements in exertional dyspnoea in moderate to severe COPD are predicted by improvements in respiratory system conductance, measured by FOT, independent of improvements in spirometry or hyperinflation. The findings suggest that FOT may be useful for measuring response to bronchodilator in COPD [11363].

For the past 40 years, beta-blockers have been widely used in cardiovascular medicine, reducing morbidity as well as mortality. Beta-blockers are currently used in a number of cardiovascular conditions such as systolic heart failure, postmyocardial infarction, and in prevention and treatment of arrhythmias. They are not recommended as the first line antihypertensive therapy, particularly in the elderly, unless there are specific indications. Despite the benefits of beta-blockers, tolerability concerns in patients with co-morbidities have limited their use. Some of these problems were overcome with the discovery of cardioselective beta-blockers. The third generation beta-blockers have additional properties of vasodilatation and advantages in terms of minimizing the adverse effects of beta-blockers. Some of the advantages include improvement of insulin resistance, decrease in cholesterol as well as alleviation of erectile dysfunction. Acute treatment with beta-blockers modifies local muscular metabolic properties and impairs endurance exercise capacity whereas the influence of chronic is debated controversially [13403].

Beta2-adrenergic agonists, or beta2-agonists, are considered essential bronchodilator drugs in the treatment of bronchial asthma, both as symptom-relievers and, in combination with inhaled corticosteroids, as disease-controllers. The use of beta2-agonists is prohibited in
sports by the World Anti-Doping Agency (WADA) due to claimed anabolic effects, and also, is prohibited as growth promoters in cattle fattening in the European Union. One paper reviewed the last seven-year (2006-2012) literature concerning the development of novel beta2-agonists molecules either by modifying the molecule of known beta2-agonists or by introducing moieties producing indole-, adamantyl- or phenyl urea derivatives. New emerging beta2-agonists molecules for future therapeutic use are also presented, intending to emphasize their potential use for doping purposes or as growth promoters in the near future.

Impact of WADA regulations

To investigate how changes to the World Anti-Doping Agency (WADA) guidelines on asthma medication requests have impacted the management of asthmatic athletes in Portugal a retrospective analysis of asthma medication requests submitted in 2008 to 2010 was performed. Yearly changes in number of asthma medication requests and diagnostic procedures was analyzed in 326 requests: 173 abbreviated Therapeutic Use Exemptions (TUEs) in 2008 (objective tests not required), 9 Declaration of Use (DoU) and 76 TUEs in 2009, and 39 DoU and 29 TUEs in 2010. Spirometry was performed in 87 and 37 percent of athletes in 2009 and 2010, respectively; the corresponding figures for bronchoprovocation were 59 and 16 percent, almost all positive in both years. Applications for inhaler use have decreased by approximately half since objective asthma testing became mandatory. The findings show that WADA guidelines have an impact on asthmatic athletes care: In 2009 a more rigorous screening was possible, leading to withdrawal of unnecessary medication. Constant changes, however, jeopardize this achievement and nowadays introduce safety issues stemming from the unsupervised use of inhaled beta2-agonists.

Finland

The class of beta2-agonists has been subject of numerous studies related to sports drug testing. In Finland, a considerable increase in using asthma medications (including corticosteroids and beta2-agonists) was recognized over the last few years; however, the increase was observed among elite athletes only and not the general public. The reason(s) for this are yet unclear and might not or not entirely be due to assumed performance-enhancing effects in the light of recent data indicating no improvement in aerobic capacity or oxygen intake in endurance-trained athletes having received supra-therapeutic doses of inhaled salbutamol. Nevertheless, it was questioned how especially exercise-induced asthma in elite athletes is to be managed and treated, concluding that individual therapies as with any non-elite athlete are recommended, starting with actions preventing inflammatory response of lung tissue (e.g. heat masks for winter sport) and combined beta2-agonist and corticosteroid treatment. In order to enable high-profile athletes, being subject of doping controls, the adequate therapy for bronchoconstriction, WADA's Prohibited List allows the use of selected beta2-agonists up to defined amounts (and resulting urinary threshold levels) with limitations concerning the route of administration.

Therapy or doping?

Asthma, exercise-induced bronchoconstriction, and airway hyper-responsiveness are often found in elite athletes, perhaps as a consequence of their sport or maybe because asthma is a common disorder in young adults. Inhaled beta2-agonists (IBA) are frequently used in elite
athletes, but due to regulations introduced by the International Olympic Committee, the use of anti-asthmatic therapy might change. Drugs that make ergogenic effect persist are prohibited in all athletes, whether or not they take part in competitions and systemic steroids and beta₂-agonists are among such drugs. On the other hand, opinion is more divided about the use of inhaled corticosteroids (ICS) and IBA. In humans, no effect has been found on the oxygen uptake, performance or distance run with therapeutic doses of IBA, either in asthmatics or non-asthmatics, whereas others report an ergogenic effect and better lung function of high doses of a beta₂-agonist in non-asthmatics. Anti-asthmatic treatment is necessary for asthmatics, but should not be used by non-asthmatic elite athletes due to both possible systemic effects and furthermore, side effects of both ICS and IBA [07186].

**Improves swim ergometer sprint performance?**

There is a high prevalence of asthma and airway hyperresponsiveness (AHR) in elite athletes, which leads to a major use of beta₂-agonists. In a randomized double-blinded crossover study, it was investigated the effects of combined inhalation of beta₂-agonists (salbutamol, formoterol, and salmeterol), in permitted doses within the World Anti-Doping Agency 2013 prohibited list, in elite swimmers with (AHR, n=13) or without (non-AHR, n=17) AHR. Maximal voluntary isometric contraction of m. quadriceps (MVC), sprint performance on a swim ergometer and performance in an exhaustive swim test at 110% of VO₂max were determined. Venous plasma interleukin-6 (IL-6) and interleukin-8 (IL-8) were measured post-exercise. No improvement was observed in the exhaustive swim test, but swim ergometer sprint time was improved in both groups from 57 ± 1.7 to 56 ± 1.8 s in AHR and 58.3 ± 1 to 57.4 ± 1 s in non-AHR. MVC and post-exercise plasma IL-6 increased with beta₂-agonists in both groups, whereas IL-8 only increased in AHR. In summary, inhalation of beta₂-agonists, in permitted doses, did not improve swim performance in elite swimmers. However, swim ergometer sprint performance and MVC were increased, which should be considered when making future anti-doping regulations [13406].

**Doping efficiency of beta₂-agonists**

The question whether beta-2-agonists (or beta₂-agonists) should be considered performance-enhancing or not has not yet been answered. A meta-analysis was conducted aiming for the assessment of the effects of beta₂-agonists on physical performance in healthy, non-asthmatic subjects when administered either via inhalation or systemically. Considering scientific data published up to August 2009, a total of 26 studies concerning inhaled beta₂-agonists was evaluated and no significant effects on endurance, strength or sprint performance in healthy athletes were observed. With systemically administered salbutamol (predominantly orally), endurance time to exhaustion (at 80-85% VO₂(max)) was found to be significantly increased while the same parameter at 70 percent VO₂(max) together with two additional recorded parameters including VO₂(max) and power output at 90 percent VO₂(max) were not influenced. Overall, the advantageous effect of beta₂-agonists on the performance of healthy athletes was considered modest. The controversy concerning the general use of beta₂-agonists in cases of exercise-induced asthma was further kindled by suggestions to substitute these therapeutics by inhaled corticosteroids in acute situations [12016].

**Physiology**

Traditional views of the determinants of physical performance in healthy individuals
document the importance of oxygen transport to the working skeletal muscles. It is also viewed that the capacity of the pulmonary system (respiratory muscles, airways and lung parenchyma) is more than adequate to meet the demands for ventilation and gas exchange. However, in top athletes, years of training have increased the capacity of cardiac and skeletal muscles to deliver and use oxygen such that the demands placed on the airways, chest wall and alveoli to maintain gas exchange cannot be met. During heavy exercise at sea level, in many healthy, highly trained endurance athletes, arterial oxygen concentration can fall precipitously from normal levels to values seen in patients with chronic obstructive lung disease. This is reflected in the development of exercise-induced arterial hypoxemia, which occurs transiently in about 50 percent of athletes during peak exercise [08323].

Changes in exercise-induced arterial hypoxemia have a measurable effect on maximal aerobic capacity in highly trained athletes. On average, the effect is about a 1 percent decrement in maximal aerobic capacity for each 1 percent decrement in percent arterial oxygen saturation [696]. It has also been shown that these athletes experience the greatest decrement in aerobic capacity when they perform in hypoxic environments. Even relatively small changes in altitude (about 1000 m), which does not effect arterial oxygen saturation or maximal aerobic capacity appreciably in untrained individuals, produces substantial hypoxemia at the high work rates achieved by elite athletes. This results in significant decrements in both aerobic power and endurance performance [08324, 08325].

As a result of years of training elite athletes often experience intra-and extrathoracic respiratory problems and may also experience expiratory flow limitation. The most difficult respiratory problem to treat in this population may be the sudden paradoxical narrowing of the glottis, usually a manifestation of vocal cord dysfunction. Inspiratory stridor is an important differentiating symptom. The reported prevalence among athletes competing outdoors is 8.3 percent. Vocal cord dysfunction usually occurs during very heavy exercise, when the inspiratory flow rates are extreme. This condition immediately results in dyspnea, hypoxemia, retention of carbon dioxide and compromised performance. Treatment is not easy because it often involves a variety of therapies and modification of activity. Approaches that may be considered include the management of any associated conditions (e.g. gastroesophageal reflux disease) as well as speech and relaxation therapy. Other treatments that have been used include Botox injections, inhalation of heliox (21 % oxygen in helium) and training of the respiratory muscles and breathing mechanics with a resistive device [08326].

Respiratory symptoms cannot be relied on to make a diagnosis of asthma and airways hyperresponsiveness (AHR) in elite athletes. For this reason, the diagnosis should be confirmed with bronchial provocation tests. Asthma management in elite athletes should follow established treatment guidelines (e.g. “Global Initiative for Asthma”) and should include education, an individually tailored treatment plan, minimization of aggravating environmental factors, and appropriate drug therapy that must meet the requirements of the World Anti-Doping Agency. Asthma control can usually be achieved with inhaled corticosteroids and inhaled beta2-agonists to minimize exercise-induced bronchoconstriction and to treat intermittent symptoms. The rapid development of tachyphylaxis to beta2-agonists after regular daily use poses a dilemma for athletes. Long-term intense endurance training, particularly in unfavorable environmental conditions, appears to be associated with an increased risk of developing asthma and airways hyperresponsiveness in elite athletes. Globally, the prevalence of asthma, exercise-induced bronchoconstriction, and AHR in Olympic athletes reflects the known prevalence of asthma symptoms in each country. The policy of requiring Olympic athletes to demonstrate the presence of asthma, exercise-induced bronchoconstriction, or AHR to be approved to inhale beta2-agonists will continue [08327].
In general, the airways are capable of meeting the respiratory demands of heavy work within the limits of the normal maximal flow volume loop. However, in some athletes, the increase in minute ventilation may cause a portion of the tidal breathing loop to intersect with the maximal flow volume loop. This expiratory flow limitation and may be present because of the substantial demand on the pulmonary system with intense exercise. Trained female athletes may be particularly susceptible to expiratory flow limitation because their airways are smaller in diameter, they have lower peak flow rates and their lung volumes are smaller than those of their male counterparts [08328]. Expiratory flow limitation may influence performance by contributing to exercise-induced arterial hypoxemia and by increasing the work of breathing at the expense of meeting the metabolic needs of working skeletal muscles [08309].

Asthma and sympathomimetica

The prevalence of asthma and bronchial hyper-responsiveness is greater in elite athletes than in the general population, and its association with mild airway inflammation has recently been reported. To study the relationship between the type of sport practised at the highest levels of competition (on land or in water) and sputum induction cell counts in a group of healthy people and people with asthma 50 athletes were enrolled. Medical history, results of methacholine challenge tests and sputum induced by hypertonic saline were analysed. Full results were available for 43 athletes, who were classified by asthma diagnosis and type of sport (land or water sports). Nineteen were healthy (10 land and 9 water athletes) and 24 had asthma (13 land and 11 water athletes). Although the eosinophil counts of healthy people and people with asthma were significantly different (mean difference 3.1%, 95% confidence interval 0.4 to 6.2), analysis of variance showed no effect on eosinophil count for either diagnosis of asthma or type of sport. However, an effect was found for neutrophil counts. There was also a significant correlation between neutrophil counts and both duration of training and bronchial hyper-responsiveness among athletes exposed to water. It was concluded that elite athletes who practice water sports have mild neutrophilic inflammation, whether or not asthma is present, related to the degree of bronchial hyper-reactivity and the duration of training in pool water [07187].

In asthmatic subjects, either bronchodilatation or bronchoconstriction may develop during exercise. It was studied two groups of asthmatic subjects with baseline mild-to-moderate airflow obstruction with the aims to quantify the bronchodilator effect of incremental maximum exercise hyperpnea in comparison with inhaled albuterol, to assess the impact of this effect on airway response to prolonged constant-load exercise, and to evaluate if the bronchodilator effects of exercise and albuterol are additive. Changes in airway caliber were measured by changes in partial forced expiratory flow. Incremental-load exercise was associated with a bronchodilatation that was similar to that obtainable with 400 microg of albuterol but about 60 percent of the maximal bronchodilatation obtainable with 1,500 microg albuterol. Constant-load exercise was associated with an initial moderate bronchodilatation, which was followed by airway re-narrowing. With both exercise protocols, premedication with inhaled albuterol (400 microg) promoted sustained bronchodilatation during exercise, which was additive to that caused by exercise hyperpnea. In conclusion, the data show that exercise hyperpnea has a potent bronchodilator effect, but less that inhaled albuterol. This effect seems to contribute to delay the development of exercise-induced bronchoconstriction and is additive with that of albuterol [09235].

The aim of one study was to identify the prevalence of allergic disease in young soccer players compared to age-matched students and to evaluate if this prevalence changes as the
intensity of training increases. A modified ECRHS questionnaire was administered to 194 soccer players divided by age as "beginners" (8-11 years), "juniors" (12-16 years) and "under 21" (17-20 years) to evaluate the prevalence of allergic diseases and symptoms as well as drug consumption. Subjects with a positive personal history of allergic diseases underwent skin prick and/or patch tests. Age-matched students (n=136) were used as a control group. The prevalence of allergic diseases was 35 percent in soccer players and 32 percent in control subjects. Skin sensitization to inhalant allergens was detected in 14 percent of symptomatic soccer players and in 19 percent of control students. Patch tests were positive in 36 percent of soccer players and 23 percent of controls with allergic dermatitis. The prevalence of allergic diseases did not significantly change in relation to the intensity of training. Although the relative prevalence of sensitization to perennial allergens and asthma was less frequent in soccer players than in controls, and the occurrence of exercise-induced bronchoconstriction was similar in the two groups, soccer players used twice as many anti-allergic and anti-asthmatic drugs as control students. The authors concluded that increasingly intensive training programme is not associated with greater risk of allergic disease in soccer players. Therapy regimens of allergic athletes and exercisers should be monitored more closely to guarantee adequate treatment yet avoid inappropriate drug use and doping practices [09236].

Approximately 10 to 50 percent of competitive athletes experience asthma symptoms with exercise, due to either chronic asthma or exercise-induced bronchospasm. Early recognition and management of asthma symptoms may improve athletic performance and quality of life for athletes with asthma or exercise-induced bronchospasm. Athletic trainers may have frequent opportunities to identify asthma symptoms and assist athletes with management. To survey athletic trainers about their experience and comfort with evaluation and management of asthma symptoms in athletes and identify athletic trainer characteristics associated with higher comfort levels a questionnaire was sent to athletic trainers web-based. Response rate was 14 percent (304 of 2,175). At least 23 percent of respondents evaluated asthma symptoms five or more times the previous year. Respondents working exclusively with junior high and/or high school athletes evaluated asthma symptoms more frequently than those working exclusively with college and/or professional athletes. Fifty-eight percent of respondents were unsatisfied with their asthma education. Only 25 percent were "very" comfortable managing asthma. Respondents with higher comfort levels evaluated asthma symptoms more frequently and were more likely to be satisfied with their asthma education. Over 95 percent of respondents endorsed more asthma education in athletic training curricula. Results of this pilot study indicate that athletic trainers have opportunities to help athletes manage asthma symptoms that can compromise athletic performance or limit sports participation. However, few athletic trainers are very comfortable managing asthma, and most are unsatisfied with their asthma education [09237].

The aim of one study was to investigate the prevalence of asthma with or without exercise induced symptoms among elite and elite aspiring swimmers and to compare sport specific exercise provocation with mannitol provocation. 101 adolescent swimmers were investigated with mannitol provocation and sport specific exercise challenge test. Mannitol positivity was defined as either direct FEV₁ PD15 (ordinary criteria) or as beta₂-reversibility ≥15 percent after challenge (extended criteria). A direct positive exercise test was defined as a drop in FEV₁ of 10 percent (ordinary criteria) or a difference in FEV of ≥15 percent either spontaneous, variability, or with beta₂-agonist, reversibility (extended criteria). It was found a high prevalence of mannitol and/or exercise positivity. Twenty-six swimmers were mannitol direct positive and 14 were direct exercise positive using ordinary criteria. Using extended criteria 43 were mannitol positive and 24 were exercise positive. When including reversibility and variability to define a positive test the sensitivity for current asthma with or without exercise induced symptoms increased while the specificity remained roughly unchanged.
Direct positivity for mannitol and exercise poorly overlapped using ordinary criteria but improved using extended criteria. Thus, the use of variability and reversibility (liability) as additional criteria to define a positive test provided to our mind relevant information and should be considered [11479].

Athletes active in endurance sports are at an increased risk of acquiring asthma through their sports activities, especially so for cross-country skiers, biathlon skiers, swimmers and athletes of other endurance sports. Asthma may be present from early childhood or develop while in active sports. This article focuses on the physical activity and sports activities in children and adolescents. Exercise-induced asthma (EIA) is found in 8-10 percent of a normal child population of school age and in about 35 percent of children with current asthma. EIA is caused by the markedly increased ventilation during exercise, with increased heat and water loss through respiration, leading to bronchial constriction. The risk of developing asthma in the young athlete is related to the repeated daily training activity with increased epithelial damage of the airways, delayed repair due to the daily repetition of the training and increased airway mucosal inflammation. The increased environmental exposure through the sports activity to environmental agents, such as cold, dry air in skiers and chlorine compounds in swimmers, increases symptoms and signs of asthma and bronchial hyper-responsiveness, either worsening an existing asthma or leading to a novel disease in a previously healthy athlete. Several specific aspects of daily training life, environmental exposure, diagnostic procedures and aspects of treatment related to the regulations of medication use in sports need particular attention when addressing the adolescent athlete with respiratory symptoms [11480].

It was postulated that high level swimming can promote airway inflammation and thus asthma by enhancing local vascular permeability. It was aimed to test this hypothesis by a cross-sectional study comparing swimmers (n=13, 17 ± 3 years, competing 7 ± 4 years, training 18 ± 3 h per week), asthmatic-swimmers (n=6, 17 ± 2 years, competing 8 ± 3 years, training 16 ± 4 h per week), and asthmatics (n=19, 14 ± 3 years). Subjects performed induced sputum and had exhaled nitric oxide, lung volumes, and airway responsiveness determined. Airway vascular permeability index was defined as the ratio of albumin in sputum and serum. Results from the multiple linear regression showed each unit change in airway vascular permeability index was associated with an increase of 0.97 percent in sputum eosinophilis, and of 2.64 percent in sputum neutrophils after adjustment for confounders. In a general linear model no significant differences between airway vascular permeability between index study groups existed, after controlling for sputum eosinophilis and neutrophils. In conclusion, competitive swimmers training in chlorine-rich pools have similar levels of airway vascular permeability than asthmatics. Although competitive swimming has been associated with asthma, airway inflammation and airway hyperresponsiveness do not seem to be dependent on increased airway vascular permeability [11481].

One article reviewed the diagnostic challenge methods-both exercise and surrogate for diagnosis of exercise-induced bronchoconstriction (EIB) and EIB with known asthma. Indirect challenges that release the entire repertoire of mediators representative of EIB and asthma are more specific for diagnosis and are recommended over direct challenges such as methacholine challenge, which are sensitive but nonspecific. Self-reported history and empiric therapeutic trials are not adequate for diagnosis of EIB with or without known asthma. Objective pulmonary function documentation with bronchodilator reversibility or exercise or surrogate challenge are optimal for diagnosis of EIB or EIB with known asthma. Such objective pulmonary function documentation is optimal for the proper management and healthy lifestyle of the exercising athlete or individual [11482].
Elite athletes, particularly those engaged in endurance sports and those exposed chronically to airborne pollutants/irritants or allergens, are at increased risk for upper and lower airway dysfunction. Airway epithelial injury may be caused by dehydration and physical stress applied to the airways during severe exercise hyperpnoea and/or by inhalation of noxious agents. This is thought to initiate an inflammatory cascade/repair process that, ultimately, could lead to airway hyperresponsiveness (AHR) and asthma in susceptible athletes. The authors review the evidence relating to prevention or reduction of the risk of AHR/asthma development. Appropriate measures should be implemented when athletes exercise strenuously in an attempt to attenuate the dehydration stress and reduce the exposure to noxious airborne agents. Environmental interventions are the most important. Non-pharmacological strategies can assist, but currently, pharmacological measures have not been demonstrated to be effective. Whether early prevention of airway injury in elite athletes can prevent or reduce progression to AHR/asthma remains to be established [12304].

**Definition of asthma in sports**

To investigate how changes to the World Anti-Doping Agency (WADA) guidelines on asthma medication requests have impacted the management of asthmatic athletes in Portugal. It was made a retrospective analysis of asthma medication requests submitted in 2008 to 2010 of athletes requesting the use of inhaled corticosteroids and/or beta2-agonists. It was analyzed 326 requests: 173 abbreviated Therapeutic Use Exemptions in 2008 (objective tests not required), 9 Declaration of Use (DoU) and 76 Therapeutic Use Exemptions (TUEs) in 2009, and 39 DoU and 29 TUEs in 2010. Spirometry was performed in 87 and 37 percent of athletes in 2009 and 2010, respectively; the corresponding figures for bronchoprovocation were 59 percent and 16 percent, almost all positive in both years. It was concluded that applications for inhaler use have decreased by approximately half since objective asthma testing became mandatory. The findings show that WADA guidelines have an impact on asthmatic athletes care: In 2009 a more rigorous screening was possible, leading to withdrawal of unnecessary medication. Constant changes, however, jeopardize this achievement and nowadays introduce safety issues stemming from the unsupervised use of inhaled beta2-agonists [12303].

**Extended diagnostic criteria**

The aim of one study was to investigate the prevalence of asthma with or without exercise induced symptoms among elite and elite aspiring swimmers and to compare sport specific exercise provocation with mannitol provocation. 101 adolescent swimmers were investigated with mannitol provocation and sport specific exercise challenge test. Mannitol positivity was defined as either direct FEV$_1$ PD15 (ordinary criteria) or as beta$_2$-reversibility ≥15 percent after challenge (extended criteria). A direct positive exercise test was defined as a drop in FEV$_1$ of 10 percent (ordinary criteria) or a difference in FEV of ≥15 percent either spontaneous, variability, or with beta$_2$-agonist, reversibility (extended criteria). It was found a high prevalence of mannitol and/or exercise positivity. Twenty-six swimmers were mannitol direct positive and 14 were direct exercise positive using ordinary criteria. Using extended criteria 43 were mannitol positive and 24 were exercise positive. When including reversibility and variability to define a positive test the sensitivity for current asthma with or without exercise induced symptoms increased while the specificity remained roughly unchanged. Direct positivity for mannitol and exercise poorly overlapped using ordinary criteria but improved using extended criteria. It was found a high prevalence of asthma among elite swimmers. The use of variability and reversibility (liability) as additional criteria to define a positive test provided to our mind relevant information and should be considered [12305].
Diagnosis of asthma

To review the methacholine tests performed in a laboratory up until 2008, to compare them with previously reported data in 2006, and to examine if the anti-doping rules are meeting the needs of asthmatic athletes who really need bronchodilator treatment a study was performed between 2004 and 2008 of 89 high-level athletes who were examined in the laboratory in order to obtain an abbreviated therapeutic use exemption for beta-2 agonists. Of these, 50 men (23 years) and 23 women (21 years) performed a methacholine inhalation test with increasing concentrations of methacholine (0.025, 0.25, 2.5, 5, 10 and 25 mg/mL) until a fall of 20 percent in forced expiratory volume in 1 s (FEV\textsubscript{1}) was achieved. Thirty-one candidates (43 %) had a provocative concentration causing a 20 percent fall in FEV\textsubscript{1}, PC\textsubscript{20} <2 mg/ml; 12 (16 %) were between 2 and 4 mg/ml; 8 (11 %) between 4.1 and 8 mg/ml and 22 candidates (30 %) had a PC\textsubscript{20} >8 mg/ml. Seven of the 73 candidates had an obstructive pattern in the spirometry at rest, demonstrated by a FEV\textsubscript{1}% <70 percent but with a FEV\textsubscript{1}>70 percent of the reference value. It was concluded that the anti-doping regulations with respect to beta-2 agonists need to be reviewed, and measures should be adopted to include a fall of 70 percent in FEV\textsubscript{1}% as an obstruction criterion to indicate a bronchodilation test and to extend the criterion for a positive methacholine test to a PC\textsubscript{20} of 8 mg/ml [10172].

How should bronchial hyperreactivity be defined?

The regulations for doping control prohibit the use of beta\textsubscript{2}-agonist bronchodilators (salbutamol, salmeterol, formoterol, and terbutaline) unless the subject follows the procedure known as abbreviated therapeutic use exemption (ATUE). To highlight how the interest in discovering possible cheats may result in damage to athletes who really need bronchodilator treatment 31 high level athletes (18 men and 13 women) with a previous diagnosis of asthma were examined in our laboratory in order to obtain an ATUE for beta\textsubscript{2}-agonist. All the subjects underwent spirometry at rest. If the results were normal, the subjects underwent an effort test and, if negative, a methacholine test inhaling progressive doses of methacholine until a fall of 20 percent in forced expiratory volume in one second (FEV1) was achieved. The international anti-doping regulations require that the fall in FEV1 occurs with a concentration of methacholine (PC20) lower than 2 mg/mL (4 mg/mL for Torino 2006). In clinical practice, a test is positive if the response occurs with a PC20 lower than 8 mg/mL. Only one subject met the criterion for the bronchodilation test at rest. The remaining 30 athletes underwent an effort test, which was positive in nine of them. In 21 cases (13 men and 8 women) the effort test was negative so a methacholine test was carried out. Seven (33 %) were negative for ATUE with a PC20 higher than 8 mg/ml, seven (33 %) were positive for ATUE with a PC20 less than 2 mg/mL, in four (19 %) the PC20 was 2-4 mg/mL, and in three (14 %) it was 4-8 mg/mL. Thus, strict vigilance of fair play should be pursued, but excessive control can lead to situations of inequality for asthmatic athletes such that a third of athletes cannot be treated with beta\textsubscript{2}-agonist. Therefore under current regulations, asthmatic athletes are often denied the most effective therapeutic option [06201].

Testing of asthma in athletes

Beta-2 Agonists bronchodilators (salbutamol, salmeterol, formoterol and terbutaline), as well as inhaled corticoids, are prohibited in sports unless the participant follows the procedure known as Therapeutic Use Exemption (TUE). An abbreviated form (ATUE) was accepted until 2008. In a revised version from 2009, the concept of an ATUE has been abolished. With the abbreviated form, the athlete could start (or continue) the treatment while the formal
process was in progress (taking into account that this process used to be very long), while with the standard form, treatment can only start after receiving the authorisation notice. It is the International Olympic Committee Medical Commission who established the criteria for accepting the use of inhaled beta-2 agonists late in 2001 for the Salt Lake City Winter Olympic Games. Due to the success of its application, these criteria were renewed in 2004 for the Athens Summer Olympic Games and assumed by WADA. These criteria require an athlete to prove the existence of bronchial hyper-responsiveness and the measures of forced expiratory volume in 1 s (FEV₁) at rest, as well as changes in FEV₁ in response to either an inhaled bronchodilator or to a bronchial provocation test. These are the essential criteria that must be completed on the TUE form for beta-2 agonists. Between 2004 and 2008, 89 high-level athletes were examined in order to obtain an abbreviated therapeutic use exemption for beta-2 agonists. Of these, 50 men and 23 women performed a methacholine inhalation test with increasing concentrations of methacholine (0.025, 0.25, 2.5, 5, 10 and 25 mg/ml) until a fall of 20 percent in forced expiratory volume in 1 s (FEV₁) was achieved. Thirty candidates (43 %) had a provocative concentration causing a 20 percent fall in FEV₁ (PC₂₀) <2 mg/mL; 12 (16 %) were between 2 and 4 mg/mL; 8 (11 %) between 4.1 and 8 mg/mL, and 22 candidates (30 %) had a PC₂₀ >8 mg/mL. Seven of the 73 candidates had an obstructive pattern in the spirometry at rest, demonstrated by a FEV₁% <70 percent but with a FEV₁ >70 percent of the reference value. The anti-doping regulations with respect to beta-2 agonists need to be reviewed, and measures should be adopted to include a fall of 70 percent in FEV₁% as an obstruction criterion to indicate a bronchodilation test and to extend the criterion for a positive methacholine test to a PC₂₀ of 8 mg/mL [11187].

**Predictive values of tests**

To study the predictive value of allergy and pulmonary function tests for the diagnosis of asthma in athletes 98 national preOlympic athletes underwent an accurate medical examination including a validated questionnaire for asthma and rhinitis, spirometric recordings and skin prick testing with a panel of the most frequent inhalant allergens. Bronchodilator and/or exercise challenge were also performed in asthmatic subjects. Clinical asthma was present in 20 percent of athletes, rhinitis in 35 percent (in 21 % of cases alone and in 14 % associated with asthma). Positive prick tests were recorded in 44 percent of athletes (in 61 % of asthmatics, in 95 % of rhinitics and in 21 % of nonasthmatic-nonrhinitic subjects). Mean spirometric values and distribution of abnormal values were not different among asthmatics, rhinitics and nonasthmatics-nonrhinitic patients. Skin-tests positivity was not related to the abnormal spirometric data found in individual cases. Provocation tests with bronchodilators or exercise did not appear sensitive enough to diagnose mild forms of asthma in subjects with normal basal spirometric values. It was concluded that allergy testing and spirometry should be performed routinely in athletes because of the high prevalence of allergy, rhinitis and asthma in this population. However, the predictive value of these tests and of the bronchial provocation tests performed in this study seems too low to document mild or subclinical asthma in athletes [07192].

**Asthma in sports**

Athletes attempt to improve performance with drugs that act on the beta-adrenergic system directly or indirectly. Of three beta-adrenoceptor subtypes, the beta₂-adrenoceptor is the main target in sport; they have bronchodilator and anabolic actions and enhance anti-inflammatory actions of corticosteroids. Although demonstrable in animal experiments and humans, there is little evidence that these properties can significantly improve performance in trained athletes. Their actions may also be compromised by receptor desensitization and
by common, naturally occurring receptor mutations (polymorphisms) that can influence receptor signalling and desensitization properties in individuals. Indirectly acting agents affect release and reuptake of noradrenaline and adrenaline, thereby influencing all adrenoceptor subtypes including the three beta-adrenoceptors. These agents can have potent psychostimulant effects that provide an illusion of better performance that does not usually translate into improvement in practice. Amphetamines and cocaine also have considerable potential for cardiac damage. beta-adrenoceptor antagonists (beta-blockers) are used in sports that require steadiness and accuracy, such as archery and shooting, where their ability to reduce heart rate and muscle tremor may improve performance. They have a deleterious effect in endurance sports because they reduce physical performance and maximum exercise load. Recent studies have identified that many beta-adrenoceptor antagonists not only block the actions of agonists but also activate other (mitogen-activated PK) signalling pathways influencing cell growth and fate. The concept that many compounds previously regarded as "blockers" may express their own spectrum of pharmacological properties has potentially far-reaching consequences for the use of drugs both therapeutically and illicitly [08329].

Asthma, a chronic inflammatory disorder of the airways is associated with variable obstruction to the airways and is provoked by many triggers including exercise. The management of asthma is primarily pharmacological, but exercise, despite causing bronchoconstriction in almost all asthmatics, is an important adjunct to treatment. With adequate control of the hyperresponsive airways obtained with inhaled corticosteroids (ICS) and inhaled beta 2 agonists (IBA), used as both a pre-exercise preventive agent and a reliever if necessary, all asthmatics should benefit from an exercise program. Some have realised this benefit with such success as to become Olympic and world champions in many sports. Exercise programs should be individually tailored, follow established guidelines and result in similar benefits to those obtained by non-asthmatics. However asthmatics must try to avoid or minimise triggers whenever possible. A specific benefit of a physical training program is that it allows asthmatics to exercise with less bronchoconstriction at the same exercise stress, although it does not abolish or reduce airway hyperresponsiveness (AHR) [11181].

In a meta-analysis, studies on swimming and asthma were divided into four groups: Group I compared frequency of asthma among elite swimmers to that of other athletes; Group II examined the association between asthma and swimming during childhood; Group III evaluated effects of swimming programs on asthma severity and pulmonary function; and Group IV compared immediate respiratory effects of swimming to those of other types of exercise. The summary results were expressed as meta-odds ratios (ORs) for binary endpoints such as presence of asthma, and meta-differences for continuous endpoints such as changes in post-exercise pulmonary function tests (PFTs). All summary measures of effect were calculated using random effects models accompanied by a corresponding 95 percent confidence interval (CI) and a test for heterogeneity. In the analysis comparing frequency of asthma among elite swimmers to that among other athletes (Group I), meta-ORs ranged from 2.3 to 2.6 with all 95 percent confidence interval excluding 1.0. The corresponding meta-ORs reflecting the association between asthma and swimming pool use during childhood (Group II) were in the 0.63-0.82 range and were not statistically significant. In comparison to swimming, running and/or cycling was associated with a statistically significant four-to six-fold increase in exercise induced bronchospasm. Although asthma is more commonly found among elite swimmers than among other high-level athletes, it is premature to draw conclusions about the causal link between swimming and asthma because most studies available to date used cross-sectional design, because the association is not confirmed among non-competitive swimmers, and because asthmatics may be more likely to select swimming as the activity of choice because of their condition [08330].
Common conditions affecting the lower airway, is for example asthma. Asthma is highly prevalent in the general population, and even more prevalent among athletes [08331, 08332]. Airway hyperresponsiveness has been reported to occur in 25-79 percent of athletes in endurance sports, and in about 20 percent of athletes in power and speed sports [08333, 08334]. The prevalence of asthma and airway hyperresponsiveness, therefore, seems to be influenced by the type of sport or the environmental conditions during training, or both.

Asthma is a chronic inflammatory disorder of the airways associated with airway hyperresponsiveness and variable airway obstruction and is considered responsible for recurrent episodes of breathlessness, wheezing, chest tightness, phlegm production and coughing. Asthma can be triggered by various stimuli, including allergens in sensitized individuals, respiratory irritants (e.g. air pollutants), cold air and exercise. Exercise-induced asthma is considered to be due to the cooling, but mostly the drying, of the airways following exercise-induced hyperventilation. The result is the development of a hyperosmolar airway fluid. Rewarming of the airways due to a post-exercise increase in airway blood flow and transient plasma transudation may also contribute to the induced bronchoconstriction [08335, 08336].

Physical activity is often recommended to patients with asthma, and regular exercise can improve asthma control. However, there is mounting evidence that frequent, intense exercise by highly trained athletes contributes to the development of asthma. Indeed, long-term endurance training may influence airway structure and function and lead to airway hyperresponsiveness. Various factors could contribute to the development of asthma in athletes, including environmental exposures, mechanical stress to the airways, increased prevalence of respiratory infections and dysautonomia [08337]. The mechanisms by which these factors could influence asthma development may be related to their ability to induce airway inflammation and structural changes (remodelling). These processes are considered to be responsible for the development of airway hyperresponsiveness and variable or persistent airway obstruction [08338, 08339].

Airway inflammation and remodelling have been observed in athletes, even those without asthma [08340, 08341]. In general, bronchial inflammation is of a mixed type, with increased neutrophil and eosinophil counts. In swimmers, airway inflammation and potentiation of the effects of allergens on airways from chlorine derivatives result in epithelial damage, which has been proposed as a mechanism that leads to airway hyperresponsiveness, respiratory symptoms and asthma [08341]. Furthermore, mechanical stress caused by intense breathing efforts may also damage the airway epithelium and promote airway remodeling [08308].

Current recommendations indicate that asthma in highly trained athletes should be managed according to existing general national and international asthma management guidelines 08[327, 08342]. Special consideration, however, should be given to the diagnosis of asthma in athletes and assessment of asthma control; preventive measures; the management of exercise-induced asthma; and medication use, which should comply with the criteria of regulatory agencies. However, pharmacotherapy for asthma in athletes should be individualized. It should be based on reduction of inflammation, mainly with the use of inhaled corticosteroid therapy (the best drug therapy for asthma control), and optimization of airway control with other add-on therapies as needed. Rapid-acting beta₂-agonists are the most frequently used medications to relieve acute intermittent symptoms and to prevent exercise-induced bronchoconstriction. Protection against exercise-induced bronchoconstriction diminishes, however, when these agents are used regularly. The minimum dose and frequency required to minimize this loss of effect are recommended. Such tachyphylaxis to their bronchoprotective effects occurs after only a few days of use and
is not antagonized by inhaled corticosteroid therapy, which can reduce exercise-induced bronchoconstriction in most people with asthma after a few weeks of regular use [08343-08346].

**Hard training as a cause of bronchial hyperreactivity**

The different approaches that the International Olympic Committee (IOC) had adopted to beta2-agonists and the implications for athletes are reviewed by a former Olympic team physician who later became a member of the Medical Commission of the IOC (IOC-MC). Steadily increasing knowledge of the effects of inhaled beta2-agonists on health, is concerned with the fact that oral beta2-agonists may be anabolic, and rapid increased use of inhaled beta2-agonists by elite athletes has contributed to the changes to the IOC rules. Since 2001, the necessity for athletes to meet IOC criteria (i.e., that they have asthma and/or exercise-induced asthma, EIA) has resulted in improved management of athletes. The prevalence of beta2-agonist use by athletes mirrors the known prevalence of asthma symptoms in each country, although athletes in endurance events have the highest prevalence. The age-of-onset of asthma/EIA in elite winter athletes may be atypical. Of the 193 athletes at the 2006 Winter Olympics who met th IOC's criteria, only 32 percent had childhood asthma and 49 percent of athletes reported onset at age 20 years or older. These findings lead to speculation that years of intense endurance training may be a causative factor in bronchial hyperreactivity. The distinction between oral (prohibited in sports) and inhaled salbutamol is possible, but athletes must be warned that excessive use of inhaled salbutamol can lead to urinary concentrations similar to those observed after oral administration. One article provided justification that athletes should provide evidence of asthma or EIA before being permitted to use inhaled beta2-agonists [06202].

**Prevalence of asthma in sports**

One review article concerned physical activity and sports in asthmatic adolescents. Exercise induced asthma (EIA) is found in 8-10 percent of a normal child population and in approximately 35 percent of children with current asthma as reported from a population based birth cohort study. The mechanisms of EIA are related to markedly increased ventilation during exercise, causing increased heat and water loss through respiration, leading to bronchial constriction. In athletes and especially in endurance athletes, the repeated daily physical activity during training will over time cause epithelial damage and increase inflammation in the respiratory mucosa. With increased exposure to environmental agents as cold air in skiers and chlorine compounds in swimmers, the athlete may contract symptoms and signs of asthma and bronchial hyperresponsiveness, either worsening an existing asthma or causing symptoms in a previous healthy adolescent athlete. There are several causes of breathlessness in adolescents including EIA, vocal cord dysfunction, poor physical fitness and others, important to consider in the diagnostic procedure. The asthmatic athlete should follow the same guidelines for treating his/her asthma as the ordinary asthmatic patient with concern made to the special diagnostic rules given for the use of asthma drugs in sports, especially for inhaled beta2-agonists [11182].

Asthma is the most common chronic medical condition that school-teachers are likely to encounter among their pupils. This study aimed to identify the needs of physical education teachers in dealing with adolescents with exercise-induced asthma, study their self-reported knowledge of asthma and identify future topics for education about exercise-induced asthma. A questionnaire was drawn up on the basis of the requirements that had emerged in the course of interviews with 18 physical education teachers. One hundred and six physical education teachers at secondary schools in the city of Trondheim and colleges in Sør-
Trøndelag County in Norway answered the questionnaire (65 % response rate). Eighty-two physical education teachers (78 %) had pupils with asthma in their sports classes, and 89 percent answered positively regarding their need for advice on teaching pupils with asthma. Twenty-seven (26 %) reported that they had sufficient knowledge to teach adolescents with asthma. Topics about asthma, its management and activities suitable for asthmatics were given high priority by the teachers [11183].

The objective of one study was to determine the prevalence of asthma and use of asthma medications in elite athletes compared with an age-matched non-athlete population. Data were collected from the respiratory component of annual medical screening of 424 elite athletes. Measures included the prevalence of current asthma and ever doctor-diagnosed asthma, and the prevalence of use of treatment for asthma including beta-agonists and inhaled corticosteroid medication. The prevalence of current asthma in athletes aged 18-29 years was 14 percent (95 % confidence interval 9 to 19 %), which did not differ significantly from the prevalence in the non-athlete control population (11 %; 95 % confidence interval 9 to 12 %). Of athletes with current asthma, 27 percent were not taking any medications for asthma, and 25 percent were treated with short-acting beta-agonist medications alone and were not taking inhaled corticosteroids. These data indicate that the overall cumulative and period prevalence of asthma in athletes is similar to that in the general age-matched population. Athletes use beta-agonists with a frequency similar to the general population [07188].

**Asthma in elite athletes**

Asthma is frequently found among elite athletes performing endurance sports such as swimming, rowing and cross-country skiing. Although these athletes often report symptoms while exercising, they seldom have symptoms at rest. Moreover, compared with nonathletic asthmatic individuals, elite athletes have been shown to have a different distribution of airway inflammation and unequal response to bronchial provocative test. Elite athletes display signs of exercise-induced symptoms, for example, nonasthmatic inspiratory wheeze, vocal cord dysfunction and cardiac arrhythmias, which could limit their physical capacity. Elite athletes should undergo comprehensive assessment to confirm an asthma diagnosis and determine its degree of severity. Treatment should be as for any other asthmatic individual, including the use of beta2-agonist, inhaled steroid as well as leukotriene-antagonist. It should, however, be noted that daily use of beta-agonists could expose elite athletes to the risk of developing tolerance towards these drugs. Use of beta2-agonist should be replaced with daily inhaled corticosteroid treatment, the most important treatment of exercise-induced asthma. All physicians treating asthma should be aware of the doping aspects. Systemic beta2-agonist intake is strictly prohibited, whereas inhaled treatment is allowed in therapeutic doses when asthma is documented and dispensation has been granted when needed [11184].

**In elite swimmers**

An increased risk of developing asthma has been reported among swimmers exposed to chloramine in pool arenas. The aim of one study was to compare the prevalence of asthma and respiratory symptoms among elite aspiring swimmers compared with age-matched controls with different degrees of physical activity. It was also aimed to relate these findings to mental and psychosocial factors. One hundred and one elite swimmers and 1628 age-matched controls answered a questionnaire containing questions about respiratory symptoms, lifestyle factors, mental and physical well-being. The controls were divided into three different groups according to the degree of physical activity, no physical activity,
recreational training and elite training. Swimmers reported significantly more asthma symptoms, with 37 percent having physician-diagnosed asthma, compared with 16 percent among the controls. Use of regular medication was more common (15 % vs 8 %) and more swimmers reported an exacerbation of their asthma during the previous 12 months (17 %) versus (6 %) for the controls. Despite an increased prevalence of asthma symptoms, the swimmers reported best physical performance and best mental and physical well-being. They also had a healthier lifestyle without smoking and low alcohol consumption [12306].

In canoe- and kayak athletes

Asthma is common in elite athletes, but the knowledge of asthma in elite canoe and kayak athletes is limited. The aim of one prospective cross-sectional study was therefore to investigate the prevalence of asthma, including asthma-like symptoms, exhaled nitric oxide, and airway reactivity to mannitol in Danish elite canoe and kayak athletes. The study group consisted of 29 (of 33 eligible) elite athletes aged 17-43 years, and the examination programme consisted of questionnaires, including the Asthma Control Questionnaire, fraction of exhaled nitric oxide (FENO), spirometry and airway reactivity to mannitol. Asthma was defined as a history of doctor-diagnosed asthma and/or elevated FENO and airway reactivity. Seven of the elite athletes (24 %) were found to have asthma, including four subjects with previously doctor-diagnosed asthma. Of the four athletes (all treated with inhaled corticosteroids) with doctor-diagnosed asthma, all reported asthma-symptoms and two had elevated FENO, but none had airway hyperresponsiveness (AHR) to mannitol. All three athletes with previously undiagnosed asthma had elevated FENO and AHR to mannitol, but reported no asthma-like symptoms. Asthma is common in elite canoe and kayak athletes, and classical signs of asthmatic airway inflammation are also found in asymptomatic athletes [12307].

Misdiagnosis of exercise-induced bronchoconstriction in professional soccer players

Physicians typically rely heavily on self-reported symptoms to make a diagnosis of exercise-induced bronchoconstriction (EIB). However, in elite sport, respiratory symptoms have poor diagnostic value. In 2009, following a change in international sports regulations, all elite athletes suspected of asthma and/or EIB were required to undergo pulmonary function testing (PFT) to permit the use of inhaled beta2-agonists. The aim of one study was to examine the diagnostic accuracy of physician diagnosis of asthma/EIB in English professional soccer players. Sixty-five players with a physician diagnosis of asthma/EIB were referred for pulmonary function assessment. Medication usage and respiratory symptoms were recorded by questionnaire. A bronchial provocation test with dry air was conducted in 42 players and a mannitol challenge in 18 players. Five players with abnormal resting spirometry performed a bronchodilator test. Of the 65 players assessed, 57 (88 %) indicated regular use of asthma medication. Respiratory symptoms during exercise were reported by 57 (88 %) players. Only 33 (51 %) of the players tested had a positive bronchodilator or bronchial provocation test. Neither symptoms nor the use of inhaled corticosteroids were predictive of pulmonary function tests’ outcome. It was concluded that a high proportion of English professional soccer players medicated for asthma/EIB (a third with reliever therapy only) do not present reversible airway obstruction or airway hyperresponsiveness to indirect stimuli. This underlines the importance of objective PFT to support a symptoms-based diagnosis of asthma/EIB in athletes [12308].

Use of antiasthmatic medicine in athletes
Use of asthma medication is common among athletes. In 2009, the World Anti-Doping Committee (WADA) and the International Olympic Committee removed the need to document asthma by lung function tests before the use of inhaled beta2-agonists. It was assessed the changes in asthma medication use in Finnish Olympic athletes 8 years apart in 2002 (n=446) and 2009 (n=372). The athletes filled out a questionnaire on asthma symptoms, diagnosis, and medication. The use of asthma medication increased from 9.4 percent in 2002 to 12.6 percent in 2009 (adjusted odds ratio (OR) = 1.71, 95 % confidence interval 1.08 to 2.69). Fixed combinations of inhaled long-acting beta2-agonists (LABAs) and inhaled corticosteroids (ICSs) were used three times more in 2009 versus 2002 (OR = 3.38, 95 % confidence interval 1.26 to 9.12). At the same time, no significant changes were observed in the occurrences of physician-diagnosed asthma (13.9 % vs 15.9 %) or wheezing (10.3 % vs 10.2 %). In 2002, all athletes on asthma medication also had a physician-diagnosed disease, but in 2009, 11.8 percent of the athletes on medication were lacking it. It was concluded that especially, the use of combination therapy of LABAs and ICSs is increasing among Finnish Olympic athletes. This trend is worrying as it is not based on increasing occurrence of symptoms, asthma diagnoses, or objective lung function measurements [12309].

Allergic rhinitis in athletes

The aim of one review was to highlight the prevalence of allergic rhinitis in athletes and the impact this condition may have on their athletic performance. Furthermore, the optimal management of medical conditions in the elite athlete forms an important part of protecting the health of the athlete. The use of pharmacological intervention in the treatment of allergic rhinitis in elite athletes requires careful planning. A variety of factors must be considered prior to prescription such as drug efficacy and safety both at rest and in conjunction with strenuous exercise, associated side effects on athletic performance and well being and whether the chosen drug is on the World Anti-Doping Agency (WADA) banned list. Allergic rhinitis is, however, common in elite athletes and may impair athletic performance and recovery. We advise that all elite and professional athletes should be screened for allergic rhinitis using validated questionnaire such as the Allergy Questionnaire for Athletes (AQUA) alongside skin prick testing or specific IgE blood tests for inhalant allergens. Intranasal corticosteroids are highly recommended as the management drug of choice for athletes in conjunction with second-generation antihistamines (severity dependant) and when practicable, allergen avoidance [11185].

During Olympic games

The asthmatic athlete has a long history in competitive sport in terms of success in performance and issues related to doping. Well documented are detailed objective tests used to evaluate the athlete with symptoms of asthma or airway hyperresponsiveness and the medical management. Initiated at the 2002 Salt Lake City Games, the International Olympic Committee's Independent Asthma Panel required testing to justify the use of inhaled beta-2 agonists in Olympic athletes and has provided valuable guidelines to the practicing physician. This program was educational and documented the variability in prevalence of asthma and/or airway hyperresponsiveness and of inhaled beta-2 agonists use between different sports and different countries. It provided a standard of care for the athlete with respiratory symptoms and led to the discovery that asthmatic Olympic athletes outperformed their peers at both Summer and Winter Olympic Games from 2002 to 2010. Changes to the World Anti-Doping Agency's Prohibited List in 2010 permitted the use of 2 of inhaled beta-2 agonists produced by the same pharmaceutical company. All others remain prohibited. However, there is no pharmacological difference between the permitted and prohibited of
inhaled beta-2 agonists. As a result of these changes, asthmatic athletes are being managed differently based on a World Anti-Doping Agency directive that has no foundation in pharmacological science or in clinical practice [11186].

Prevalence of use beta-2-agonists

The prevalence of asthma and the use of anti-asthmatic medication is high among elite athletes. Elite athletes require a TUE certificate (Therapeutic Use Exemption) if they require anti-asthmatic medication which is on the prohibited list. The aim of the study was to determine the distribution of Danish TUE certificates and to examine the use of anti-asthmatic medication among Danish elite athletes. A cross-sectional study of all applications for TUE certificates in 2005 was carried out. It was focused on applications including anti-asthmatic medication. All applications resulted in certificates being issued. A total of 694 TUE certificates were issued. Of these, 445 (64 %) concerned anti-asthmatic medication. Short-acting beta-2-agonists (SABA) were the most frequent medication (79 %). Only 2 percent received long-acting beta-2-agonists (LABA) as single therapy. Inhaled steroids were used by 69 percent. Swimmers received significantly higher doses of inhaled steroids compared to all others athletes (1031 microg/day). The applications for TUE certificates were generally handled by general practitioners (78 %). It was concluded that most TUE certificates issued in 2005 concerned anti-asthmatic medication. Only a few athletes were treated with non-recommended anti-asthmatic medication like LABA as single therapy. Swimmers received higher doses of inhaled steroids compared to all others athletes. Most of the applications for TUE certificates were handled by general practitioners [07193].

Exercise-induced bronchoconstriction (EIB)

Exercise-related respiratory symptoms in the diagnosis of exercise-induced bronchoconstriction (EIB) have poor predictive value. The aim of one study was to evaluate how athletes presenting with these symptoms are diagnosed and managed in primary care. An electronic survey was distributed to a random selection of family practitioners in England. The survey was designed to assess the frequency with which family practitioners encounter adults with exercise-related respiratory symptoms and how they would approach diagnostic work-up and management. The survey also evaluated awareness of and access to diagnostic tests in this setting and general knowledge of prescribing asthma treatments to competitive athletes. 257 family practitioners completed the online survey. One-third of respondents indicated they encountered individuals with this problem at a frequency of more than one case per month. Over two-thirds of family practitioners chose investigation as an initial management strategy, while one-quarter would initiate treatment based on clinical information alone. PEFR pre- and post-exercise was the most commonly selected test for investigation (44 %), followed by resting spirometry pre- and post-bronchodilator (35 %). Short-acting beta2-agonists were the most frequently selected choice of treatment indicated by respondents (90 %). Family practitioners encounter individuals with exercise-related respiratory symptoms commonly and although objective testing is often employed in diagnostic work-up, the tests most frequently utilised are not the most accurate for diagnosis of EIB. This diagnostic approach may be dictated by the reported lack of access to more precise testing methods, or may reflect a lack of dissemination or awareness of current evidence. Overall the findings have implications both for the management and hence welfare of athletes presenting with this problem to family practitioners and also for the competitive athletes requiring therapeutic use exemption [09238].

To investigate the incidence of asthma symptoms in 171 young amateur swimmers (6-14
years of age), and to describe the clinical treatment of the children with asthma in a private
sports club in the city of São Paulo, Brazil all of the participants or their legal guardians were
asked to complete the International Study of Asthma and Allergies in Childhood (ISAAC)
questionnaire, and 119 were submitted to pulmonary function testing at rest. The overall
incidence of asthma symptoms (ISAAC score > 6) among the swimmers was 17 percent. Of
the 119 swimmers submitted to spirometry, 39 (33 %) presented spirometric alterations
(FEV1/FVC < 0.75). Among those with an ISAAC score > 6, there were 10 (31 %) who stated
that they were receiving no asthma treatment. Of those who reported receiving
pharmacological treatment, 24 percent made use of bronchodilators but not of
corticosteroids. The incidence of asthma symptoms and pulmonary function alterations
among amateur swimmers within the 6-14 age bracket was high. In addition, a relevant
proportion of these athletes were receiving no treatment [09239].

Exercise induced bronchoconstriction (EIB) describes the acute transient airway narrowing
that occurs during and most often after exercise, and is prevalent in elite athletes. Prolonged
hyperventilation of dry or cold air and increased inhalation of pollutants or allergens could
account for the bronchoconstrictive reaction. The subsequent airway inflammation seems to
differ from typical asthma. Objective measures of lung function and provocation tests should
be used for an accurate and reliable diagnosis. EIB is currently treated with inhalation of
beta2-agonists or, as second choice, sodium cromoglycate approximately 15 min before
exercise. If this proves to be insufficient then inhaled steroids should be added. Leukotriene
receptor antagonists can be used in patients whose symptoms do not respond to inhaled
steroids. The screening of high risk populations such as swimmers, cyclists, rowers and
winter athletes is recommended by some authors [08348].

The International Olympic Committee-Medical Commission (IOC-MC) accepts a number of
bronchial provocation tests for the diagnosis of exercise-induced bronchoconstriction (EIB) in
elite athletes, none of which have been studied in elite swimmers. With the suggestion of a
different pathogenesis involved in the development of EIB in swimmers, there is a possibility
that the recommended test for EIB in elite athletes, the eucapnic voluntary hyperpnoea
(EVH) challenge, may be missing the diagnosis in elite swimmers. The aim of one study was
to assess the effectiveness of the EVH challenge, the field swim challenge and the laboratory
cycle challenge in the diagnosis of EIB in elite swimmers. Thirty-three elite swimmers were
evaluated on separate days for the presence of EIB using 3 different bronchial provocation
challenge tests: an 8 minute field swim challenge, a 6 minute laboratory EVH challenge, and
an 8 minute laboratory cycle challenge. Only 1 of the 33 subjects (3 %) had a positive field
swim challenge with a fall in FEV1 of 16 percent from baseline. Eighteen of the 33 subjects
(55 %) had a positive EVH challenge, with a mean fall in FEV1 of 20 percent from baseline.
Four of the subjects (12 %) had a positive laboratory cycle challenge, with a mean fall in
FEV1 of 15 percent from baseline. Only 1 of the 33 subjects was positive to all 3 challenges.
These results suggest that the EVH challenge is a highly sensitive challenge for identifying
EIB in elite swimmers, in contrast to the laboratory and field-based exercise challenge tests,
which significantly underdiagnose the condition. The EVH challenge, a well-established and
standardised test for EIB in elite winter and summer land-based athletes, should thus be
used for the diagnosis of EIB in elite swimmers, as recommended by the IOC-MC [10173].

The role of mast cells in the airway response to exercise and the benefit of sodium
cromoglycate (SCG) in athletes are unclear. The purpose of one study was to clarify the role
of mast cell mediators in the airway response to exercise in athletes and to investigate the
effect of SCG. Eleven athletes with exercise-induced bronchoconstriction (EIB+) and 11
without (EIB-) performed a eucapnic voluntary hyperpnea (EVH) test (a surrogate for
exercise) 10 min after inhalation of a placebo or 40 mg of the mast cell stabilizing agent
sodium cromoglycate. The urinary concentrations of 9a,11β-PGF2 (a metabolite of PGD2

1335
and a marker of mast cell activation) and leukotriene E4 (LTE4) were measured by enzyme immunoassay 60 min before and for 90 min after the challenge. The results support mast cell activation with release of bronchoconstrictive mediators after hyperpnea in athletes with and without EIB and inhibition by SCG. The degree of airway responsiveness to the specific mediator released is likely to determine whether or not bronchoconstriction will occur after EVH [10486].

Prevalence of exercise-induced bronchoconstriction

To assess the progression of bronchial reactivity (BR) and incidence of bronchial hyperreactivity (BH), exercise-induced bronchoconstriction (EIB) and asthma in triathletes over 2 years seven athletes from the Swiss national triathlon team (mean age 24 years), who initially were not asthmatic, not treated with antiasthmatic medication, and who had performed at international level for more than 3 consecutive years (2001-2003). To assess BR, BH and EIB, subjects ran on a 400 m track for 8 min at intensities equal to the anaerobic threshold. Tests were conducted in ambient temperatures of 4 degrees C, -9 degrees C and 4 degrees C, and humidity of 52, 83, and 93 percent. Forced expiratory volume in 1 s (FEV1) was measured before and at 2, 5, 10 and 15 min after EIB, and 5 min after inhalation of a beta2-agonist. Two methods were used to calculate the incidence: the standard assessment and extrapolation of the decrease in FEV1 to the BH limit. BR increased significantly in the seven athletes. Within 2 years, BR had increased significantly and even reached BH in some athletes. Three athletes exhibited BH. After extrapolation of the decrease in FEV1 in all seven athletes, the limit of 10 percent by definition for BH was determined to occur within 2-5 years, resulting in 21-57 percent of athletes with newly developed BH per year. It was concluded that athletes develop EIB quickly, a rate of increase 195-286 times that of the normal rate for development of asthma [07189].

Exercise-induced bronchospasm (EIB) occurs more commonly in elite athletes than in the general population. There have been relatively few prevalence studies examining EIB in college athletes despite studies which have shown significant morbidity from asthma attacks related to exercise occurring in athletes in this age group. None of the previous studies utilized euopnic voluntary hyperpnea (EVH) testing, which is the currently recommended test to document EIB in Olympians. One hundred seven athletes from 22 sports participated. Forty-two of 107 athletes (39 %) were EIB positive according to EVH results. Thirty-six of 42 EIB-positive athletes (86 %) had no prior history of EIB or asthma. There were no significant differences in the prevalence of EIB according to sex of the athlete or ventilation demands of the sport. Symptoms were not predictive of EIB. The prevalence of EIB was 36 percent in athletes with negative symptoms and 35 percent for those with positive symptoms. Athletes in high-ventilation sports were significantly more symptomatic (48 %) than athletes in low-ventilation sports (25 %); however, there was no difference in the prevalence of EIB between the two groups. It was concluded that varsity athletes show a high incidence of EIB when objectively diagnosed by a variety of pulmonary function criteria. Sex of the athlete or ventilation demands of the sport does not affect the prevalence of EIB. The use of symptoms to diagnose EIB is not predictive of whether athletes have objectively documented EIB. Empiric diagnosis and treatment of EIB on the basis of subjective symptoms alone may lead to an increased number of inaccurate diagnoses and increased morbidity [07190].

The prevalence of exercise-induced bronchospasm (EIB) is significantly higher in athletes than that in the general population and can result in significant morbidity in young, competitive athletes. Guidelines emphasize that education and written treatment protocols improve clinical outcomes for asthmatics. Evidence also supports objective testing when EIB is suspected, when there is immediate availability of rescue inhalers, and when there is
involvement of asthma specialists in the care of asthmatic athletes. It was sought to determine how EIB is managed at the National Collegiate Athletic Association (NCAA) sports medicine programs. A survey consisting of multiple-choice questions related to EIB in athletes was sent electronically to 3200 athletic trainers affiliated with NCAA sports medicine programs. Five hundred and forty-one athletic trainers responded. A minority of athletic trainers surveyed (21%) indicated that an asthma management protocol exists at their institution. Twenty-two percent indicated that pulmonologists are on staff or consultants to the sports medicine department. Many indicated that a short-acting beta-agonist is not required to be available at all practices (39%) and games (41%), and few athletic trainers indicated that their programs use objective testing to diagnose EIB (17%). Regression modeling demonstrated education about EIB, and involvement of pulmonologists significantly improved adherence to current consensus guidelines. On the basis of these data, many NCAA sports medicine programs do not manage athletes with EIB according to current consensus guidelines. This may result in inaccurate diagnoses and may be detrimental to clinical outcomes and overall health of student athletes. Providing education about EIB and involvement of pulmonologists significantly increase adherence to guidelines that likely improves clinical care of athletes and potentially athletic performance [09240].

**Eucapnic voluntary hyperpnea**

Exercise challenge testing is the typical method for diagnosing exercise induced bronchoconstriction; however, alternate tests have been developed. The purpose of this paper was to summarize the current literature comparing eucapnic voluntary hyperpnea and mannitol with standard exercise challenge testing to determine whether either test is a suitable alternative to standard exercise testing for the diagnosis of exercise-induced bronchoconstriction. Using valid systematic review methods, a comprehensive search strategy to avoid publication bias, it was identified 10 studies that compared exercise challenge testing with either eucapnic voluntary hyperpnea or mannitol. For the 7 diagnostic cross-sectional studies that examined eucapnic voluntary hyperpnea, the sensitivity and specificity values were heterogeneous, ranging from 25 to 90% for sensitivity and 0 to 71% for specificity. In the 3 diagnostic cross-sectional studies that evaluated mannitol, the sensitivity and specificity ranged from 58 to 96 percent and 65 to 78 percent, respectively. For most studies, a representative spectrum of participants being tested was not used. Given the heterogeneity in sensitivity and specificity of eucapnic voluntary hyperpnea studies and the relatively small number of studies that have examined mannitol, insufficient evidence is available to conclude that either of these tests are suitable alternatives to exercise challenge testing to detect exercise-induced bronchoconstriction [11364].

**In children**

Exercise-induced bronchoconstriction (EIB) is defined as acute, reversible bronchoconstriction induced by physical exercise. It is widely believed that EIB occurs after exercise. However, in children with asthma the time to maximal bronchoconstriction after exercise is short, suggesting that the onset of EIB in such children occurs during exercise. In one study the authors investigated pulmonary function during exercise in cold air in children with asthma. Thirty-three children with asthma with a mean age of 12 years and a clinical history of exercise induced symptoms, underwent a prolonged, submaximal, exercise test of 12 min duration at approximately 80 percent of the predicted maximum heart rate. Pulmonary function was measured before and each minute during exercise. If EIB occurred (fall in forced expiratory volume in 1 s >15% from baseline), exercise was terminated and salbutamol was administered. Nineteen children showed EIB. In 12 of these children bronchoconstriction occurred during exercise (breakthrough EIB), while seven children
showed bronchoconstriction immediately after exercise (non-breakthrough EIB). Breakthrough EIB occurred between 6 and 10 min of exercise (mean 8 min). It was concluded that in the majority of children with EIB in this study (i.e. 12 out of 19), bronchoconstriction started during, and not after, a submaximal exercise test [11365].

A lot of emphasis has been placed in screening individuals with exercise-induced bronchospasm in order to avoid persistence bronchial hyperactivity and consequent chronic silent inflammation of the respiratory tract. The purpose of one study was to evaluate the effect of interval training on the respiratory function and endurance in children with exercise-induced asthma (EIA) participating in the sport of soccer. Twenty-nine boys ages 10-14, who developed EIA after a 6-minute free running test (decline in forced expiratory volume in 1 second: \(FEV_1\) 10 %), participated in the study. They were divided into 2 groups (experimental: \(n=18\), and control: \(n=11\)), fulfilling the same criteria (i.e. age, body height and weight, and severity of asthma). The experimental group exercised with the interval training method for a period of 8 weeks, (3 sessions per week), whereas the control group exercised with the usual football program. Measurements were made for \(FEV_1\) and endurance in both groups, before and after the application of training (8 weeks). Following the implementation of the training program, a significant improvement in \(FEV_1\) and endurance was documented in the experimental group, as well as significant differences between the 2 groups. In conclusion, duration and aerobic training via the interval method seems to be beneficial to soccer players with EIA [07191].

**Comparison of blood and urin levels between routes of administration**

It was aimed to assess the plasma and urine concentrations of beta\(_2\)-agonists and evaluate the difference between three routes of administration in trained adults in order to distinguish doping from prevention of exercise-induced asthma. Ten young healthy Caucasian male subjects received during a four treatment period study: 1) inhaled salbutamol (S(I)) 2 x 100 microg t.i.d. for 3 days, 2) inhaled formoterol (F(I)) 2 x 12 microg b.i.d. for 3 days, 3) a single subcutaneous injection of salbutamol (S(S)) 0.5 mg, and 4) salbutamol 2 x 2 mg t.i.d. orally for 3 days (S(O)). Blood samples were taken during the first and the third day of experimentation at baseline, 30 min, 1 h, 2 h, 4 h and 6 h after administration; additional blood samples were drawn at 15 min for S(I), S(S) and F(I) and at 12 h for F(I). Urinary samples were collected at baseline, 2 h, 4 h, 6 h and 12 h after administration. Urinary concentrations were 20 to almost 50 times higher after S(O) than after S(I). Mean urinary concentration after S(O) increased to above 800 ng/mL within the two hours and above 1000 ng/mL at 6 to 12 hours post-drug administration. Urinary concentrations after S(S) were maximal during the first 2 hours (mean: 340 ± 172 ng/mL). Plasma concentrations were very low, whatever the routes of administration. Results showed that we could eliminate the use of S(I) (authorized) and S(S) administration when individual urinary concentrations are higher than 230 ng/mL and 615 ng/mL, respectively. Therefore, at rest, the cut-off value used to discriminate therapeutic from doping salbutamol intake could be fixed at 250 ng/mL instead of the 1000 ng/mL still authorized by international committees [06203].

**Ergogenic effects**

*In chronic pulmonary disease*

Chronic obstructive pulmonary disease is currently considered a systemic disease, presenting structural and metabolic alterations that can lead to skeletal muscle dysfunction.
This negatively affects the performance of respiratory and peripheral muscles, functional capacity, health-related quality of life and even survival. The decision to prescribe ergogenic aids for patients with chronic obstructive pulmonary disease is based on the fact that these drugs can avert or minimize catabolism and stimulate protein synthesis, thereby reducing the loss of muscle mass and increasing exercise tolerance. One review summarized the available data regarding the use of anabolic steroids, creatine, L-carnitine, branched-chain amino acids and growth hormones in patients with chronic obstructive pulmonary disease. The advantage of using these ergogenic aids appears to lie in increasing lean muscle mass and inducing bioenergetic modifications. Within this context, most of the data collected deals with anabolic steroids. However, to date, the clinical benefits in terms of increased exercise tolerance and muscle strength, as well as in terms of the effect on morbidity and mortality, have not been consistently demonstrated. Dietary supplementation with substances of ergogenic potential might prove to be a valid adjuvant therapy for treating patients with advanced chronic obstructive pulmonary disease, especially those presenting loss of muscle mass or peripheral muscle weakness [06204].

In non-asthmatics

To provide an overview of the current literature on the use of inhaled beta2-agonists in non-asthmatic competitive athletes, and to assess the performance enhancing effect of inhaled beta2-agonists. Twenty randomised, placebo controlled studies (19 double blind, one single blind) were located. Only three studies reported a performance enhancing effect of inhaled beta2 agonists. However, methodological shortcomings were most likely responsible for these findings (for example, non-elite athletes, inconsistent results in different tests, subgroups with above-average responsiveness). The review reveals that there is no ergogenic potential of inhaled beta2-agonists in non-asthmatic athletes. In view of the epidemiology of asthma in athletes and the considerable workload involved in provision of therapeutic use exemptions the inclusion of inhaled beta2-agonists on the list of prohibited substances should be reconsidered [06205].

Treatment

The aims of one report was to review the current recommended treatment of exercise-induced asthma (EIA), respiratory and allergic disorders in sports, and to review the evidence on possible improvement of performance in sports by asthma drugs and to make recommendations for their treatment. To assess the evidence of the literature regarding use of beta2-agonists related to athletic performance, the Task Force searched Medline for relevant papers up to November 2006 using the present search words: asthma, bronchial responsiveness, exercise-induced bronchoconstriction, athletes, sports, performance and beta2-agonists. Treatment recommendations for EIA and bronchial hyper-responsiveness in athletes are set forth with special reference to controller and reliever medications. Evidence for lack of improvement of exercise performance by inhaled beta2-agonists in healthy athletes serves as a basis for permitting their use. There is a lack of evidence of treatment effects of asthma drugs on EIA and bronchial hyper-responsiveness in athletes whereas extensive documentation exists in treatment of EIA in patients with asthma. The documentation on lack of improvement on performance by common asthma drugs as inhaled beta2-agonists with relationship to sports in healthy individuals is of high evidence, level (1+). It was concluded that exercise induced asthma should be treated in athletes along same principles as in ordinary asthma patients with relevance to controller and reliever treatment after careful diagnosis. There is very high level of evidence for the lack of improvement in athletic performance by inhaled beta2-agonists [08347].
Meta-analysis of beta₂-agonists effects

Inhaled beta₂-agonists are commonly used as bronchodilators in the treatment of asthma. Their use in athletes, however, is restricted by anti-doping regulations. Controversies remain as to whether healthy elite athletes who use bronchodilators may gain a competitive advantage. The aim of one systematic review and meta-analysis was to assess the effects of inhaled and systemic beta₂-agonists on physical performance in healthy, non-asthmatic subjects. To this end, MEDLINE, EMBASE, and the Cochrane Central Register of Controlled Trials (CENTRAL) were searched up to August 2009. Reference lists were searched for additional relevant studies. The search criteria were for randomized controlled trials examining the effect of inhaled or systemic beta₂-agonists on physical performance in healthy, non-asthmatic subjects. Two authors independently performed the selection of studies, data extraction and risk of bias assessment. Parallel-group and crossover trials were analysed separately. Mean difference (MD) and 95 percent confidence intervals were calculated for continuous data and, where possible, data were pooled using a fixed effects model. Twenty-six studies involving 403 participants (age range 7-30 years) compared inhaled beta₂-agonists with placebo. No significant effect could be detected for inhaled beta₂-agonists on maximal oxygen consumption (VO₂max) (mean difference -0.14 mL/kg/min; 95 confidence interval -1.07 to 0.78; 16 studies), endurance time to exhaustion at 105-110 percent VO₂max (mean difference -1.5 s; 95 % confidence interval -15.6, 12.6; four studies), 20-km time trial duration (mean difference -4.4 s; 95 % confidence interval -23.5 to 14.7; two studies), peak power (mean difference -0.14 W/kg; 95 % confidence interval -0.54 to 0.27; four studies) and total work during a 30-second Wingate test (mean difference 0.80 J/kg; 95 % confidence interval -2.44 to 4.05; five studies). Thirteen studies involving 172 participants (age range 7-22 years) compared systemic beta₂-agonists with placebo, with 12 studies involving oral and one study involving intravenous salbutamol. A significant effect was detected for systemic beta₂-agonists on endurance time to exhaustion at 80-85% VO₂max (mean difference 402 s; 95 % confidence interval 34 to 770; two studies), but not for VO₂max (placebo 42.5 ± 1.7 mL/kg/min, salbutamol 42.1 ± 2.9 mL/kg/min, one study), endurance time to exhaustion at 70 percent VO₂max (mean difference 400 s; 95 % confidence interval -408 to 1208; one study) or power output at 90 % VO₂max (placebo 234.9 ± 16 W, salbutamol 235.5 ± 18.1 W, one study). A significant effect was shown for systemic beta₂-agonists on peak power (mean difference 0.91 W/kg; 95 % confidence interval 0.25 to 1.57; four studies), but not on total work (mean difference 7.8 J/kg; 95 percent confidence interval -3.3 to 18.9; four studies) during a 30-second Wingate test. There were no randomized controlled trials assessing the effects of systemic formoterol, salmeterol or terbutaline on physical performance. In conclusion, no significant effects were detected for inhaled beta₂-agonists on endurance, strength or sprint performance in healthy athletes. There is some evidence indicating that systemic beta₂-agonists may have a positive effect on physical performance in healthy subjects, but the evidence base is weak [11189].

Designer beta2-agonists

Beta2-adrenergic agonists, or beta2-agonists, are considered essential bronchodilator drugs in the treatment of bronchial asthma, both as symptom-relievers and, in combination with inhaled corticosteroids, as disease-controllers. The use of beta2-agonists is prohibited in sports by the World Anti-Doping Agency (WADA) due to claimed anabolic effects, and also, is prohibited as growth promoters in cattle fattening in the European Union. One paper reviewed the last seven-year (2006-2012) literature concerning the development of novel
beta2-agonists molecules either by modifying the molecule of known beta2-agonists or by introducing moieties producing indole-, adamantyl- or phenyl urea derivatives. New emerging beta2-agonists molecules for future therapeutic use are also presented, intending to emphasize their potential use for doping purposes or as growth promoters in the near future [13407].

Salbutamol

The World Anti-doping Agency stipulates that athletes who declare salbutamol use should not exceed 1600 microg over a 24 h period. No studies have investigated the physiological effect of 1600 microg of inhaled salbutamol on intermittent sports. This study investigated the physiological effect of inhaling 800 microg and 1600 microg of salbutamol prior to a football specific run at high ambient temperatures. Five male non-asthmatic football players (mean ± SD; age 24 ± 4 years; weight 71 ± 4 kg; height 175 ± 5 cm) volunteered. Participants attended the lab on three occasions to inhale either a placebo (PLA), 800 microg of salbutamol (800 SAL) or 1600 microg of salbutamol (1600 SAL) 15 min prior to performing a football-specific run for 45 min at 30°C. The football specific run consisted of seven stages which included two high intensity stages and five low intensity stages. During each stage VO\textsubscript{2}, RER, HR and blood lactate was measured. Repeated measures ANOVA were performed to investigate the changes in VO\textsubscript{2}, RER, HR and blood lactate during each stage. During stage one and two HR was significantly higher in 800 SAL (143 ± 10 b/min; 148 ±12 b/min) and 1600 SAL (142 ± 3 b/min; 147 ± 4.0 b/min) when compared to PLA (119 ± 12 b/min; 129 ± 10 b/min). Following stage two blood lactate was significantly higher in 800 SAL (4.2 ± 1.8 mmol/L) and 1600 SAL (4.5 ± 1.6 mmol/L) when compared with PLA (2.4 ± 1.2 mmol/L). Following stage five blood lactate was significantly higher in 800 SAL (4.2 ± 1.6 mmol/L) and 1600 SAL (5.0 ± 1.4 mmol/L) when compared with PLA (2.8 ± 1.0 mmol/L). The increased HR (stages one and two) and increased blood lactate concentration (stages two and five) observed following salbutamol inhalation, suggests supratherapeutic doses of salbutamol may increase the physiological cost during a football match [11483].

The World Anti-doping Agency stipulates that athletes who declare the use of salbutamol should not exceed 1600 microg over a 24 h period. Most studies investigating the effect of inhaled salbutamol on maximal flow volume values, such as FEV\textsubscript{1}, have investigated doses of up to 800 microg of salbutamol. The purpose of this study was to investigate the effect of inhaling 800 microg or 1600 microg on the FEV\textsubscript{1} in non-asthmatic football players. Five male non-asthmatic football players (mean ± SD; age 24 + 4 year; weight 71 ± 4 kg; height 175 ± 5 cm) volunteered. Participants attended the laboratory on six occasions to inhale either a placebo, 800 microg of salbutamol or 1600 microg salbutamol and complete a football-specific run. Maximal Flow Volume Loops were measured at baseline, 10 min following inhalation of salbutamol or placebo and 5 min following the football specific run. Repeated measures ANOVA were performed to investigate the changes in FEV\textsubscript{1} 10 min post salbutamol and post football specific treadmill run. There was a significant difference between FEV\textsubscript{1} following the inhalation of either 800 microg (4.4 ± 0.5 L) or 1600 microg (4.4 ± 0.4 L) salbutamol when compared to placebo (4.3 ± 0.5 L) prior to the football-specific run. This significant difference was maintained following the completion of the football specific run following the inhalation of either 800 microg or 800 μg (4.5 ± 0.5 L) or 1600 microg (4.5 ± 0.5 L) salbutamol when compared placebo (4.3 ± 0.5 L). The improvements in FEV\textsubscript{1} following the inhalation of 800 microg or 1600 microg salbutamol prior to the football-specific treadmill run appears to suggest there is a potential for significant bronchodilation in the airways of non-asthmatic football players. The difference in FEV\textsubscript{1} between placebo and salbutamol appears to be maintained following the completion of a football-specific treadmill run [11484].
One study examined the effects of an oral acute administration of the beta2-agonist salbutamol (Sal) (6 mg) versus placebo on muscle strength and fatigability in 12 non-asthmatic recreational male athletes in a randomized double-blind protocol. Contractile properties of the right quadriceps muscle were measured during electrical stimulations, i.e. twitch, 1-s pulse trains at 20 (P(20)) and 80 Hz (P(80)) and during maximal voluntary isometric contraction (MVIC) before (PRE) and after (POST) a fatigue-producing protocol set by an electromyostimulation (30 contractions, frequency: 75 Hz, on-off ratio: 6.25-20s). In addition, the level of muscle voluntary activation was measured. In PRE and POST conditions, the peak torque (PT) of twitch, P(80) and MVIC were not modified by the treatment. The PT in POST P(20) was slightly, although not significantly, less affected by fatigue in Sal compared with placebo condition. Moreover, twitch half-relaxation time at PRE was smaller under Sal than under placebo. No significant changes in the degree of voluntary activation were observed with Sal treatment in PRE or POST condition. It was concluded that although these findings did not exclude completely an effect of Sal on peripheral factors of human skeletal muscle, oral acute administration of the beta2-agonist salbutamol seems to be without any relevant ergogenic effect on muscle contractility and fatigability in non-asthmatic recreational male athletes [11485].

Salbutamol is sometimes misused or abused, which can result in various cardiovascular adverse effects. We report one case of fatal salbutamol misuse or abuse in a 36-year-old poorly controlled female asthmatic patient with a past medical history of alcoholism and a recent smoking cessation. She died shortly after hospital admission following acute dyspnea and sudden collapse at home. Toxicological analyses evidenced salbutamol overdose, and necropsy showed acute lung edema and marked dysplasia of the right ventricle and revealed the patient was pregnant. The involvement of an initial disorder of the ventricular rhythm leading to cardiac failure is suggested by the presence of several combined pro-arrhythmogenic factors, such as arrhythmogenic right ventricle dysplasia, hypoxemia related to bronchospasm and salbutamol overdose [11486].

One study was designed to examine the dose-response relationship of inhaled salbutamol and its concentration in the urine while resting at various times after inhalation, and to compare these values against the current World Anti-Doping Code limits. Eight healthy, nonasthmatic males participated in the study. Administration of three different doses of inhaled salbutamol (800, 400, and 200 microg) in a randomized fashion separated by at least 72 hours gave highly variable urine concentrations between subjects and increased as dose increased, with a significant difference noted between 800 and 200 microg at 30, 60, and 120 minutes after inhalation. Urine concentrations of salbutamol peaked at 60 minutes for all doses. No samples exceeded the doping criterion of 1000 ng/mL, and the maximum value observed was 904 ng/mL. These results indicate that after inhalation of doses up to 800 microg, urinary concentrations of salbutamol are well below the limits used in doping control [08349].

A fast and reliable quantitative method for salbutamol using direct analysis of the urine sample by ultraperformance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) has been developed. Urine samples were spiked with salbutamol-d6 (internal standard), and, then, they were diluted with ultrapure water (1:1, v/v). Aliquots of 1 microl of the mixture were directly analyzed by UPLC/MS/MS. The chromatographic separation was performed in a UPLC BEH C18 (100 mm x 2.1 mm, 1.7 microm) column with a mobile phase contained 0.01 percent formic acid in ultrapure water (v/v) and 0.01 percent formic acid in acetonitrile (v/v), using gradient elution at 0.6 ml/min. The temperature of the column was set to 45 degrees C. The total run time was 3.2 min. Electrospray ionization in positive ion mode was used under multiple reaction monitoring (MRM) at different collision
energies. Nitrogen and argon were used as desolvation and collision gas, respectively. The method was shown to be linear from 200 to 5000 ng/ml. The limit of quantitation was estimated in 200 ng/ml. Intra-assay precision and accuracies, evaluated by using quality control samples containing 550 and 1100 ng/ml salbutamol, were always better than 8.4 percent. The intermediate precision was estimated to be in the range of 5.6-8.9 percent. The method was shown to be reliable when applying to routine samples, and the short analysis time resulting from a simple sample preparation and a fast instrumental analysis makes it of great interest for antidoping control purposes [09241].

Data on blood and urinary concentrations of salbutamol after inhalation and oral administration in healthy subjects are scarce. Accordingly, it was examined the pharmacokinetics of inhaled and oral salbutamol in asthmatic subjects. It was enrolled 10 men aged 18-45 years in an open-label study in which 0.8 mg of inhaled or 8 mg of systemic salbutamol was administered in a crossover design. All subjects had doctor-diagnosed asthma, used beta2-agonist when needed, and abstained from any medicine, beta2-agonist inclusive, for 14 d before visit. Urine was collected from all subjects (0-4, 4-8, and 8-12 h), and blood samples were taken at 0, 0.5, 1, 2, 3, 4, and 6 h after salbutamol administration. Maximum urine concentration was reached during the first 4 h after administration of both inhaled and oral salbutamol. It was found significant differences in median urinary concentrations (C_{max}) of 261 and 2422 ng/mL, respectively. Urinary concentrations show high individual variability irrespective of the route of administration. Blood analyses showed a systemic exposure of salbutamol after both inhaled and oral salbutamol with peak concentration after inhalation before the oral intake. A significant difference in median C_{max} after inhalation and oral treatment was found: 1.75 and 18.77 ng/mL, respectively. It was concluded that median urinary concentrations after oral administration of 8 mg of salbutamol were significantly higher than those after inhalation of 0.8 mg of salbutamol [10169].

It was examined blood and urine concentrations of repetitive doses of inhaled salbutamol in relation to the existing cut-off value used in routine doping control. It was compared the concentrations in asthmatics with regular use of beta2-agonists prior to study and healthy controls with no previous use of beta2-agonists. It was enrolled 10 asthmatics and 10 controls in an open-label study in which subjects inhaled repetitive doses of 400 microgram salbutamol every second hour (total 1600 microgram), which is the permitted daily dose by the World Anti-Doping Agency (WADA). Blood samples were collected at baseline, 30 min, 1, 2, 3, 4, and 6 h after the first inhalations. Urine samples were collected at baseline, 0-4 h, 4-8 h, and 8-12 h after the first inhalations. Median urine concentrations peaked in the period 4-8 h after the first inhalations in the asthmatics and between 8-12 h in controls and the median ranged from 268 to 611 ng/mL. No samples exceeded the WADA threshold value of 1000 ng/mL when corrected for the urine specific gravity. When not corrected one sample exceeded the cut-off value with urine concentration of 1082 ng/mL. In conclusion it was found no differences in blood and urine concentrations between asthmatic and healthy subjects. It was found high variability in urine concentrations between subjects in both groups. The variability between subjects was still present after the samples were corrected for urine specific gravity [11188].

The prevalence of asthma is higher in elite athletes than in the general population. The risk of developing asthmatic symptoms is the highest in endurance athletes and swimmers. Asthma seems particularly widespread in winter-sport athletes such as cross-country skiers. Asthmatic athletes commonly use inhaled beta2-agonists to prevent and treat asthmatic symptoms. However, beta2-agonists are prohibited according to the Prohibited List of the World Anti-Doping Agency. An exception can be made only for the substances formoterol, salbutamol, salmeterol and terbutaline by inhalation, as long as a therapeutic use exemption has been applied for and granted. In this context, the question arises of whether beta2-
agonists have ergogenic benefits justifying the prohibition of these substances. In 17 of 19 randomised placebo-controlled trials in non-asthmatic competitive athletes, performance-enhancing effects of the inhaled beta$_2$-agonists formoterol, salbutamol, salmeterol and terbutaline could not be proved. This is particularly true for endurance performance, anaerobic power and strength performance. In three of four studies, even supratherapeutic doses of salbutamol (800-1200 microg) had no ergogenic effect. In contrast to inhaled beta$_2$-agonists, oral administration of salbutamol seems to be able to improve the muscle strength and the endurance performance. There appears to be no justification to prohibit inhaled beta$_2$-agonists from the point of view of the ergogenic effects [07194].

Data on pharmacokinetics of inhaled and oral salbutamol in elite athletes with asthma are needed to differentiate between therapeutic use and doping in doping control. Eight elite athletes with asthma and 10 nonasthmatic subjects aged 18 to 33 years were given 0.8 mg of inhaled salbutamol and 8 mg of oral salbutamol separated by 14 days and it was measured urine concentration of free salbutamol. Maximum urine concentrations peaked in the period of 0 to 4 hours after the administration of inhaled and oral salbutamol in both groups. Median concentrations after inhaled salbutamol and oral salbutamol were 402 and 2108 ng/mL in healthy subjects and 335 and 2975 ng/mL in elite athletes with asthma. There were no significant statistical differences between the groups. One sample exceeded the World Anti-Doping Agency threshold value of 1000 ng/mL with a urinary salbutamol concentration of 1057 ng/mL 4 hours after inhalation, when no correction for urine specific gravity was done. When this sample was corrected for urine specific gravity, the result was 661 ng/mL. It was thus concluded that there was no significant difference in pharmacokinetic profile of inhaled and oral salbutamol between elite athletes with asthma and nonasthmatic subjects. The results indicate that urine salbutamol concentrations should be corrected for urine specific gravity when evaluating doping cases [12310].

The prevalence of asthma is higher among elite athletes than in the general population. This has resulted in the frequent use of anti-asthmatic medication such as beta2-agonists among asthmatic athletes. Beta2-agonists are on the prohibited list of WADA. The use of the beta2-agonist salbutamol is only permitted in therapeutic inhaled doses. Most studies have reported the lack of ergogenic effects of therapeutic doses of inhaled beta2-agonists measured in maximal oxygen uptake. No previous studies have examined any possible effects of high-dose inhaled salbutamol on oxygen uptake kinetics. It was enrolled nine healthy well-trained men in a randomized, blinded, placebo-controlled crossover study. Subjects were randomized to inhalation of 40 puffs of 0.2 mg salbutamol or two placebo tablets and performed an incremental test to exhaustion and three submaximal tests at 75% of peak power to determine oxygen uptake kinetics. During the incremental test, there were no effects of inhaled salbutamol on VO$_2$$_{max}$ in absolute or relative terms, and no effect on peak power and lactate threshold. During the submaximal test, we found no effects on the time constant, time delay, the mean response time or O$_2$ deficit related to oxygen uptake kinetics. In conclusion, no ergogenic effect of a high dose of salbutamol on aerobic capacity was found [12311].

The renal elimination of salbutamol in asthmatic and non-asthmatic subjects was investigated, who received either 0.8 mg of salbutamol via inhalation or 8 mg in tablet formulation orally. The inhaled salbutamol did not result in urinary concentrations exceeding the threshold of 1000 ng/mL when correction for specific gravity was applied. In contrast, the oral application of 8 mg yielded peak values of free urinary salbutamol of more than 6000 ng/mL. The comparison of pharmacokinetic profiles of elite athletes and non-asthmatic individuals did not reveal a statistically significant difference. In order to provide a means to differentiate a potential misuse of beta$_2$-agonists from therapeutic usage, two studies focusing on either inhaled formoterol (18 mg) or inhaled salmeterol (100 mg) were presented.
Demonstrating limits of quantification (LOQs) at sub-ng/mL concentrations in urine, both analytes were sensitively determined in administration study urine samples and authentic doping control specimens. In case of formoterol peak values of 11.4 ng/mL were observed while salmeterol did not exceed 1.3 ng/mL in elimination studies; doping control specimens concentrations below 30 ng/mL and 2 ng/mL prevailed for formoterol and salmeterol, respectively [12017].

Prevalence of exercise-induced bronchoconstriction (EIB) is high in elite athletes, especially after many years training in cold and dry air conditions. The primary treatment of EIB is inhaling a short-acting beta-2-agonist such as salbutamol. However, professional speed skaters also inhale nebulized isotonic saline or tap water before and after a race or intense training. The use of nebulized isotonic saline or tap water to prevent EIB has not been studied before, raising questions about safety and efficacy. The aim of one study was to analyze the acute effect of nebulized isotonic saline or salbutamol on EIB in elite speed skaters following a 1,500-meter race. The randomized controlled trial compares single dose treatment of 1 mg nebulized salbutamol in 4 mL of isotonic saline, or with 5 mL of isotonic saline. A minimum of 13 participants will be allocated in each treatment group. Participants should be between 18 and 35 years of age and able to skate 1,500 m in less than 2 min 10 s (women) or 2 min 05 s (men). Repeated measurements of spirometry, forced oscillation technique, andelectromyography will be performed before and after an official 1,500-m race. Primary outcome of the study is the difference in fall in FEV1 after exercise in the different treatment groups. The trial is currently enrolling participants. Elite athletes run the risk of pulmonary inflammation and remodeling as a consequence of their frequent exercise, and thus increased ventilation in cold and dry environments. Although inhalation of nebulized isotonic saline is commonplace, no study has ever investigated the safety or efficacy of this treatment [13408].

According to WADA, all beta$_2$-agonists are per se categorized as prohibited with the exemptions of inhaled salbutamol (maximum allowed dosage 1600 microg/day), inhaled formoterol (maximum allowed dosage 54 microg/day) and inhaled salmeterol. Further to the permitted routes and daily dosages of salbutamol and formoterol, urinary threshold levels have been enforced [13009].

**Effect on performance**

Salbutamol may affect lung function and exercise performance differently in individuals with and without asthma. To compare the effects of inhaled salbutamol on lung function, exercise performance and respiratory parameters during cycling exercise in athletes with a positive response to a eucapnic voluntary hyperpnoea (EVH+) and negative (EVH-) challenge, indicative of exercise-induced bronchoconstriction was investigated. In a randomised controlled trial with a crossover design, a total of 49 well-trained male athletes (14 EVH+ and 35 EVH-) performed two simulated 10 km time-trials on a cycle ergometer 60 min after the inhalation of either 400 microg of salbutamol or a placebo. Lung function, assessed by forced expiratory volume in 1 s, was measured immediately before and 30 min after inhalation. Performance was measured by mean power output. Despite a significant increase in lung function after the inhalation of salbutamol compared to the placebo, salbutamol did not affect athletes' perceptions of dyspnoea or leg exertion during exercise. Salbutamol did not affect mean power output: EVH+ and EVH- athletes averaged 4.0 (0.5) and 4.1 (0.5) W/kg after salbutamol and 4.0 (0.5) W/kg and 4.0 (0.4) W/kg after placebo, respectively. It was concluded that the inhalation of salbutamol induced a significant increase in resting lung function in EVH+ and EVH- athletes but this improvement in lung function did not translate to improved exercise performance. Salbutamol had no discernible effect on key ventilatory and exercise parameters regardless of EVH challenge outcome [13409].
Neuromuscular function

The potential ergogenic effects of therapeutic inhaled salbutamol doses in endurance athletes have been controversially discussed for decades. It was hypothesized that salbutamol inhalation may increase peripheral muscle contractility, reduce fatigability, and improve force recovery after a localized exercise in endurance athletes. Eleven healthy, nonasthmatic male athletes with high aerobic capacities were recruited to be compared in a double-blinded, randomized crossover study of two dose levels of salbutamol (200 and 800 microg) and a placebo administered by inhalation before a quadriceps fatigue test. Subjects performed an incremental exercise protocol consisting in sets of 10 intermittent isometric contractions starting at 20 percent of maximum voluntary contraction (MVC) with 10 percent MVC increment until exhaustion. Femoral nerve magnetic stimulation was used during and after MVC to evaluate neuromuscular fatigue after each set, at task failure, and after 10 and 30 min of recovery. Initial MVC and evoked muscular responses were not modified with salbutamol. The total number of submaximal contractions until task failure significantly differed between treatments. MVC and evoked muscular responses were similarly reduced with all treatments during the fatiguing task. Voluntary activation was unaffected by the fatiguing task and treatments. It was concluded that supratherapeutic inhaled doses of beta2-agonists increased quadriceps endurance during an incremental and localized fatiguing task in healthy endurance-trained athletes without significant effect on neuromuscular fatigue. Further studies are needed to clarify the underlying mechanisms [13410].

After intense exercise in endurance athletes

The objective of one study was to investigate urine concentrations of 8 mg oral salbutamol in samples collected after intense exercise in endurance athletes. Nine male endurance athletes with a VO2max of 70 mL/min/kg (mean) took part in the study. Two hours after administration of 8 mg oral salbutamol, subjects performed submaximal exercise for 15 min followed by two, 2-min exercise bouts at an intensity corresponding to 110 percent of VO2max and a bout to exhaustion at same intensity. Urine samples were collected 4, 8, and 12 h following administration of salbutamol. Samples were analyzed by the Norwegian World Anti-doping Agency (WADA) laboratory. Adjustment of urine concentrations of salbutamol to a urine specific gravity (USG) of 1.020 g/mL was compared with no adjustment according to WADA's technical documents. It was observed greater urine concentrations of salbutamol 4 h after administration when samples were adjusted to a USG of 1.020 g/mL compared with no adjustment (3089 ± 911 vs 1918 ± 1081 ng/mL). With the current urine decision limit of 1200 ng/mL for salbutamol on WADA's 2013 list of prohibited substances, fewer false negative urine samples were observed when adjusted to a USG of 1.020 g/mL compared with no adjustment. In conclusion, adjustment of urine samples to a USG of 1.020 g/mL decreases risk of false negative doping tests after administration of oral salbutamol [043]. Adjusting urine samples for USG might be useful when evaluating urine concentrations of salbutamol in doping cases [13411].

Renal elimination

The renal elimination of salbutamol in asthmatic and non-asthmatic subjects was investigated, who received either 0.8 mg of salbutamol via inhalation or 8 mg in tablet formulation orally. The inhaled salbutamol did not result in urinary concentrations exceeding the threshold of 1000 ng/ml when correction for specific gravity was applied. In contrast, the oral application of 8 mg yielded peak values of free urinary salbutamol of more than
6000 ng/ml. The comparison of pharmacokinetic profiles of elite athletes and non-asthmatic individuals did not reveal a statistically significant difference [13012].

**Acute effects on muscles**

The potential ergogenic effects of therapeutic inhaled salbutamol doses in endurance athletes have been controversially discussed for decades. It was hypothesized that salbutamol inhalation may increase peripheral muscle contractility, reduce fatigability, and improve force recovery after a localized exercise in endurance athletes. Eleven healthy, nonasthmatic male athletes with high aerobic capacities were recruited to be compared in a double-blinded, randomized crossover study of two dose levels of salbutamol (200 and 800 microg) and a placebo administered by inhalation before a quadriceps fatigue test. Subjects performed an incremental exercise protocol consisting in sets of 10 intermittent isometric contractions starting at 20 percent of maximum voluntary contraction (MVC) with 10 percent MVC increment until exhaustion. Femoral nerve magnetic stimulation was used during and after MVC to evaluate neuromuscular fatigue after each set, at task failure, and after 10 and 30 min of recovery. Initial MVC and evoked muscular responses were not modified with salbutamol. The total number of submaximal contractions until task failure significantly differed between treatments. MVC and evoked muscular responses were similarly reduced with all treatments during the fatiguing task. Voluntary activation was unaffected by the fatiguing task and treatments. Supratherapeutic inhaled doses of the beta2-agonists increased quadriceps endurance during an incremental and localized fatiguing task in healthy endurance-trained athletes without significant effect on neuromuscular fatigue. Further studies are needed to clarify the underlying mechanisms [13412].

**Side effects**

**Effects on bone remodeling**

Animal studies suggest that bone remodeling is under beta-adrenergic control via the sympathetic nervous system. The impact of beta-agonist substances, at doping doses, has not been studied in adult rats. The purpose of one study was to examine the effects of salbutamol injections with or without treadmill exercise on trabecular and cortical bone in adult rats. Adult (36 week of age) female Wistar rats (n=56) were treated with salbutamol (3 mg/kg/day sc, 5 days/week) or vehicle (sham) with or without subsequent treadmill exercise (13 m/min, 60 min/day, 5 days/week) for 10 week. Tibial and femoral bone mineral density was analyzed by dual-energy X-ray absorptiometry. Metaphysic trabecular bone structure was analyzed by micro-CT at the time of the animals' death. Bone cell activities were assessed histomorphometrically. After 10 wk, the increase in bone mineral density was less in salbutamol-treated than in sham rats (+3.3 % vs +12.4 %), and trabecular parameters were altered and bone resorption was increased in salbutamol-treated rats compared with controls. The negative effect on bone architecture in salbutamol-treated rats persisted, even with treadmill exercise. These results confirm the deleterious effect of beta2-agonists on bone mass during chronic treatment and describe its effects on bone mechanical properties in adult rats. Bone loss occurred independently of a salbutamol-induced anabolic effect on muscle mass and was equally severe in sedentary and exercising rats, despite a beneficial effect of exercise on the extrinsic and intrinsic energy to ultimate strain. These bone effects may have important consequences in athletes who use salbutamol as a doping substance [06206].

**Experimental**

A key question regarding the biochemical basis of potential salbutamol-mediated anabolic
effects concerns whether salbutamol activates such effects through beta-adrenoreceptor stimulation or by other mechanisms. Beta-2 agonist-mediated anabolic effects on muscle have been reported to be dependent and independent of actions on beta-adrenoreceptors. Salbutamol has been shown to mediate anabolic effects after intravenous administration. However, the mechanism responsible for the anabolic actions of salbutamol remains unknown. The potential androgenic activity of salbutamol was investigated in vitro by the A-Screen assay that measures androgen-dependent inhibition of proliferation of the androgen receptor (AR)-positive human mammary carcinoma cell line, MCF7-AR1. The results indicate that salbutamol exerts anabolic effects through androgen receptor agonistic activity in vitro [07185].

**Laboratory techniques**

Salbutamol is commonly used in asthma treatment, being considered a short-effect bronchodilator. This drug poses special interest in certain fields of chemical analysis, such as food, clinical and doping analyses, in which it needs to be analyzed with quantitative precision and accuracy. Salbutamol, however, is known to degrade under certain conditions and this is critical if quantitative results must be generated. One study aimed to investigate salbutamol extraction from urine samples, to determine whether salbutamol is unstable in other solvents as well as in urine samples, to elucidate the structures of the possible degradation products and to validate an analytical method using the extraction procedure evaluated. Stability investigations were performed in urine at different pH values, in methanol and acetone at different temperatures. Semi-preparative liquid chromatography was performed for the isolation of degradation products, and gas chromatography coupled to mass spectrometry as well as nuclear magnetic resonance were used for identification. Three unreported methylation products were detected in methanolic solutions and had their structures elucidated. Urine samples showed a reduction in salbutamol concentration of up to 26 percent after 5 weeks. These results show that special care must be taken regarding salbutamol quantitative analyses, since degradation either in standard solutions or in urine could lead to incorrect values [13413].

Matrix effects in determination of three beta-receptor agonists including salbutamol (SAL), clenbuterol, and terbutaline in animal-derived foodstuffs were studied by ultra-performance LC-MS/MS with cleanup of immunoaffinity SPE column (IAC). Some animal tissue samples including pig liver, swine muscle, and fish muscle were hydrolyzed by the mixed enzyme solution or HCl solution, and the cleanup efficiencies with SAL IAC, MCX SPE column, and C(18)-SCX tandem columns were examined and compared by using spiked experiments. The results showed that the matrix effects in the determination of SAL and terbutaline can be eliminated with SAL IAC cleanup, and the average recoveries of SAL were 77-82, 79-83, and 85-87 percent in pig liver, swine muscle, and fish muscle, respectively. The decision limit (ccalpha) and detection capability (ccbeta) for SAL in pig liver were 0.02 and 0.05 microg/kg, respectively [12312].

**Terbutaline**

Terbutaline is a fast-acting beta2-adrenergic agonist used in the treatment of obstructive pulmonary diseases. Doping control for beta2-agonists, which are forbidden in sports by the World Anti-doping Agency (WADA), is performed in screening by liquid chromatography/mass spectrometry after hydrolysis of phase-II metabolites. In one study, the mono-sulfoconjugated phase-II metabolite of terbutaline was synthesized and the chemical structure was characterized by 1H-nuclear magnetic resonance spectrometry and
high resolution/high accuracy Orbitrap mass spectrometry. The metabolite was designated as the phenolic esterified compound, which has been mentioned in most literature reports but has not been verified so far. The benzylic esterified compound was also synthesized and characterized by high-resolution/high accuracy Orbitrap mass spectrometry but was not detectable in urine samples from an excretion study performed after a single application of one terbutaline capsule (7.5 mg terbutaline sulfate salt). The phenolic sulfate of terbutaline was detected for two to four days after administration, whereas the unchanged terbutaline was detected for four to five days. A glucuronidated, disulfated or trisulfated phase-II metabolite of terbutaline was not found. The measurement of phase-II metabolites is planned to be incorporated into existing screening procedures to allow a faster sample preparation.

It was examined urine and serum concentrations after therapeutic use of single and repetitive doses of inhaled and supratherapeutic oral use of terbutaline. It was compared the concentrations in 10 asthmatics and 10 healthy subjects in an open-label, cross-over study with 2 mg inhaled and 10 mg oral terbutaline on 2 study days. Further, 10 healthy subjects were administrated 1 mg inhaled terbutaline in 4 repetitive doses with total 4 mg. Blood samples were collected at baseline and during 6 h after the first inhalations. Urine samples were collected at baseline and during 12 h after the first inhalations. Median (IQR) urine concentrations peaked in the period 0-4 h after inhalation with Cmax 472 (324) ng/mL in asthmatics and 661 (517) ng/mL in healthy subjects, and 4-8 h after oral use with Cmax 666 (877) ng/mL in asthmatic and 402 (663) ng/mL in healthy subjects. In conclusion it was found no significant differences in urine and serum concentrations between asthmatic and healthy subjects. It was compared urine and serum concentrations after therapeutic inhaled doses and supratherapeutic oral doses and observed significant statistical differences in both groups but found it impossible to distinguish between therapeutic and prohibited use based on doping tests with urine and blood samples.

One study aimed to investigate the effects on a possible improvement in aerobic and anaerobic performance of oral terbutaline (TER) at a supra-therapeutic dose in 7 healthy competitive male athletes. On day 1, ventilatory threshold, maximum oxygen uptake and corresponding power output were measured and used to determine the exercise load on days 2 and 3. On days 2 and 3, 8 mg of TER or placebo were orally administered in a double-blind process to athletes who rested for 3 h, and then performed a battery of tests including a force-velocity exercise test, running sprint and a maximal endurance cycling test at delta 50 percent. Lactatemia, anaerobic parameters and endurance performance were raised during the corresponding tests. It was found that TER administration did not improve any of the parameters of aerobic performance. In addition, no change in kinetic parameters was found with TER compared to placebo. Moreover, no enhancement of the force-velocity relationship was observed during sprint exercises after TER intake and, on the contrary, maximal strength decreased significantly after TER intake but maximal power remained unchanged. In conclusion, oral acute administration of TER at a supra-therapeutic dose seems to be without any relevant ergogenic effect on anaerobic and aerobic performances in healthy athletes. However, all participants experienced adverse side effects such as tremors.

Repeated injury of the airway epithelium caused by hyperpnoea of poorly conditioned air has been proposed as a key factor in the pathogenesis of exercise-induced bronchoconstriction (EIB) in athletes. In animals, the short-acting beta2-agonist terbutaline has been shown to reduce dry airflow-induced bronchoconstriction and the associated shedding of airway epithelial cells. Our aim was to test the efficacy of inhaled terbutaline in attenuating hyperpnoea-induced bronchoconstriction and airway epithelial injury in athletes. Twenty-seven athletes with EIB participated in a randomized, double-blind, placebo-controlled,
crossover study. Athletes completed an 8-min eucapnic voluntary hyperpnoea (EVH) test with dry air on two separate days 15 min after inhaling 0.5 mg terbutaline or a matching placebo. Forced expiratory volume in 1 s (FEV1) and urinary concentration of the club cell (Clara cell) protein 16 (CC16, a marker of airway epithelial perturbation) were measured before and up to 60 min after EVH. The maximum fall in FEV1 of 17 ± 8 percent on placebo was reduced to 8 ± 5 percent following terbutaline. Terbutaline gave bronchoprotection (i.e. post-EVH FEV1 fall <10%) to 22 (81 %) athletes. EVH caused an increase in urinary excretion of CC16 in both conditions, and terbutaline significantly reduced this rise (pre-to postchallenge CC16 increase 416 ± 495 pg/micromol creatinine after placebo versus 315 ± 523 pg/micromol creatinine after terbutaline). These results suggest that the inhalation of a single therapeutic dose of terbutaline offers significant protection against hyperpnoea-induced bronchoconstriction and attenuates acute airway epithelial perturbation in athletes [13415].

Using an analytical set-up with modified internal standard, a study allowing to assess the possible differentiation of inhaled versus orally administered terbutaline was conducted with healthy (10 male volunteers) as well as asthmatic (10 male volunteers) individuals. While the expected differences in urinary concentrations were observed in both groups following inhalation and oral application of 2-4 mg and 10 mg, respectively, no significant difference was found between the healthy and asthmatic persons. Moreover it remained impossible to adequately distinguish between therapeutic and non-therapeutic use of the beta2-agonist [13009].

**Urine and serum concentrations of inhaled and oral terbutaline**

It was examined urine and serum concentrations after therapeutic use of single and repetitive doses of inhaled and supratherapeutic oral use of terbutaline. It was compared the concentrations in 10 asthmatics and 10 healthy subjects in an open-label, cross-over study with 2 mg inhaled and 10 mg oral terbutaline on 2 study days. Further, 10 healthy subjects were administrated 1 mg inhaled terbutaline in 4 repetitive doses with total 4 mg. Blood samples were collected at baseline and during 6 h after the first inhalations. Urine samples were collected at baseline and during 12 h after the first inhalations. Median (IQR) urine concentrations peaked in the period 0-4 h after inhalation with Cmax 472 (324) ng/mL in asthmatics and 661 (517) ng/mL in healthy subjects, and 4-8 h after oral use with Cmax 666 (877) ng/mL in asthmatic and 402 (663) ng/mL in healthy subjects. In conclusion we found no significant differences in urine and serum concentrations between asthmatic and healthy subjects. It was compared urine and serum concentrations after therapeutic inhaled doses and supratherapeutic oral doses and observed significant statistical differences in both groups but found it impossible to distinguish between therapeutic and prohibited use based on doping tests with urine and blood samples [12314].

**Formoterol**

It was examined urinary and serum concentrations of formoterol in asthmatic and healthy individuals after a single dose of 18 µg inhaled formoterol and after repeated inhaled doses in healthy individuals. Results were evaluated with the WADA 2012 threshold for formoterol. On the day of this open-label, crossover study, 10 asthmatic subjects who regularly used beta2-agonists and 10 healthy participants with no previous use of beta2-agonists received a single dose of 18 µg formoterol. Further, 10 non-asthmatic participants inhaled 18 µg formoterol every second hour until obtaining a total of 72 µg, which is twice the maximum daily dose (36 microg formoterol) permitted by the World Anti-Doping Agency (WADA). Blood
samples were collected at baseline, 30 min, 1, 2, 3, 4, and 6 hours after the first inhalation. Urine samples were collected at baseline, 0-4, 4-8, and 8-12 hours after the first inhalation. Median urine concentration, corrected for specific gravity, after the single-dose administration peaked during 0-4 hours after inhalation at a maximum of 7.4 ng/mL in asthmatic subjects and 7.9 ng/mL in healthy subjects. Median urine concentration after repeated doses peaked during 4-8 hours after inhalation of a total of 72 microg formoterol at a maximum of 16.8 ng/mL in healthy participants. The maximal individual concentration of 25.6 ng/mL was found after inhalation of a total of 72 microg formoterol. It was thus not found any significant differences in urinary and serum concentrations of formoterol between asthmatic and healthy subjects. It was found high inter-individual variability in the concentrations in all groups. The data support the WADA 2012 urinary threshold of 30 ng/mL formoterol as being an adverse analytical finding [12511].

Formoterol is a frequently prescribed beta2-agonist used for the treatment of asthma. Due to performance-enhancing effects of some beta2-agonists, formoterol appears on the prohibited list, published by the World Anti-doping Agency (WADA). Its therapeutic use is allowed but restricted to inhalation. Since the data on urinary concentrations originating from therapeutic use is limited, no discrimination can be made between use and misuse when a routine sample is found to contain formoterol. Therefore the urinary excretion of six volunteers after inhalation of 18 microg of formoterol was investigated. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the quantification of formoterol in urine samples. Sample preparation consists of an enzymatic hydrolysis of the urine samples, followed by a liquid-liquid extraction at pH 9.5 with diethyl ether/isopropanol (5/1, v/v). Analysis was performed using selected reaction monitoring after electrospray ionization. The method was linear in the range of 0.5-50 ng/mL. The limit of quantification (LOQ) was 0.5 ng/ml. The bias ranged between -1.0 and -6.8 percent. Results for the urinary excretion show that formoterol could be detected for 72 h. The maximum urinary concentration detected was 8.5 ng/ml without and 11.4 ng/ml after enzymatic hydrolysis. Cumulative data showed that maximum 12 and 23 percent of the administered dose is excreted as parent drug within the first 12 h, respectively, non-conjugated and conjugated. Analysis of 82 routine doping samples, declared positive for formoterol during routine analysis, did not exhibit concentrations which could be attributed to misuse [12315].

Formoterol is a long-acting beta2-adrenoceptor agonist used for the treatment of bronchial asthma, prevention of exercise-induced bronchospasm, and for chronic obstructive pulmonary disease (COPD). It is formulated as a fumarate salt, and consists of a racemic mixture of two enantiomers. It is normally administered by inhalation either alone or in combination with a glucocorticosteroid. Formoterol has an extended duration of action (up to 12 h) compared to short-acting beta2 agonists such as salbutamol. In addition to the desired pharmacological action, there is some evidence indicating that systemic beta2-agonists may have a positive effect on physical performance in healthy subjects and, for this reason, its use in sports is restricted by the World Anti-Doping Agency (WADA). All beta2-agonists are prohibited with the exception of salbutamol, formoterol, and salmeterol when taken by inhalation in accordance to the manufacturer's recommended therapeutic regime. Recently, a threshold concentration of 30 ng/mL was defined by the World Anti-Doping Agency (WADA) to distinguish between therapeutic and forbidden use of formoterol. The objective of one work was to evaluate that threshold concentration. Concentrations of formoterol were measured in urine samples collected after administration of 18 microg of inhaled formoterol to five healthy volunteers, and in samples collected in routine doping tests belonging to athletes having declared inhaled formoterol use. Formoterol was detected up to 8 h after administration in all volunteers with concentrations up to 19.6 ng/mL. From 28 routine samples, 27 had less than 10 ng/mL of formoterol and only in one of the samples the concentration was 25 ng/mL. Therefore, administration of formoterol by inhalation at the
maximum dose allowed by WADA will not produce false positive results using a threshold concentration of 30 ng/mL, and the experience up to now in routine doping tests indicates that the probability of obtaining urines with concentrations greater than 30 ng/mL is close to nil. For this reason, sports authorities should re-evaluate the need of a threshold concentration for formoterol and its practical usefulness [13416].

In order to provide a means to differentiate a potential misuse of beta2-agonists from therapeutic usage, two studies focusing on either inhaled formoterol (18 microg) or inhaled salmeterol (100 microg) were presented. Demonstrating limits of quantification (LOQs) at sub-ng/mL concentrations in urine, both analytes were sensitively determined in administration study urine samples and authentic doping control specimens. In case of formoterol peak values of 11.4 ng/mL were observed while salmeterol did not exceed 1.3 ng/mL in elimination studies; doping control specimens concentrations below 30 ng/mL and 2 ng/mL prevailed for formoterol and salmeterol, respectively [13012].

Since 1992, formoterol is included in the prohibited list of doping substances and methods, presently reviewed and updated by the World Anti-Doping Agency. Recently a threshold value of 40 ng/mL has been established to differentiate between the prohibited (oral) and the permitted (inhalatory) administration of formoterol to athletes. This paper considers the urinary excretion profile of formoterol and its main metabolites after inhalation of different doses of two of the most used medicaments, available in Italy, containing formoterol fumarate bihydrate (12 and 36 microg twice a day of Foradil® or 9 and 27 microg twice a day of Symbicort®), focusing also on the effects, on the measured levels of formoterol, of potential alteration processes (thermal and/or microbiological) that may take place after the collection of the urine samples. Urine sample preparation included an enzymatic hydrolysis and a dilution step. Detection of analytes was performed by a newly developed and validated direct LC-ESI-MS/MS procedure, using a triple quadrupole mass spectrometer under positive ion electro-spray ionization conditions and selected reaction monitoring acquisition mode. The results showed the capability and suitability of the direct LC-ESI-MS/MS analysis for the quantitative confirmation analysis of formoterol in urine samples. The data from the analysis of the urine samples obtained in the excretion studies showed that formoterol is excreted mainly as unmodified drug and to a lesser degree as O-demethylated metabolite. The urinary levels of formoterol (40-60 %) and its metabolites (O-demethylated metabolite 5-25 %; glucuronide metabolites 25-40%) vary significantly depending both on the administered drug formulation and the subject tested. The maximum urinary concentration reached in this study was 15 ng/mL (free+glucuronide), that is significantly lower than the threshold value fixed to report an adverse analytical finding. Finally, the results also showed that formoterol is stable for at least 4 weeks in urine samples correctly collected and stored [13417].

The use of formoterol in sports is allowed by inhalation at the maximum recommended therapeutic dose. Recently, a threshold concentration of 30 ng/mL was defined by the World Anti-Doping Agency (WADA) to distinguish between therapeutic and forbidden use of formoterol. The objective of this work was to evaluate that threshold concentration. Concentrations of formoterol were measured in urine samples collected after administration of 18 microg of inhaled formoterol to five healthy volunteers, and in samples collected in routine doping tests belonging to athletes having declared inhaled formoterol use. Formoterol was detected up to 8 h after administration in all volunteers with concentrations up to 19.6 ng/mL. From 28 routine samples, 27 had less than 10 ng/mL of formoterol and only in one of the samples the concentration was 25 ng/mL. Therefore, administration of formoterol by inhalation at the maximum dose allowed by WADA will not produce false positive results using a threshold concentration of 30 ng/mL, and the experience up to now in routine doping tests indicates that the probability of obtaining urines with concentrations greater than 30
ng/mL is close to nil. For this reason, sports authorities should re-evaluate the need of a threshold concentration for formoterol and its practical usefulness [13418].

It was examined urinary and serum concentrations of formoterol in asthmatic and healthy individuals after a single dose of 18 microg inhaled formoterol and after repeated inhaled doses in healthy individuals. Results were evaluated using the World Anti-Doping Agency (WADA) 2012 threshold for formoterol. On the day of an open-label, crossover study, 10 asthmatic subjects who regularly used beta2-agonists and 10 healthy participants with no previous use of beta2-agonists received a single dose of 18 microg formoterol. Further, 10 nonasthmatic participants inhaled 18 microg formoterol every second hour until obtaining a total of 72 microg, which is twice the maximum daily dose (36 microg formoterol) permitted by the WADA. Blood samples were collected at baseline, 30 min, 1, 2, 3, 4, and 6 h after the first inhalation. Urine samples were collected at baseline, 0-4, 4-8, and 8-12 h after the first inhalation. Median urine concentration, corrected for specific gravity, after the single-dose administration peaked during 0-4 h after inhalation at a maximum of 7.4 ng/mL in asthmatic subjects and 7.9 ng/mL in healthy subjects. Median urine concentration after repeated doses peaked during 4-8 h after inhalation of a total of 72 microg formoterol at a maximum of 16.8 ng/mL in healthy participants. The maximum individual concentration of 25.6 ng/mL was found after inhalation of a total of 72 microg formoterol. It was found no significant differences in urinary and serum concentrations of formoterol between asthmatic and healthy subjects. It was found high interindividual variability in the concentrations in all groups. The data support the WADA 2012 urinary threshold of 30 ng/mL formoterol as being an adverse analytical finding [13419].

Formoterol is a new threshold substance in the prohibited list 2012 according to World Anti-Doping Agency. Extracted by ethyl acetate using formoterol-D6 as internal standard, formoterol underwent a constant flow rate gradient elution separation in reversed-phase liquid chromatography. Subsequently, mass spectrometry analysis was conducted by tandem mass spectrometry in the multiple reaction monitoring mode. According to the proposed method, a calibration curve was constructed in the range of 0.2-500 ng/mL with a limit of quantification 0.2 ng/mL. The mean extracted recovery assessed at three different concentrations (1, 30 and 100 ng/mL) was more than 80 percent. The method was validated by the analysis of three quality control samples from World Association of Anti-Doping Scientists. In conclusion, the developed and validated method was sensitive, accurate and precise for the quantification of formoterol in human urine for doping control purposes [13420].

Since 1992, formoterol is included in the prohibited list of doping substances and methods, presently reviewed and updated by the World Anti-Doping Agency. Recently a threshold value of 40 ng/mL has been established to differentiate between the prohibited (oral) and the permitted (inhalatory) administration of formoterol to athletes. One paper considers the urinary excretion profile of formoterol and its main metabolites after inhalation of different doses of two of the most used medicaments, available in Italy, containing formoterol fumarate bihydrate (12 and 36 microg twice a day of Foradil® or 9 and 27 microg twice a day of Symbicort®), focusing also on the effects, on the measured levels of formoterol, of potential alteration processes (thermal and/or microbiological) that may take place after the collection of the urine samples. Urine sample preparation included an enzymatic hydrolysis and a dilution step. Detection of analytes was performed by a newly developed and validated direct LC-ESI-MS/MS procedure, using a triple quadrupole mass spectrometer under positive ion electro-spray ionization conditions and selected reaction monitoring acquisition mode. The results showed the capability and suitability of the direct LC-ESI-MS/MS analysis for the quantitative confirmation analysis of formoterol in urine samples. The data from the analysis of the urine samples obtained in the excretion studies showed that formoterol is excreted
mainly as unmodified drug and to a lesser degree as O-demethylated metabolite. The urinary levels of formoterol (40-60 %) and its metabolites (O-demethylated metabolite 5-25 %; glucuronide metabolites 25-40 %) vary significantly depending both on the administered drug formulation and the subject tested. The maximum urinary concentration reached in this study was 15ng/mL (free + glucuronide), that is significantly lower than the threshold value fixed to report an adverse analytical finding. Finally, the results also showed that formoterol is stable for at least 4 weeks in urine samples correctly collected and stored [13421].

To the permitted routes and daily dosages of salbutamol and formoterol, urinary threshold levels have been enforced. For formoterol, this threshold was increased from 30 ng/mL to 40 ng/mL as of January 2013, and various studies were conducted to demonstrate the capability of doping control analytical assays to quantify the target analyte in sports drug testing samples as well as to probe for the rationale of the 2012 and 2013 thresholds for urinary formoterol [13009].

**Procaterol**

While the use of oral beta₂-agonists by athletes is prohibited because of their anabolic effects, some inhaled beta₂-agonists can be used in accordance with the World Anti-Doping Agency regulations. It was examined the dose disparity between the bronchodilating effect and anabolic effect of inhaled procaterol, a beta₂-agonists, to determine if the drug might be effective for athletes with asthma. Intact rats were given nebulized procaterol at 0.001, 0.01, 0.1 and 1 mg/mL by inhalation, and its inhibitory effect on carbachol-induced bronchoconstriction was evaluated. Castrated rats were given nebulized procaterol at 0.03, 0.1, 0.3 and 1 mg/mL by inhalation 3 times a day for 14 days, and anabolic markers (body weight gain, weight of the levator ani muscle and gastrocnemius muscle) were measured. At 0.01 mg/mL and higher, procaterol dose-dependently inhibited carbachol-induced bronchoconstriction with a significant effect. At doses of up to 0.3 mg/mL, there were no signs indicating an anabolic effect of procaterol. At 1 mg/mL, however, a slight but statistically significant increase in the weight of the levator ani muscle was observed with no significant changes in other anabolic markers. It was suggested that inhaled procaterol might be useful for athletes with asthma because of the big dose disparity between its bronchodilating effect and anabolic effect in rats [08350].

**Clenbuterol**

Rapid screening of clenbuterol in urine was performed by combining desorption electrospray ionization (DESI) and tandem mass spectrometry (MS/MS). Optimization experiments were carried out including the selection of substrates, spray solutions, nebulizing gas pressures, high-voltage power supplies and flow rates of spray solution. The limit of detection (LOD), defined as the lowest quantity that can be detected, was 5.0 pg for the pure compound. Using DESI coupled with solid-phase extraction (SPE), the linear response range was from 10 to 400 ng/mL and the concentration LOD for urine sample was 2.0 ng/mL. The analysis for one spiked urine sample was achieved within 4 min. In addition to the fast analysis speed, MS/MS provided structural information for the confirmation of clenbuterol. Urine samples from different people were investigated and the recoveries were within 100 ± 20 percent [08351].

Beta-agonists such as ractopamine (RAC) and clenbuterol (CLEN), have similar effects as anabolic steroids i.e. they promote growth of muscular tissue and reduce body fat. They have
been used successfully with animals and humans but have also been banned in many countries principally, because of their serious side effects. However, their illegal use persists. Thus, their interaction with biomolecules such as bovine serum albumin (BSA) is of significance, especially the co-operative reaction of mixed ligands with the protein. Fluorescence and UV-vis spectra of complex mixtures of individual ligands, binary and ternary complexes with BSA resulted in significantly overlapping spectral profiles. Qualitative and quantitative information about the various complex ligand-protein species formed, was obtained with the resolution of the excitation-emission fluorescence three-way data matrices by chemometrics methods-MCR-ALS and PARAFAC. Individual spectra of the ligands, their binary complexes with BSA and their ternary complexes were extracted, and quantitative concentration profiles for each species in a particular interaction were constructed. Such analyses made it possible to interpret the role and behaviour of each reaction component. It was found that both ligands, RAC and CLEN, bound co-operatively in site I of the BSA. This was confirmed with the use of site markers such as warfarin (site I) and ibuprofen (site II). However, CLEN formed a 1:1 CLEN-BSA complex, while RAC formed a 2:1 RAC(2)-BSA binary species. Interestingly, when CLEN or RAC was added to RAC(2)-BSA or CLEN-BSA, respectively, ternary complexes were produced such as RAC(2)-BSA-CLEN. Significantly, the presence of the second ligand in such an interaction in excess, appeared to increase the affinity of the added ligand for BSA. This may have consequences on the amount of steroid required to achieve a desired tissue growth effect [10375].

The beta-adrenergic signaling pathway represents a novel therapeutic target for skeletal muscle wasting and weakness due to its role in the mechanisms controlling protein synthesis and degradation and in modulating fiber type. Stimulation of the pathway with beta-adrenoceptor agonists (beta-agonists) has therapeutic potential for muscle wasting disorders including: sarcopenia, cancer cachexia, disuse and inactivity, unloading or microgravity, sepsis and other metabolic disorders, denervation, burns, HIV-AIDS, chronic kidney or heart failure, and neuromuscular diseases. However, there are also pitfalls associated with beta-agonist administration and clinical applications have so far been limited, largely because of cardiovascular side effects. In rats and mice, newer generation beta-agonists (such as formoterol) can elicit an anabolic response in skeletal muscle even at very low doses, with reduced effects on the heart and cardiovascular system compared with older generation beta-agonists (such as fenoterol and clenbuterol). However, the potentially deleterious cardiovascular side effects of beta-agonists have not been obviated completely and so it is important to refine their development and therapeutic approach in order to overcome these obstacles. A review describes the therapeutic potential of stimulating the beta-adrenergic signaling pathway with beta-agonists, highlighting the beneficial effects on skeletal muscle structure and function and identifying some of the pitfalls associated with short- and long-term beta-agonist administration [08352].

The beta2-agonist clenbuterol [4-amino-alpha(t-buty-l-amino)methyl-3,5-dichlorobenzyl alcohol] is used as a non-steroidal anabolic drug for sports doping. The effects of clenbuterol on the transcriptional process and mRNA stability of beta-adrenoceptor in skeletal and cardiac muscles are still unknown. Therefore, it was investigated the effects of clenbuterol on beta1- and beta2-adrenoreceptor mRNA expressions of fast-twitch fiber-rich extensor digitorum longus, slow-twitch fiber-rich soleus, and left ventricle muscles by real-time RT-PCR. Adult male Sprague Dawley rats were divided into the clenbuterol-administered group and control group. The administration (1.0 mg/kg body weight/day, subcutaneously) of clenbuterol was maintained for 10 days. The administration of clenbuterol significantly increased the weight, RNA concentration, and total RNA content of extensor digitorum longus muscle. No effects of clenbuterol on those of soleus and left ventricle muscles, however, were observed. The administration of clenbuterol significantly decreased beta1-AR mRNA expression of left ventricle muscle. Furthermore, the administration of clenbuterol
significantly decreased beta2-AR mRNA expression of extensor digitorum longus and left ventricle muscles. No effect of clenbuterol on beta2-AR mRNA expression of soleus muscle, however, was observed. These results suggest that the effects of clenbuterol on beta1- and beta2-AR mRNA expressions and muscle hypertrophy depend on muscle fiber types [08353].

The aim of one study was to evaluate the effect of joint immobilization on morphometric parameters and glycogen content of soleus muscle treated with clenbuterol in male Wistar (3-4 months old) rats. The clenbuterol group showed an increase in glycogen without alteration in weight, cross-sectional area or connective tissue compared with the control group. The immobilized group showed a reduction in muscle weight, glycogen content, and cross-sectional area, and an increase in connective tissue. However, the immobilized + clenbuterol group showed an increase in weight, glycogen, and cross-sectional area, and a reduction in connective tissue. This study emphasizes the importance of anabolic pharmacological protection during immobilization to minimize skeletal muscle alterations resulting from disuse [09246].

Enantiomers of clenbuterol were separated by a new HPLC method on a chiral column. Enantiomeric resolution was achieved on a vancomycyin macrocyclic antibiotic chiral stationary phase known as chirobiotic V with UV detection at 247 nm. The polar ionic mobile phase consisting of methanol-triethylamine-glacial acetic acid was used at a flow rate of 1.0 mL/min. The method was validated for linearity, accuracy, precision, and robustness. Standard linear calibration curves were established for the R-(-) and S-(+) enantiomers over the range of 0.2-20 microg/mL, and an average recovery of 98 percent and a mean relative standard deviation of 1.5 percent were obtained at 5.0 microg/mL. The lower limit of detection was 0.05 microg/mL for each enantiomer. The mean recovery for R-(-) and S-(+) clenbuterol enantiomers from plasma was 91.0-97.0% at 0.20-20 microg/mL. The method was successfully used to identify and quantify the clenbuterol enantiomers in human plasma [09247].

The aim of one study was to determine the level of clenbuterol residues in muscle tissue of pigs after repeat administration in a growth-promoting dose. An anabolic dose of clenbuterol (20 mug/kg body mass per day) was administered orally to experimental group (n=12) for 28 days, whereas control animals (n=3) were left untreated. Clenbuterol treated pigs were randomly sacrificed (n=3) on days 0, 3, 7 and 14 of treatment discontinuation and clenbuterol residues determined in muscle tissue. Determination of residual clenbuterol was by enzyme-linked immunosorbent assay (ELISA) as a screening method and liquid chromatography tandem mass spectrometry (LC-MS/MS) as a confirmation method. The highest clenbuterol content in the muscle of treated animals was recorded on day 0 of treatment cessation and significantly exceeded the maximum residue limit (MRL) of 0.1 ng/g. On day 3 of withdrawal, it was 0.49 ng/g and on day 7 0.1 (at MRL); on day 14 of treatment discontinuation, clenbuterol content was below the limit of detection (<0.1ng/g) in all samples. Administration of clenbuterol as a growth promoter in pig production could lead to residues in meat for human consumption up to 7 days after treatment discontinuation [10170].

In one report, it was presented a novel approach to detect clenbuterol based on competitive surface-enhanced Raman scattering (SERS) immunoassay. Herein, a SERS nanoprobe that relies on gold nanoparticle (GNP) is labeled by 4,4'-dipyridyl (DP) and clenbuterol antibody, respectively. The detection of clenbuterol is carried out by competitive binding between free clenbuterol and clenbuterol-BSA fastened on the substrate with their antibody labeled on SERS nanoprobe. The present method allows us to detect clenbuterol over a much wider concentration range (0.1-100 pg/mL) with a lower limit of detection (about 0.1 pg/mL) than the conventional methods. Furthermore, by the use of this new competitive SERS immunoassay, the clenbuterol-BSA (antigen) is chosen to fasten on the substrate instead of
the clenbuterol antibody, which could reduce the cost of the assay. Results demonstrate that the proposed method has the wide potential applications in food safety and agonist control.

beta-Adrenoceptor agonists are reported to induce skeletal muscle hypertrophy and hence serve as valuable adjunct to the treatment of wasting disorders. In the present study, we attempted to find out whether metabolic and physiologic characteristics of fibres are important in determining skeletal muscle response to clenbuterol (an adrenergic receptor agonist) therapy, as proposed in the treatment of wasting disorders. The treatment of mice with clenbuterol (2 mg/kg body wt for 30 days) resulted in skeletal muscle hypertrophy, more common amongst fast-twitch glycolytic fibres/muscle, with increase in body mass and a parallel rise in muscle mass to body mass ratio. Measurement of fibre diameters in soleus (rich in slow-twitch oxidative fibres), ALD or anterior latissimus dorsi (with a predominance of fast-twitch glycolytic fibres) and gastrocnemius (a mixed-type of muscle) from clenbuterol-treated mice for 30 days revealed noticeable increase in the per cent population of narrow slow-twitch fibre and a corresponding decline in white-type or fast-twitch glycolytic fibres in gastrocnemius and ALD. As revealed by counting of muscle cells in soleus, narrow red fibres declined with corresponding increase in white-type glycolytic fibres population. A significant decline in the succinic dehydrogenase activity was observed, thereby suggesting abnormality in oxidative activity of skeletal muscles in response to clenbuterol therapy.

**Induction of IGF and myostatin**

Clenbuterol, a beta₂-adrenergic agonist, increases the hypertrophy of skeletal muscle. Insulin-like growth factor (IGF) is reported to work as a potent positive regulator in the clenbuterol-induced hypertrophy of skeletal muscles. However, the precise regulatory mechanism for the hypertrophy of skeletal muscle induced by clenbuterol is unknown. Myostatin, a member of the TGFβ super family, is a negative regulator of muscle growth. The aim of the present study is to elucidate the function of myostatin and IGF in the hypertrophy of rat masseter muscle induced by clenbuterol. To investigate the function of myostatin and IGF in regulatory mechanism for the clenbuterol-induced hypertrophy of skeletal muscles, we analysed the expression of myostatin and phosphorylation levels of myostatin and IGF signaling components in the masseter muscle of rat to which clenbuterol was orally administered for 21 days. Hypertrophy of the rat masseter muscle was induced between 3 and 14 days of oral administration of clenbuterol and was terminated at 21 days. The expression of myostatin and the phosphorylation of smad2/3 were elevated at 21 days. The phosphorylation of IGF receptor 1 (IGFR1) and akt1 was elevated at 3 and 7 days. These results suggest that myostatin functions as a negative regulator in the later stages in the hypertrophy of rat masseter muscle induced by clenbuterol, whereas IGF works as a positive regulator in the earlier stages.

**Clenbuterol in food**

The therapeutic agent clenbuterol [(RS)-1-(4-Amino-3,5-dichlorphenyl)-2-(tert-butylamino)ethanol] is an approved bronchodilator and tocolytic agent, which has been further investigated as a potential means in therapy concerning various different conditions including, for example, amyotrophic lateral sclerosis, muscle atrophy, Pompe disease, and heart failure. However, the particular growth-promoting properties of clenbuterol, whose mechanism(s) of action have recently been further clarified, have also been a temptation to abuse as proven, for example, in stock farming/meat production and elite sport, which resulted in the prohibition of the drug in animal husbandry and sport. The issue of illicit administration of clenbuterol to animals destined for food production has evidently resulted in
several cases and epidemics of poisoning, stressing particularly the corresponding health problems. In addition, the contamination of dietary products with clenbuterol can affect an athlete’s integrity and career when the drug is inadvertently ingested, resulting in an adverse analytical finding during doping controls. Despite the continuously improving analytical options and methods in sports drug testing programmes especially by means of liquid chromatography/(tandem) mass spectrometry [LC-MS/(MS)], the differentiation of therapeutic/deliberately administered clenbuterol from ingested unintentionally residues remains a complex challenge. Consequently, any adverse analytical finding (AAF) necessitates careful investigation and evaluation of circumstantial evidence that might prove the athlete’s innocence. In the present communication, the inquest into a food contamination issue concerning elite soccer players who competed in Mexico in 2011 is presented. Induced by five AAFs of the Mexican seniors’ national soccer team with clenbuterol in May of 2011, the Fédération Internationale de Football Association (FIFA) recognized the need for a thorough investigation of the circumstances of these and potential future anti-doping rule violations, particularly in the light of the FIFA U-17 World Cup 2011 to be held between 18 June and 10 July 2011 in Mexico. Hence, during the period of this U-17 competition, samples from meals containing meat were collected at team-hosting hotels concomitantly to doping control specimens routinely sampled at competition sites from randomly selected players. While urine samples were subjected to regular doping controls, meat specimens underwent dedicated clenbuterol analyses [13424].

The illicit use of growth promoters in animal husbandry has frequently been reported in the past. Among the drugs misused to illegally increase the benefit of stock farming, clenbuterol has held a unique position due to the substance’s composition, mechanism of action, metabolism, and disposition. Particularly clenbuterol’s disposition in animals’ edible tissues destined for food production can cause considerable issues on consumption by elite athletes registered in national and international doping control systems as demonstrated in this case-related study. Triggered by five adverse analytical findings with clenbuterol among the Mexican national soccer team in out-of-competition controls in May 2011, the Fédération Internationale de Football Association (FIFA) initiated an inquest into a potential food contamination (and thus sports drug testing) problem in Mexico, the host country of the FIFA U-17 World Cup 2011. Besides 208 regular doping control samples, which were subjected to highly sensitive mass spectrometric test methods for anabolic agents, 47 meat samples were collected in team hotels during the period of the tournament and forwarded to Institute of Food Safety. In 14 out of 47 meat samples (30 %), clenbuterol was detected at concentrations between 0.06 and 11 microg/kg. A total of 109 urine samples out of 208 doping control specimens (52 %) yielded clenbuterol findings at concentrations ranging from 1–1556 pg/ml, and only 5 out of 24 teams provided urine samples that did not contain clenbuterol. At least one of these teams was on a strict “no-meat” diet reportedly due to the known issue of clenbuterol contamination in Mexico. Eventually, owing to the extensive evidence indicating meat contamination as the most plausible reason for the extraordinary high prevalence of clenbuterol findings, none of the soccer players were sanctioned. However, elite athletes have to face severe consequences when testing positive for a prohibited anabolic agent and sufficient supporting information corroborating the scenario of inadvertent ingestion are required to be acquitted from anti-doping rule violations. Hence, governmental contribution is urgently needed to combat the illegal use of clenbuterol in stock breeding [13424].

_Clenbuterol in hair_

The illegal use of clenbuterol has been an increasingly serious issue in today's livestock products industry. It becomes an important project to develop a reliable approach to detect its content in food animals. A simple and sensitive LC-MS/MS method was developed to
detect clenbuterol residue in hair, with the low limit of quantitation (LLOQ) about 0.5 ng/g. Hogs fed with 340 microg/day of clenbuterol for 2 weeks were found a high clenbuterol residue in their hair approximately at 1-2 months after withdrawal. There remained 3.31 ng/g clenbuterol in hog hair approximately 5 months after the last administration, focused on the tip of the hair (mainly in hogs with dark hair). An extensive contamination was observed in twenty investigated market hogs whose dark hair obviously had a higher clenbuterol residue than the light ones. Volunteers (60%) from Xuhui district (Shanghai) were found to have a detectable amount of clenbuterol in their hair (>0.5 ng/g). In conclusion, hair residue detection is a reliable method to evaluate the clenbuterol contamination in animals and humans. Meat supply in the Xuhui district might have serious potential safety risks which should be further investigated and discussed to determine the safety range of clenbuterol residue [13425].

Clenbuterol and heart problems

Clenbuterol is an orally administered long-acting beta-2 adrenergic agonist closely related to albuterol that, in recent years, has become a substance of abuse in the bodybuilding and athletic community. It was repored two cases of acute myocardial ischemia associated with clenbuterol abuse in two healthy young male body builders. Two male bodybuilders, ages 18 and 22 years, presented to the Emergency Department with palpitations, nausea, vomiting, chest pain, diaphoresis, and tachycardia shortly after ingesting clenbuterol. Both patients experienced prolonged sinus tachycardia that, in one patient, was relatively resistant to both beta- and calcium channel blockade. Both patients were found to have elevated troponin levels, the first patient as high as 4.71 ng/mL (normal <0.04 ng/mL). Further investigation revealed normal coronary arteries at catheterization and normal cardiac magnetic resonance imaging in the first patient, and normal echocardiograms for both patients. The tachycardia gradually resolved and both patients recovered uneventfully. The etiology of cardiac ischemia in these patients is uncertain. Thus, emergency physicians should be aware of the clinical presentation of clenbuterol abuse and overdose, and the possibility of related cardiac ischemia and rhythm disturbances. Suggested treatment includes intravenous fluids, oxygen, aspirin, beta-blockers, and benzodiazepines, although efficacy remains unproven [13426].

A novel, sensitive, and reliable LC-MS/MS method for multiresidue analysis of nine beta-agonists (salbutamol, terbutaline, cimaterol, fenoterol, clorprenaline, ractopamine, tulobuterol, clenbuterol, and penbuterol) in four farm animal muscles was developed. Muscle matrix was extracted with acetonitrile-10 percent sodium carbonate solution, and then was subjected to cleanup using a SPE cartridge packed with new polymer synthesized in acetone. Chromatographic separation of the components was performed on a Luna C18 column using 0.1 percent of formic acid in water and acetonitrile. The mass spectrometer was operated in the positive electrospray mode. Good precision and accuracy were obtained for all analytes (except for fenoterol) at the spiked three levels of 1.0, 10, and 50 microg/kg. The decision limit and detection capability of nine beta-agonists ranged from 0.04 to 0.18 and 0.15 to 0.69 microg/kg, respectively. The method developed was successfully applied to the monitoring of nine beta-agonists in pork, beef, mutton, and chicken from Chinese markets [13427].

Clenbuterol is a beta2-agonist approved in the United States for veterinary use in nonfood animals. Clenbuterol use is emerging among bodybuilders and fitness enthusiasts attracted to the hypertrophic and lipolytic effects. It was presented a retrospective chart review of clenbuterol exposures reported to 2 poison control centers. Misuse of clenbuterol for weight loss and bodybuilding was reported in 11 of 13 clenbuterol users. Reported clinical effects included tachycardia, widened pulse pressure, tachypnea, hypokalemia, hyperglycemia, ST changes on electrocardiogram (ECG), elevated troponin, elevated creatine phosphokinase
(CPK), palpitations, chest pain, and tremor. Measured serum clenbuterol concentration was 2983 pg/mL post 4.5 mg ingestion. Co-ingestants included T3 and anabolic steroids. Treatments included activated charcoal, benzodiazepines, beta-blockers, potassium replacement, and intravenous (IV) fluid. There is an increasing use of the Internet for illicit drug use for bodybuilding and weight loss purposes. These patients may not present as the stereotype of illicit drug abusers, but as healthy athletic low-risk patients. Clinical effects persisted greater than 24 hours with evidence of myocardial injury in 2 patients. Clenbuterol is increasingly being abused within the bodybuilding subculture. These cases illustrate the hidden dangers of clenbuterol abuse among bodybuilders and fitness enthusiasts [13428].

**Supression of bacterial phagocytosis**

Splenic marginal zone macrophages expressing macrophage receptor with collagenous structure (MARCO) contribute to the clearance of blood-borne pathogens. It was determined a splenic adherent cell fraction abundantly containing cells expressing a higher level of MARCO by flow cytometry, and examined the effects of daily administration of an anabolic dose of beta2-agonist clenbuterol on the phagocytic capacity of the cells in mice. After 6 weeks of clenbuterol (1.0 mg/kg body weight/d) or vehicle administration to the mice, splenic adherent cells were isolated. These cells were separated into three cell-size subpopulations. Among them, the small-cell subpopulation contained abundantly the cells with markedly higher levels of MARCO and exhibited more intense phagocytic capacity against Escherichia coli, as compared with the other subpopulations. The phagocytic capacity of the small cells was significantly reduced after clenbuterol administration. These results suggest that the utilization of clenbuterol as doping drug impairs bacterial clearance in the spleen [13434].

**Salbutamol and clenbuterol**

The aim of the study was to evaluate the adequacy of enzyme-linked immunosorbent assay (ELISA) in the post-exposure determination of the beta-agonists clenbuterol and salbutamol in animal plasma and serum. Experimental guinea pigs (n=20) were treated with two doses (0.25 and 2.5 mg/kg) of clenbuterol (n=10) and salbutamol (n = 10) for seven days, whereas the control animal group (n=10) was left untreated. Validation of the applied method yielded acceptable recovery (mean > 70 %) and repeatability rates, showing ELISA to be applicable for the semi-quantitative determination of both analytes in both matrices, preferably in plasma. In both matrices, clenbuterol concentrations were proven to be significantly (14-fold) higher than those of salbutamol. Concentrations of both analytes were higher in plasma than in serum. The application of a 10-fold higher clenbuterol and salbutamol dose (2.5 mg/kg) resulted in concentrations 3- to 4-fold higher for clenbuterol and 2- to 3-fold higher for salbutamol, indicating a different release rate of these two beta-agonists [13433].

**Laboratory technique**

An ultrasensitive electrochemiluminescence (ECL) immunosensor based on CdSe quantum dots (QDs) has been designed for the detection of clenbuterol. The immunosensor was fabricated by layer by layer and characterized with atomic force microscopic images (AFM) and electrochemical impedance spectra (EIS). In oxygen-saturated pH=9.0 Tris-HCl buffer, a strong ECL emission of QDs could be observed during the cathodic process due to the H2O2 product from electrochemical reduction of dissolved oxygen. Upon the formation of immunocomplex, the second antibody labeled with horseradish peroxidase was simply immobilized on the electrode surface. The ECL emission decreased since steric hindrance of the immunocomplex slowed down the electron-transfer speed of dissolved oxygen, and also could be greatly amplified by an enzymatic cycle to consume the self-produced coreactant.
Using clenbuterol as model analyte, the ECL intensity was determined by the concentration of competitive immunoassay of clenbuterol with a wide calibration in the range of 0.05 ng/mL to 1000 ng/mL, and a low detection limit was 0.02 ng/mL. The immunosensor shows good stability and fabrication reproducibility. It was applied to detecting practical samples with the satisfactory results. This immunosensing strategy opens a new avenue for detection of residue and application of QDs in ECL biosensing [13422].

Clenbuterol is a beta-2 agonist agent with anabolic properties given by the increase in the muscular mass in parallel to the decrease of the body fat. For this reason, the use of clenbuterol is forbidden by the World Anti-Doping Agency (WADA) in the practice of sport. This compound is of particular interest for anti-doping authorities and WADA-accredited laboratories due to the recent reporting of risk of unintentional doping following the eating of meat contaminated with traces of clenbuterol in some countries. In this work, the development and the validation of an ultra-high pressure liquid chromatography coupled to electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS/MS) method for the quantification of clenbuterol in human urine is described. The analyte was extracted from urine samples by liquid-liquid extraction (LLE) in basic conditions using tert butyl-methyl ether (TBME) and analyzed by UHPLC-MS/MS with a linear gradient of acetonitrile in 9 min only. The simple and rapid method presented here was validated in compliance with authority guidelines and showed a limit of quantification at 5 pg/mL and a linearity range from 5 pg/mL to 300 pg/mL. Good trueness (86-105 %), repeatability (5.7-10.6 % RSD) and intermediate precision (5.9-14.9 % RSD) results were obtained. The method was then applied to real samples from eighteen volunteers collecting urines after single oral doses administration (1, 5 and 10 μg) of clenbuterol-enriched yogurts [13423].

Detecting the misuse of clenbuterol in sports drug testing has been a challenge to anti-doping laboratories ever since the drug was banned in the early 1990s. Initially, meeting the minimum required performance levels (MRPLs) and detection limits required for efficient clenbuterol testing was a complex task due to the comparably poor GC-MS properties of the analyte. However, various strategies including derivatization, high resolution and/or tandem mass spectrometry allowed for the analysis at sub-ng/mL levels. Urine samples were enzymatically hydrolyzed, liquid-liquid extracted, and the residue was trimethylsilylated with in situ generated trimethylidodosilane to yield clenbuterol-bis-TMS. By means of chiral liquid chromatography and high resolution/high accuracy tandem mass spectrometry, doping control as well as elimination study urine samples containing clenbuterol were analyzed, demonstrating that such enantiomeric analyses can support the interpretation of clenbuterol findings; however, inconclusive results were also obtained, demonstrating the need for further research [13009].

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One study demonstrates the development of a gas chromatography-triple quadrupole tandem mass spectrometry (GC-MS-MS) assay to detect clenbuterol in human urine and the comparison of this method with GC-MS techniques and gas chromatography-high resolution mass spectrometry (GC-HRMS) techniques. Urine samples were hydrolyzed with β-glucuronidase, extracted with methyl tert-butyl ether and dried under nitrogen. The derivative reagent was N-methyl-N-(trimethylsilyl)-trifluoroacetamide with NH₄I and was analyzed by GC-MS, GC-MS-MS and GC-HRMS. A validation study was conducted by GC-MS-MS. The analyses of clenbuterol using different mass spectrometric techniques were compared. The limit of detection (LOD) for clenbuterol in human urine was 2 ng/mL by GC-MS (selected ion monitoring mode: SIM mode), 0.06 ng/mL by GC-HRMS and 0.03 ng/mL by GC-MS-MS, respectively, while the LOD by GC-HRMS was 0.06. With GC-MS-MS, the intra-assay and inter-assay precisions were less than 15%, the recoveries were 86 to 112% and the linear range was 0.06 to 8.0 ng/mL. The GC-MS under SIM mode can be used as a screening tool to detect clenbuterol at trace levels in human urine. The GC-MS-MS and GC-HRMS methods can confirm clenbuterol when its concentration is below 2 ng/mL. The results demonstrate that the GC-MS-MS method is quite sensitive, specific and reliable for the detection of clenbuterol in doping analysis [12318].

A simple and inexpensive pretreatment procedure was developed for 10 β-agonists (clenbuterol, ractopamine, salbutamol, bmbuterol, penbuterol, tulobuterol, clorprenaline, mabuterol, cimaterol and terbutaline) in swine urine using dispersive solid phase extraction (dSPE) with multi-walled carbon nanotubes (MWCNTs). The sample was analysed after purification by ultra high performance liquid chromatography-positive electrospray ionisation tandem mass spectrometry (UHPLC-ESI-MS/MS). The pH value of the swine urine, extraction time, type and amount of MWCNTs and type of eluent were optimised to increase the sample throughput and sensitivity. The samples were quantified using clenbuterol-D9, ractopamine-D6 and salbutamol-D3 as internal standards. The recoveries of the target compounds from swine urine samples at pH 10.0 were most efficient when using 20 mg of MWCNTs with a 30-50 nm outer diameter and a length of 10-30 μm, while a mixture of water/methanol (90:10, v/v) with 0.5 beta formic acid was shown to be the most suitable solvent for desorbing the compounds from the MWCNTs. The proposed method was validated according to the European Commission Decision 2002/657/EC, which determines linearity, specificity, decision limit (CCα), detection capability (CCβ), recovery, precision and stability [12319].

One study demonstrates the development of a gas chromatography-triple quadrupole tandem mass spectrometry (GC-MS-MS) assay to detect clenbuterol in human urine and the comparison of this method with GC-MS techniques and gas chromatography-high resolution mass spectrometry (GC-HRMS) techniques. Urine samples were hydrolyzed with β-glucuronidase, extracted with methyl tert-butyl ether and dried under nitrogen. The derivative reagent was N-methyl-N-(trimethylsilyl)-trifluoroacetamide with NH₄I and was analyzed by GC-MS, GC-MS-MS and GC-HRMS. A validation study was conducted by GC-MS-MS. The analyses of clenbuterol using different mass spectrometric techniques were compared. The limit of detection (LOD) for clenbuterol in human urine was 2 ng/mL by GC-MS (selected ion monitoring mode: SIM mode), 0.06 ng/mL by GC-HRMS and 0.03 ng/mL by GC-MS-MS, respectively, while the LOD by GC-HRMS was 0.06. With GC-MS-MS, the intra-assay and inter-assay precisions were less than 15%, the recoveries were 86 to 112% and the linear range was 0.06 to 8.0 ng/mL. The GC-MS under SIM mode can be used as a screening tool to detect clenbuterol at trace levels in human urine. The GC-MS-MS and GC-HRMS methods
can confirm clenbuterol when its concentration is below 2 ng/mL. The results demonstrate that the GC-MS-MS method is quite sensitive, specific and reliable for the detection of clenbuterol in doping analysis [13429].

Clenbuterol, terbutaline and salbutamol are beta2-agonists drugs included in the list of banned substances of the World Anti Doping Agency (WADA) prohibited in and out of competition. In this article, the excretion of urinary metabolites of clenbuterol, terbutaline and salbutamol have been studied using liquid chromatography electrospray time-of-flight mass spectrometry (LC-TOFMS), after a single therapeutic dose administration in rats. Urine collected was processed with solid-phase extraction prior to LC-TOFMS analyses using electrospray in the positive ion mode and pseudo MS/MS experiments from in-source collision induced dissociation (CID) fragmentation (without precursor ion isolation). The strategy applied for the identification of metabolites was based on the search of typical biotransformations with their corresponding accurate mass shift and the use of common diagnostic fragment ions from the parent drugs. The approach was satisfactory applied, achieving the identification of 11 metabolites (5 from clenbuterol, 4 from salbutamol and 3 from terbutaline), 4 of them not previously reported in urine. Novel metabolites identified in rat urine included N-oxide-salbutamol, hydroxy-salbutamol, methoxy-salbutamol glucuronide and terbutaline N-oxide, which are all reported here for the first time [13430].

The development of new approaches to study the affinity between ligands and G-protein-coupled receptors proves to be of growing interest for pharmacologists, chemists, and biologists. The aim of one work was to determine the binding of seven drugs to beta2-adrenoceptors by frontal analysis using immobilized receptor stationary phase. The dissociation constants (Kd ) were determined to be (3.16 ± 0.09) × 10⁻⁴ M for salbutamol, (4.29 ± 0.12) × 10⁻⁴ M for terbutaline, (6.19 ± 0.16) × 10⁻⁴ M for methoxyphenamine, (2.11 ± 0.07) × 10⁻⁴ M for tulobuterol, (1.82 ± 0.11) × 10⁻⁴ M for fenoterol, (9.75 ± 0.24) × 10⁻⁶ M formoterol, and (9.84 ± 0.26) × 10⁻⁵ M for clenbuterol. These results showed a good correlation with the data determined by radioligand binding assay. Further investigations revealed that the dissociation constant mainly attributed to the number of hydrogen bonds in the structures of ligands. The study indicates that affinity chromatography using immobilized receptor stationary phase can be used for the direct determination of drug-receptor binding interactions and has the potential to become a reliable alternative for quantitative studies of ligand-receptor interactions [13431].

Clenbuterol (4-amino-alpha-[(tert-butylamino)methyl]-3,5-dichlorobenzyl alcohol) is approved for human and veterinary use primarily for the treatment of pulmonary afflictions. Despite the authorized administration in cases of medical indications, the misuse of clenbuterol in animal husbandry as well as elite and amateur sport has frequently been reported, arguably due to growth-promoting properties. Due to various recent incidences of doping control specimens containing clenbuterol, strategies towards the discrimination of a surreptitious application from unintended intake via animal-derived edibles or dietary supplements were required. The enantiomeric compositions of clenbuterol in human urine samples derived from administration studies with therapeutic amounts of the beta2-agonist and authentic doping control specimens were determined. Due to the facts that therapeutic clenbuterol consists of a racemic mixture of (+)- and (-)-stereoisomers and that the first mentioned (dextrorotatory) stereoisomer is retained to a greater extent in edible animal tissue, the differentiation of a recent administration of (+)-clenbuterol from food contamination (stereoisomerically depleted clenbuterol) was considered. Employing deuterated clenbuterol as internal standard, the target analytes were extracted from human urine by means of concerted liquid-liquid and solid-phase extractions and subjected to chiral liquid chromatography hyphenated to high resolution/high accuracy mass spectrometry with electrospray ionization. Both enantiomers of clenbuterol were baseline separated and
relative abundances of corresponding labeled and unlabeled stereoisomers were determined, demonstrating that the therapeutic use of clenbuterol results in racemic mixtures in urine for at least 24 h while adverse analytical findings presumably originating from food contaminations can yield (−)-clenbuterol-depleted pairs of analytes. Thus, the determination of relative abundances of clenbuterol enantiomers can indicate the ingestion of clenbuterol via contaminated food; however, depletion of (−)-clenbuterol in edible animal tissue is time-dependent and thus results can still be inconclusive as to the inadvertent ingestion of clenbuterol when clenbuterol administration to animals was conducted until slaughter [13432].

Experimental

Spinal cord injury (SCI) is generally associated with a rapid and significant decrease in muscle mass and corresponding changes in skeletal muscle properties. Although beta2-adrenergic and androgen receptor agonists are anabolic substances clearly shown to prevent or reverse muscle wasting in some pathological conditions, their effects in SCI patients remain largely unknown. Here it was studied the effects of clenbuterol and testosterone propionate administered separately or in combination on skeletal muscle properties and adipose tissue in adult CD1 mice spinal-cord-transected (Tx) at the low-thoracic level (i.e. induced complete paraplegia). Administered shortly post-Tx, these substances were found to differentially reduce loss in body weight, muscle mass, and muscle fiber cross-sectional area (CSA) values. Although all three treatments induced significant effects, testosterone-treated animals were generally less protected against Tx-related changes. However, none of the treatments prevented fat tissue loss or muscle fiber type conversion and functional loss generally found in Tx animals. These results provide evidence suggesting that clenbuterol alone or combined with testosterone may constitute better clinically-relevant treatments than testosterone alone to decrease muscle atrophy (mass and fiber CSA) in SCI subjects [10171].

Clenbuterol, a beta2-adrenergic receptor (beta2-AR) selective agonist, has been shown to decrease body fat in animals and can induce apoptosis in adipose tissue in mice. We hypothesized that direct actions of a beta-adrenergic receptor agonist on adipocytes could trigger the observed apoptotic effect. The hypothesis was inspected by investigating the direct effect of clenbuterol on apoptosis, adipogenesis, and lipolysis in vitro using the 3T3-L1 cell line and rat primary adipocytes. Cells were treated with 10^−9 to 10^−5 M clenbuterol depending on the experiments. There was no apoptotic effect of clenbuterol both in 3T3-L1 cells and rat primary adipocytes. Adipogenesis monitored by Oil Red O staining and AdipoRed assay was modestly decreased by clenbuterol treatment. In fully differentiated primary adipocytes, clenbuterol significantly increased basal lipolysis compared with the control. In summary, direct stimulation of beta2-adrenergic receptor by clenbuterol does not cause apoptosis in adipocytes, despite a direct lipolytic stimulation and attenuation of adipogenesis [10376].

Illegal in cattle

Recent discovery of clenbuterol contamination in Portuguese food led to the specific inspection of 16 cattle farms for beta-agonists, involving the analysis of a total of 486 samples (78 feed, 106 drinking water, 168 urine and 134 hair). The samples were screened for the beta-agonists: bromobuterol, cimaterol, clenbuterol, clenpenterol, clenproperol, hydroxymethylclenbuterol, mapenterol, salbutamol and terbutaline. Only clenbuterol was found in all analyzed matrices and the most likely method of illegal administration to animals was through drinking water. Of all samples analysed, 14 percent of drinking water were
found positive in the range 0.03-3.80 mg/L clenbuterol. Inclusion of hair samples in the Portuguese plan for clenbuterol residue control in live animals is discussed [09248].

**In horses**

Clenbuterol is a beta₂-agonist and potent selective bronchodilator that is used to treat bronchospasm in the horse. The drug is normally administered to horses orally as a syrup formulation. Once absorbed into the systemic circulation, clenbuterol has the potential to cause many side effects, including a repartitioning effect and major alterations in cardiac and skeletal muscle function. Recent studies have also reported that clenbuterol can affect bone and the immune, endocrine and reproductive systems. A great deal of information has been published on the beneficial effects of short term therapeutic doses of clenbuterol on the equine respiratory system, although there is limited information about chronic administration, particularly since this has been associated with adverse physiological effects on other systems [08354].

**Methoxyphenamine**

Methoxyphenamine (o-methoxy-N,alpha-dimethylphenethylamine) used in earlier times as a bronchodilator is prohibited in sports according to the regulations of the World Anti-Doping Agency. The drug and several of its metabolites are commonly analysed in doping control screening assays using gas chromatography-mass spectrometry requiring extraction from urine specimens. A complementary method employing liquid chromatography-atmospheric pressure chemical ionisation-tandem mass spectrometry and direct injection of urine aliquots was developed, which provided a fast and sensitive alternative to confirm the presence of the prohibited compound and degradation products in sports drug testing samples. In particular, the chromatographic separation of the active drug from isomeric compounds such as the designer drug p-methoxymetamphetamine was of particular interest to unambiguously identify the applied substance and was accomplished using a C6-phenyl reverse-phase column with isocratic elution. The established procedure was validated for methoxyphenamine with regard to specificity, limit of detection (0.7 ng/mL), intraday- and interday precision (2.5-5.8 % and 10.8-16.2 %, respectively) and its applicability was demonstrated with an authentic doping control sample which tested positive for the prohibited compound early in 2008 [08355].

**Laboratory techniques**

A sensitive and specific liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) is described for quantitation of salbutamol in human urine using nadolol as the internal standard (IS). Urine samples were hydrolyzed with beta-glucuronidase followed by a solid-phase extraction procedure using Bond Elut-Certify cartridges. The HPLC column was an Agilent Zorbax SB-C(18) column. A mixture of 0.01 M ammonium formate buffer (pH 3.5)-acetonitrile (85:15, v/v) was used as the mobile phase. Analytes were quantitated using positive electrospray ionization in a quadrupole spectrometer. Selected ion monitoring (SIM) mode was used to monitor m/z 166 for salbutamol and m/z 310 for I.S. Good linearity was obtained in the range of 10.0-2000.0 ng/mL. The limit of quantification was 10.0 ng/mL. The intra- and inter-run precision, calculated from quality control (QC) samples was less than 7.3 percent. The accuracy as determined from QC samples was within ± 2.6 percent. The method was applied for determining excretion curves of salbutamol [06208].
In official doping controls, about 300 drugs and metabolites have to be screened for each sample. Moreover, the number of determinations to be routinely processed increases continuously as the number of both samples and potential illicit drugs keeps growing. As a consequence, increasingly specific, sensitive, and, above all, fast methods for doping controls are needed. One study presented an efficient fast-GC/MS approach to the routine screening of two different classes of doping agents, namely beta-adrenoceptor ligands and diuretics (belonging to the S3, P2, and S5 groups of the WADA list of prohibited substances). Narrow bore columns (100 mm id) of different lengths and coated with apolar stationary phases were successfully used to separate the derivatized analytes; preliminary experiments (results not shown) showed better performances with OV-1701 for the separation of beta-adrenoceptor ligands. On the same stationary phase some diuretics required too high a temperature or a long isothermal time for elution, in which case a DB1-MS column was preferred. Two methods of sample preparation, derivatization, and analysis were used on aqueous standard mixtures of, respectively, (i) eight beta-adrenoceptor ligands, including five beta-antagonists (acebutolol, alprenolol, atenolol, metoprolol, pindolol) and three beta2-agonists (salbutamol, clenbuterol, terbutaline) and (ii) seventeen diuretic drugs (acetazolamide, althiazide, bendroflumethiazide, bumethanide, canrenone, chlorothiazide, chlorotalidone, clopamide, ethacrinic acid, furosemide, hydrochlorothiazide, hydroflumethiazide, indapamide, indomethacine, spironolactone, triamterene, trichloromethiazide) and one masking agent (probenecid). The mixture of beta-adrenoceptor ligand derivatives was efficiently separated in about 5.6 min, while the one of 18 diuretics and masking agents required less than 5 min for analysis. Limits of detection were from 1 microg/L for pindolol, ethacrinic acid, furosemide, indomethacine, and trichloromethiazide, to 20 microg/L for terbutaline, salbutamol, and metoprolol, and 50 microg/L for clopamide; the instrumental repeatability proved to be excellent (area RSD% <2 for almost all analytes). For this work a quadrupole MS with inert ion source has been used, demonstrating that the quadrupole technology is perfectly adequate to provide precise integration of 400 ms-wide GC peaks [06209].

A method using hollow fibre-protected liquid-phase microextraction (HF-LPME) with in situ derivatization followed by gas chromatography/mass spectrometry (GC/MS) was established for the analysis of beta-agonists and beta-blockers in urine. Because it can simultaneously extract and derivatize compounds of interest by methylbenzol and N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) in HF-LPME, the approach overcomes the drawbacks of considerable time-consuming and tedious operation, meanwhile improves enrichment multiple. The optimized conditions were extraction for 20 min at 35 degrees C with 5.0 microL of mixed extraction solvent (methylbenzol/MSTFA=1:1, v/v) with stirring speed of 925 rpm in 5.0 mL sample under pH 12.0 and 14% (w/v) NaCl. The method provided very wide linear ranges (0.25-400 ng/mL) and low detection limits in the range of 0.08-0.10 ng/mL for clenbuterol, metoprolol and propranolol while enrichment factors reached up to 256. The analytes could be determined in spiked urine by the method with high extraction efficacy (93.79-109.04% recoveries) and precision (<9.70 % RSD). It has a satisfactory result for metoprolol in practical human urine samples for a single-dose administration of 50 mg after 36 h. The proposed method only needs few microliters of organic solvent and derivatizing agent; the operation is simple, convenient and rapid for the trace analysis of beta-agonists and beta-blockers in biological fluids; it can be readily generalized for high sample throughput [09243].

One paper presents a capillary electrophoresis method, developed for the detection, in human urine, of beta-adrenergic agents and phenolalkylamines. The electrophoretic separation is achieved in less than 10 min and is based on the use of CEfix kit, for the dynamic capillary coating. The effects of accelerator buffer pH and separation voltage were investigated. The optimum buffer pH was found to be 2.5 for beta2-agonists and 6.2 for beta-
blockers and phenoalkylamines with a separation voltage of 15 kV. Urine samples spiked with the compounds here studied were treated according to the standard procedure (SPE and evaporation to dryness) and analyzed by CE interfaced with an UV diode-array, set at 195 and 210 nm. The quantitative validation results, obtained analyzing samples at three different concentrations, show a good precision of peak areas that do not exceed 5 percent for intra-day assays and 10 percent for inter-day assays. Good linearity was obtained within the 50-500 ng/mL concentration range. The analytes were clearly distinguishable in urine, with LOD and LOQ in the range of 10-80 and 40-100 ng/mL, respectively [09244].

A general screening method based on solid phase extraction (SPE) and liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) was developed and investigated with 124 different doping agents, including stimulants, beta-blockers, narcotics, beta2-adrenergic agonists, agents with anti-estrogenic activity, diuretics and cannabinoids. Mixed mode cation exchange/C8 cartridges were applied to SPE, and chromatography was based on gradient elution on a C18 column. Ionization of the analytes was achieved with electrospray ionization in the positive mode. Identification by LC/TOFMS was based on retention time, accurate mass and isotopic pattern. Validation of the method consisted of analysis of specificity, analytical recovery, limit of detection and repeatability. The minimum required performance limit (MRPL), established by World Anti-Doping Agency (WADA), was attained to 97 doping agents. The extraction recoveries varied between 33 and 98 percent and the median was 58 percent. Mass accuracy was always better than 5 ppm, corresponding to a maximum mass error of 0.7 mDa. The repeatability of the method for spiked urine samples, expressed as median of relative standard deviations (RSD%) at concentrations of MRPL and 10 times MRPL, were 14 and 9 percent, respectively. The suitability of the LC/TOFMS method for doping control was demonstrated with authentic urine samples [07195].

Experimental

Systemic administration of beta-adrenoceptor (beta-AR) agonists has been found to induce skeletal muscle hypertrophy and significant metabolic changes. In the context of energy homeostasis, the importance of beta-AR signaling has been highlighted by the inability of beta(1-3)-AR-deficient mice to regulate energy expenditure and susceptibility to diet induced obesity. However, the molecular pathways and gene expression changes that initiate and maintain these phenotypic modulations are poorly understood. Therefore, the aim of one study was to identify differential changes in gene expression in murine skeletal muscle associated with systemic (acute and chronic) administration of the beta2-AR agonist formoterol. Skeletal muscle gene expression (from murine tibialis anterior) was profiled at both 1 and 4 hours following systemic administration of the beta2-AR agonist formoterol. Illumina expression profiling revealed significant expression changes in genes associated with skeletal muscle hypertrophy, myoblast differentiation, metabolism, circadian rhythm, transcription, histones, and oxidative stress. Differentially expressed genes relevant to the regulation of muscle mass and metabolism (in the context of the hypertrophic phenotype) were further validated by quantitative RT-PCR to examine gene expression in response to both acute (1-24 h) and chronic administration (1-28 days) of formoterol at multiple timepoints. In terms of skeletal muscle hypertrophy, attenuation of myostatin signaling (including differential expression of myostatin, activin receptor IIB, phospho-Smad3 etc) was observed following acute and chronic administration of formoterol. Acute (but not chronic) administration of formoterol also significantly induced the expression of genes involved in oxidative metabolism, including hexokinase 2, sorbin and SH3 domain containing 1, and uncoupling protein 3. Interestingly, formoterol administration also appeared to influence some genes associated with the peripheral regulation of circadian rhythm (including nuclear
factor interleukin 3 regulated, D site albumin promoter binding protein, and cryptochrome 2). It was concluded in this study to utilize gene expression profiling to examine global gene expression in response to acute beta_2-AR agonist treatment of skeletal muscle systemic administration of a beta_2-AR agonist had a profound effect on global gene expression in skeletal muscle. In terms of hypertrophy, beta_2-AR agonist treatment altered the expression of several genes associated with myostatin signaling, a previously unreported effect of beta-AR signaling in skeletal muscle. The study also demonstrates a beta_2-AR agonist regulation of circadian rhythm genes, indicating crosstalk between beta-AR signaling and circadian cycling in skeletal muscle. Gene expression alterations discovered in this study provides insight into many of the underlying changes in gene expression that mediate beta-AR induced skeletal muscle hypertrophy and altered metabolism [09245].
A second-order multivariate calibration method based on a combination of unfolded partial least-squares (U-PLS) with residual bilinearization (RBL) has been applied to second-order data obtained from excitation-emission fluorescence matrices for determining atenolol in human urine, even in the presence of background interactions and fluorescence inner filter effects, which are both sample dependent. Atenolol is a cardioselective beta-blocker, which is considered a doping agent in shoot practice, so that its determination in urine can be required for monitoring the drug. Loss of trilinearity due to analyte-background interactions which may vary between samples, as well as inner filter effects, precludes the use of methods like parallel factor analysis (PARAFAC) that cannot handle trilinearity deviations, and justifies the employment of U-PLS. Successful analysis required to include the background in the calibration set. Unexpected components appear in new urine samples, different from those used in calibration set, requiring the second-order advantage which is obtained from a separate procedure known as residual bilinearization. Satisfactory results were obtained for artificially spiked urines, and also for real urine samples. They were statistically compared with those obtained applying a reference method based on high-performance liquid chromatography (HPLC) [11191].
ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORS AND ANGIOTENSIN II TYPE RECEPTOR ANTAGONISTS

In the decade since the angiotensin-converting enzyme (ACE) gene was first proposed to be a “human gene for physical performance”, there have been numerous studies examining the effects of ACE genotype on physical performance phenotypes such as aerobic capacity, muscle function, trainability, and athletic status. While the results are variable and sometimes inconsistent, and corroborating phenotypic data limited, carriers of the ACE insertion allele (the presence of an alu repeat element in intron 16 of the gene) have been reported to have higher maximum oxygen uptake (VO$_{2\text{max}}$), greater response to training, and increased muscle efficiency when compared with individuals carrying the ‘deletion’ allele (absence of the alu repeat). Furthermore, the insertion allele has been reported to be over-represented in elite athletes from a variety of populations representing a number of endurance sports. The mechanism by which the ACE insertion genotype could potentiate physical performance is unknown. The presence of the ACE insertion allele has been associated with lower ACE activity (ACE$_{\text{plasma}}$) in number of studies, suggesting that individuals with an innate tendency to have lower ACE levels respond better to training and are at an advantage in endurance sporting events. This could be due to lower levels of angiotensin II (the vasoconstrictor converted to active form by ACE), higher levels of bradykinin (a vasodilator degraded by ACE) or some combination of the two phenotypes. Observations that individuals carrying the ACE insertion allele (and presumably lower ACE$_{\text{plasma}}$) have an enhanced response to training or are over-represented amongst elite athletes raises the intriguing question: would individuals with artificially lowered ACE$_{\text{plasma}}$ have similar training or performance potential? As there are a number of drugs (i.e. ACE inhibitors and angiotensin II type 1 receptor antagonists; angiotensin receptor blockers, ARBs) that have the ability to either reduce ACE$_{\text{plasma}}$ activity or block the action of angiotensin II, the question is relevant to the study of ergogenic agents and to the efforts to rid sports of doping. The article discussed the possibility that ACE inhibitors and ARBs, by virtue of their effects on ACE or angiotensin II function, respectively, have performance-enhancing capabilities; it also reviews the data on the effects of these medications on VO$_{2\text{max}}$, muscle composition and endurance capacity in patient and non-patient populations. It was concluded that, while the direct evidence supporting the hypothesis that ACE-related medications are potential doping agents is not compelling, there are insufficient data on young, athletic populations to exclude the possibility, and there is ample, albeit indirect, support from genetic studies to suggest that they should be. Unfortunately, given the history of drug experimentation in athletes and the rapid appropriation of therapeutic agents into the doping arsenal, this indirect evidence, coupled with the availability of ACE-inhibiting and ACE-receptor blocking medications may be sufficiently tempting to unscrupulous competitors looking for a shortcut to the finish line [10163].

Recent research has analyzed the genetic factors that influence world-class athletic status. Much of what we know comes from association studies, with the ACE I/D and ACTN3 R577X polymorphisms having been extensively studied. The association between the ACTN3 R577X variation and elite athlete status in power sports is strongly documented, yet whether the current body of knowledge on other variants can be extrapolated to athletic champion status remains to be determined. Athletic champion status is a complex polygenic trait in which numerous candidate genes, complex gene-gene interactions, and environment-gene interactions are involved. Besides the need for more studies and new approaches taking into account the complexity of the problem, we believe that factors beyond genetic endowment are likely to have a stronger influence in the attainment of athletic champion status [10164].
Hypertension is a prevalent disease worldwide. Its inadequate treatment leads to major cardiovascular complications, such as myocardial infarction, stroke, and heart failure. These conditions decrease life expectancy and are a substantial cost burden to health care systems. Physically active individuals and professional athletes are not risk free for developing this condition. Although the percentage of persons affected is substantially lower than the general population, these individuals still need to be thoroughly evaluated and blood pressure targets monitored to allow safe competitive sports participation. Regarding treatment, lifestyle modification measures should be routinely emphasized to athletes and active individuals with the same importance as for the general population. Medication treatment can be complicated because of restrictions by athletic organizations and possible limitations on maximal exercise performance. In addition, the choice of an antihypertensive drug should be made with consideration for salt and water losses that routinely occur in athletes, as well as preservation of exercise performance and endothelial function. First-line therapies for athletes and physically active individuals may be different from the general population. Some authorities believe that blocking the renin-angiotensin system with angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) is more beneficial compared with diuretics because of ACE inhibitors and ARBs being able to avoid salt and water losses. Dihydropyridine calcium channel blockers (CCBs) are another reasonable choice. Despite effects on heart rate, nondihydropyridine CCBs do not appear to impair exercise performance. beta-Blockers are not used as a first-line therapy in athletes because of effects on exercise and prohibition by the National Collegiate Athletic Association and World Anti-Doping Agency in certain sports [10165].

In the decade since the angiotensin-converting enzyme (ACE) gene was first proposed to be a “human gene for physical performance”, there have been numerous studies examining the effects of ACE genotype on physical performance phenotypes such as aerobic capacity, muscle function, trainability, and athletic status. While the results are variable and sometimes inconsistent, and corroborating phenotypic data limited, carriers of the ACE insertion allele (the presence of an ALU repeat element in intron 16 of the gene) have been reported to have higher maximum oxygen uptake (VO$_{2\text{max}}$), greater response to training, and increased muscle efficiency when compared with individuals carrying the deletion allele (absence of the ALU repeat). Furthermore, the insertion allele has been reported to be over-represented in elite athletes from a variety of populations representing a number of endurance sports. The mechanism by which the ACE insertion genotype could potentiate physical performance is unknown. The presence of the ACE insertion allele has been associated with lower ACE activity (ACE$_{\text{plasma}}$) in number of studies, suggesting that individuals with an innate tendency to have lower ACE levels respond better to training and are at an advantage in endurance sporting events. This could be due to lower levels of angiotensin II (the vasoconstrictor converted to active form by ACE), higher levels of bradykinin (a vasodilator degraded by ACE) or some combination of the two phenotypes. Observations that individuals carrying the ACE insertion allele (and presumably lower ACE$_{\text{plasma}}$) have an enhanced response to training or are over-represented amongst elite athletes raises the intriguing question: would individuals with artificially lowered ACE$_{\text{plasma}}$ have similar training or performance potential? As there are a number of drugs (i.e. ACE inhibitors and angiotensin II type 1 receptor antagonists; angiotensin receptor blockers - ARBs) that have the ability to either reduce ACE$_{\text{plasma}}$ activity or block the action of angiotensin II, the question is relevant to the study of ergogenic agents and to the efforts to rid sports of doping. It was concluded that, while the direct evidence supporting the hypothesis that ACE-related medications are potential doping agents is not compelling, there are insufficient data on young, athletic populations to exclude the possibility, and there is ample, albeit indirect, support from genetic studies to suggest that they should be. Unfortunately, given the history of drug experimentation in athletes and the rapid appropriation of therapeutic agents into the doping arsenal, this indirect evidence, coupled with the availability of ACE-inhibiting and ACE-receptor blocking medications may
be sufficiently tempting to unscrupulous competitors looking for a shortcut to the finish line [08362].

In the decade since the angiotensin-converting enzyme (ACE) gene was first proposed to be a 'human gene for physical performance', there have been numerous studies examining the effects of ACE genotype on physical performance phenotypes such as aerobic capacity, muscle function, trainability, and athletic status. While the results are variable and sometimes inconsistent, and corroborating phenotypic data limited, carriers of the ACE "insertion" allele (the presence of an ALU repeat element in intron 16 of the gene) have been reported to have higher maximum oxygen uptake (VO_2max), greater response to training, and increased muscle efficiency when compared with individuals carrying the "deletion" allele (absence of the ALU repeat). Furthermore, the insertion allele has been reported to be over-represented in elite athletes from a variety of populations representing a number of endurance sports. The mechanism by which the ACE insertion genotype could potentiate physical performance is unknown. The presence of the ACE insertion allele has been associated with lower ACE activity (ACE plasma) in number of studies, suggesting that individuals with an innate tendency to have lower ACE levels respond better to training and are at an advantage in endurance sporting events. This could be due to lower levels of angiotensin II (the vasoconstrictor converted to active form by ACE), higher levels of bradykinin (a vasodilator degraded by ACE) or some combination of the two phenotypes. Observations that individuals carrying the ACE insertion allele (and presumably lower ACE plasma) have an enhanced response to training or are over-represented amongst elite athletes raises the intriguing question: would individuals with artificially lowered ACE plasma have similar training or performance potential? As there are a number of drugs (i.e. ACE inhibitors and angiotensin II type 1 receptor antagonists) that have the ability to either reduce ACE plasma activity or block the action of angiotensin II, the question is relevant to the study of ergogenic agents and to the efforts to rid sports of "doping". It may be discussed the possibility that ACE inhibitors and angiotensin receptor blockers, by virtue of their effects on ACE or angiotensin II function, respectively, have performance-enhancing capabilities. It may be concluded that, while the direct evidence supporting the hypothesis that ACE-related medications are potential doping agents is not compelling, there are insufficient data on young, athletic populations to exclude the possibility, and there is ample, albeit indirect, support from genetic studies to suggest that they should be. Unfortunately, given the history of drug experimentation in athletes and the rapid appropriation of therapeutic agents into the doping arsenal, this indirect evidence, coupled with the availability of ACE-inhibiting and ACE-receptor blocking medications may be sufficiently tempting to unscrupulous competitors looking for a shortcut to the finish line [09282].

It was assessed the possible association between variants of the genes encoding for the angiotensin-converting enzyme (ACE) and alpha-actinin-3 ACTN3 (both individually and combined) and several endurance phenotypic traits, e.g., peak power output (PPO), ventilatory (VT) and respiratory compensation threshold (RCT), among others, in professional road cyclists and sedentary controls (n=46 each). It was only found a significant genotype effect with no concomitant covariate effect for ACTN3, with cyclists who were not alpha-actinin-3 deficient (RR + RX genotypes) having higher PPO and VT values than their XX counterparts. Cyclists with an "extreme" ACTN3 and ACE genotype combination, i.e. most strength/power oriented (DD + RR/RX), had higher RCT values than those with the "intermediate" combinations but similar to those with the most endurance oriented genotype (II + XX). No significant differences were found in controls. In summary, in world-class cyclists, it was only found an association between ACTN3 genotypes and VT and PPO, and between ACTN3/ACE genotype combinations and RCT [09283].

Previous studies have linked an insertion/deletion polymorphism in the angiotensin-
converting enzyme (ACE) gene with variability in muscle strength responses to strength training, though conclusions have been inconsistent across investigations. Moreover, most previous studies have not investigated the influence of sex on the association of ACE I/D genotype with muscle phenotypes. The purpose of one study was to investigate the association of ACE genotype with muscle phenotypes before and after ST in older men and women. Eighty-six inactive men and 139 inactive women, ages 50-85 years, were studied before and after 10 weeks of unilateral knee extensor strength training. The one-repetition maximum (1RM) test was used to assess knee extensor muscle strength, and computed tomography was used to measure quadriceps muscle volume (MV). Across the entire cohort at baseline, ACE genotype was significantly associated with total lean mass and body weight, with significantly higher values in DD genotype carriers. At baseline, DD genotype carriers exhibited significantly greater MV compared with II genotype carriers for both the trained leg and untrained leg with no significant genotype x sex interaction. No ACE genotype associations were observed for the 1RM or MV adaptations to strength training in either men or women. This means that ACE genotype was associated with baseline differences in muscle volume, but it was not associated with the muscle hypertrophic response to strength training [09284].

An ALU insertion (I)/deletion (D) polymorphism in the angiotensin I converting enzyme (ACE) gene has been associated with ACE activity. Opposing effects on elite athletic performance have been proposed for the I and D alleles; while the D allele favours improved endurance ability, the I allele promotes more power-oriented events. It was tested this hypothesis by determining the frequency of ACE ID alleles amongst 121 Israeli top-level athletes classified by their sporting discipline (marathon runners or sprinters). Genotyping for ACE ID was performed using polymerase chain reaction on DNA from leucocytes. The ACE genotype and allele frequencies were compared with those of 247 healthy individuals. Allele and genotype frequencies differed significantly between the groups. The frequency of the D allele was 0.77 in the marathon runners, 0.66 in the control subjects and 0.57 in the sprinters. The ACE DD genotype was significantly more prevalent among the endurance athletes than among the control subjects and the power athletes. In the group of elite athletes, the odds ratio of ACE DD genotype being an endurance athlete was 3.26 (95 % confidence interval 1.49 to 7.11), and of ACE II genotype was 0.41 (95 % confidence interval 0.14 to 1.19). It was conclude that in Israeli elite marathon runners the frequency of the ACE D allele and ACE DD genotype seems to be higher than in sprinters, suggesting a positive association between the D allele and the likelihood of being an elite endurance athlete in some ethnic groups [09285].

East African runners are continually successful in international distance running. The extent to which genetic factors influence this phenomenon is unknown. The insertion (I) rather than deletion (D) of a 287 bp fragment in the human angiotensin converting enzyme (ACE) gene is associated with lower circulating and tissue ACE activity and with endurance performance amongst Caucasians. To assess the association between ACE gene variation and elite endurance athlete status in an African population successful in distance running, DNA samples were obtained from 221 national Kenyan athletes (N), 70 international Kenyan athletes (I), and 85 members of the general Kenyan population (C). Blood samples were obtained from C and assayed for circulating ACE activity. It was concluded that ACE I/D and A22982G polymorphisms are not strongly associated with elite endurance athlete status amongst Kenyans [09286].

Angiotensin converting enzyme gene (ACE) is the most frequent investigated gene in the context of genetic conditioning of sports-predispositions. Product of this gene is a key-element in the renin-angiotensin system responsible for the regulation of blood pressure. In one study DNA polymorphism in the ACE gene was studied in Polish rowers in order to examine the hypothesis that ACE genotype is associated with athletes performance. Fifty-
five male Polish rowers including Olympic and World champions were recruited for this study. Control samples were prepared from 115 unrelated volunteers. PCR amplification of the insertion (I) or deletion (D) fragment of ACE gene was performed. Genotype distribution and allele frequencies were determined by genotype and gene counting. ACE genotype distributions amongst subjects and controls were in Hardy-Weinberg equilibrium. Compared with controls, the frequency of I allele differ significantly from that found in rower's group. The data confirm a positive association of the insertion allele of ACE gene with endurance performance [09287].

The most widely-studied candidate gene for endurance performance is the Angiotensin Converting Enzyme (ACE) gene. The best endurance runners in the world hail from Kenya and Ethiopia, so the lack of association between the ACE gene and elite endurance athlete status we previously reported in Kenyans requires replication in Ethiopians. DNA was extracted from buccal swabs collected from subjects filling four groups: elite endurance runners from the Ethiopian national athletics team specializing in 5 km to marathon distances (n=76), controls demographically matched to the elite endurance athletes (n=410) controls representing the general Ethiopian population (n=317), and sprint and power event athletes from the Ethiopian national athletics team (n=38). ACE I/D and A22982G (rs4363) genotype frequencies were determined for each of these groups. There were no significant deviations from Hardy-Weinberg equilibrium in endurance athletes or either control group. Endurance athletes did not differ significantly in ACE I/D genotype frequency when compared to the endurance athlete-matched control group, general controls, or sprint and power athletes. Similarly, no significant differences were found in ACE A22982G genotype between groups. Thus, as previously shown in elite Kenyan athletes, ACE I/D and A22982G polymorphisms are not associated with elite endurance athlete status in Ethiopians [10384].

In one study, genotype frequencies of several polymorphisms that are candidates to influence sports performance (ie, ACTN3 R577X, ACE ID, PPARGC1A Gly482Ser, AMPD1 C34T, CKMM 985bp/1170bp and GDF8 (myostatin) K153R) were compared in 123 nonathletic controls, 50 professional cyclists, 52 Olympic-class runners and 39 world-class rowers (medallists in world championships, lightweight category). Significant differences in genotype distributions among the groups were not found except for the ACE gene, that is, lower proportion of II in rowers (10 %) than in the total subject population (22 %). In summary, sports performance is likely polygenic with the combined effect of hundreds of genetic variants, one possibly being the ACE ID polymorphism (at least in the sports studied here), but many others remain to be identified [10387].

It was investigated the association between the angiotensin I-converting enzyme (ACE) gene insertion (I)/deletion (D) polymorphism and endurance running performance in Japanese elite runners, including several Olympic athletes. The frequency of the I/I genotype was not significantly higher and the frequency of the D/D genotype was not significantly lower in elite runners compared with non-athletes. However, the frequency of the I/D genotype tended to be lower in elite runners than in non-athletes. The best performance was significantly higher for runners with the D/D genotype than for those with the I/I genotype, and the average running speed was significantly higher for those with the combined D/D + I/D genotypes than for those with the I/I genotype. There were no I/I genotypes among the five fastest marathon runners. These results suggest that the D allele of the ACE gene I/D polymorphism is associated with a high level of human endurance [10386].

Angiotensin-converting enzyme (ACE) is associated with the development of cardiac hypertrophy and improved physical fitness. The objective of one study was to investigate the relationship between the ACE gene insertion/deletion (I/D) polymorphism and adaptation to sports training. The study included 299 elite Spanish athletes (193 men and 106 women)
from 32 different sports disciplines, which were grouped according to their static and dynamic components. All participants underwent body composition analysis, Doppler echocardiography at rest, and ergospirometry. Their ACE genotype was determined using the polymerase chain reaction. The most common genotype in both males and females was the deletion-insertion (DI) heterozygote (58 % and 55 %, respectively), followed by the DD homozygote (31 % and 35 %), and the II homozygote (12 % and 10 %). Differences in morphometric and functional cardiac adaptation were observed between the different sports disciplines, but there was no statistically significant relationship with the ACE I/D polymorphism. Moreover, when athletes with different genotypes were compared, the only differences observed were between the DD and DI groups in female athletes, who differed in body mass index and longitudinal right atrial dimension. The ACE I/D polymorphism did not appear to influence cardiovascular adaptation in response to training. However, the DI genotype was the most common, probably because the sample was biased by being made up of elite athletes [10385].

Regular physical activity and training are associated with reductions in blood pressure (BP), yet elevated BP is one of the most common abnormalities found during the pre-participation physical evaluation of athletes. Hypertension remains the most common cardiovascular condition encountered in athletic populations, therefore all athletes require screening for hypertension. Because athletes often have white coat hypertension, BP recordings outside the office are also necessary. The 36th Bethesda Conference classified sports according to their varying physiologic demands and provided specific recommendations for the evaluation, treatment, and sport participation of athletes with HTN. In general, angiotensin-converting enzyme inhibitors and other vasodilators are the medications of choice for active and athletic patients because of their limited interference with cardiovascular conditioning. Other agents can be used but some sports governing bodies proscribe the use of certain antihypertensive medications such as beta-blockers for elite athletes [09288].

It was examined the association between the angiotensin I-converting enzyme (ACE) gene (insertion (I) and deletion (D)) polymorphism in Japanese university track athletes and race distance, as well as to evaluate the gender effects on this association. The ACE I/D allele frequency was determined in 277 athletes (176 men, 101 women), who were then grouped on the basis of their major competitive race distances (short distance (SD), \( < 200 \) m; middle distance (MD), 400-800 m, and long distance (LD), \( \geq 1500 \) m). The ACE I allele frequency increased significantly with the distance (44 %, 48 %, and 66 % for the SD (n=107), MD (n=62), and LD (n=108) groups). On multinomial logistic regression analysis, significant associations between ACE genotype and race distance were observed only in male athletes. There was no significant association between ACE genotype and race distance in female athletes. It was concluded that the ACE I allele is overrepresented in endurance athletes, and that its frequency varies depending on gender [09289].

The most widely studied candidate gene for endurance performance is the angiotensin-converting enzyme (ACE) gene. The best endurance runners in the world hail from Kenya and Ethiopia, so the lack of association between the ACE gene and elite endurance athlete status we previously reported in Kenyans requires replication in Ethiopians. DNA was extracted from buccal swabs collected from subjects filling four groups: elite endurance runners from the Ethiopian national athletics team specializing in 5 km to marathon distances (n=76), controls demographically matched to the elite endurance athletes (n=410), controls representing the general Ethiopian population (n=317), and sprint and power event athletes from the Ethiopian national athletics team (n=38). ACE I/D and A22982G (rs4363) genotype frequencies were determined for each of these groups. There were no significant deviations from Hardy-Weinberg equilibrium in endurance athletes or either control group. Endurance athletes did not differ significantly in ACE I/D genotype frequency when compared with the
An Alu insertion (I)/deletion (D) polymorphism in the angiotensin I converting enzyme (ACE) gene has been associated with ACE activity. Opposing effects on elite athletic performance have been proposed for the I and D alleles; while the D allele favours improved endurance ability, the I allele promotes more power-orientated events. It was tested this hypothesis by determining the frequency of ACE ID alleles amongst 121 top-level athletes classified by their sporting discipline (marathon runners or sprinters). Genotyping for ACE ID was performed using polymerase chain reaction on DNA from leucocytes. The ACE genotype and allele frequencies were compared with those of 247 healthy individuals. Allele and genotype frequencies differed significantly between the groups. The frequency of the D allele was 0.77 in the marathon runners, 0.66 in the control subjects and 0.57 in the sprinters. The ACE DD genotype was significantly more prevalent among the endurance athletes (0.62) than among the control subjects (0.43) and the power athletes (0.34). In the group of elite athletes, the odds ratio of ACE DD genotype being an endurance athlete was 3.26 (95% confidence interval 1.49 to 7.11), and of ACE II genotype was 0.41 (95% confidence interval 0.14 to 1.19). It was concluded that in Israeli elite marathon runners the frequency of the ACE D allele and ACE DD genotype seems to be higher than in sprinters, suggesting a positive association between the D allele and the likelihood of being an elite endurance athlete in some ethnic groups [07196].

The aim of one study was to examine the distribution of insertion/deletion (I/D) polymorphism in the ACE gene among Korean male elite athletes. Participants of the study were 139 Korean male elite athletes (15 basketball, 41 soccer, 31 baseball, 12 gymnastics, 7 volleyball, 8 long-distance running, 8 judo, and 17 ice hockey players). The control group consisted of 163 non-athletes. Polymerase chain reaction method was used to investigate I/D polymorphism of the ACE gene. The distribution of genotype and allele frequencies did not indicate the differences between the athletes and controls. However, an excess of II genotype and I allele was shown in long distance runners, although it was not statistically significant. It may be necessary to perform further studies using a homogeneous cohort of subjects from single sporting discipline, because the probable role of I allele in athletic performance has been hard to be detected by heterogeneous groups of diverse sporting disciplines [07197].

To investigate the relationship between genetic variation in the renin-angiotensin system and the effect of 12-week endurance training in Korean women 17 women who participated in an endurance training program for 12 weeks were genotyped for the angiotensinogen M235T polymorphism, angiotensin II type 1 receptor A1166C polymorphism, angiotensin-converting enzyme (ACE) T-3892C polymorphism, and angiotensin II type 2 receptor C3123A polymorphism. The following clinical parameters were measured before and after the endurance training program: blood pressure, body composition, ventilatory response, total cholesterol, triglyceride, and glucose. Of the genetic markers investigated, the frequency of the T allele for the ACE T-3892C polymorphism was significantly associated with the change in body mass index and VO2max after 12 weeks of endurance training. None of the other polymorphisms were significantly associated with the effect of training. The significant association between ACE T-3892C and the change in body mass index and VO2max in Korean women are attributed to training, suggesting that this genetic variation is a useful genetic marker for clarifying the interindividual response to endurance training [07198].
To determine the role of the angiotensin converting enzyme, ACE, (I/D) gene polymorphism on erythropoietic response in endurance athletes after natural exposure to moderate altitude erythropoietic activity was measured in 63 male endurance athletes following natural exposure to moderate altitude (2200 m) during 48 h. Erythropoietin (EPO) levels and hemoglobin (Hb) concentrations were measured at baseline and 12, 24, and 48 h after reaching the set altitude. Reticulocyte counts were determined at baseline and 48 h thereafter. Subjects were grouped into two groups (responders and nonresponders) based on significant increase in EPO levels (median: > 16.5 ng/m) after 24 h at altitude. ACE gene polymorphism was ascertained by polymerase chain reaction (DD, 31 (49 %); ID, 24 (38 %); II, 8 (13 %)). Overall, EPO levels significantly increased at 12 (70 %) and 24 h (72 %) above baseline concentration following exposure to 2200 m. Thereafter, EPO concentration decreased at 48 h, but a significant increase in Hb levels and reticulocyte count was observed at the end of the experiment, suggesting negative feedback. There were no significant differences in EPO and Hb concentration profiles between subjects with DD genotype and those with other genotypes (ID/II). Moreover, responders (n=42; DD, 50 %; ID/II, 50 %) and nonresponders (n= 21; DD, 48 %; ID/II, 52 %) showed a similar erythropoietic profile during the experiment and the ACE gene polymorphism did not influence the time course of the erythropoietic response. It was concluded that the ACE gene polymorphism does not influence erythropoietic activity in endurance athletes after short-term exposure to moderate altitude [06218].

Myocardial tolerance to ischaemia/reperfusion (I/R) injury is improved by exercise training, but this cardioprotection is impaired by the chronic use of anabolic androgenic steroids (AAS). The present study evaluated whether blockade of angiotensin II receptor (AT\textsubscript{1}-R) with losartan and aldosterone receptor (mineralocorticoid receptor, MR) with spironolactone could prevent the deleterious effect of AAS on the exercise-induced cardioprotection. Male Wistar rats were exercised and treated with either vehicle, nandrolone decanoate (10 mg/kg/week i.m.) or the same dose of nandrolone plus losartan or spironolactone (20 mg/kg/day orally) for 8 weeks. Langendorff-perfused hearts were subjected to I/R and evaluated for the postischaemic recovery of left ventricle (LV) function and infarct size. mRNA and protein expression of angiotensin II type 1 receptor (AT\textsubscript{1}-R), mineralocorticoid receptor (MR), and K\textsubscript{ATP} channels were determined by reverse-transcriptase polymerase chain reaction and Western blotting. Postischaemic recovery of LV function was better and infarct size was smaller in the exercised rat hearts than in the sedentary rat hearts. Nandrolone impaired the exercise-induced cardioprotection, but this effect was prevented by losartan (AT\textsubscript{1}-R antagonist) and spironolactone (MR antagonist) treatments. Myocardial AT\textsubscript{1}-R and MR expression levels were increased, and the expression of the K\textsubscript{ATP} channel subunits SUR2a and Kir6.1 was decreased and Kir6.2 increased in the nandrolone-treated rat hearts. The nandrolone-induced changes of AT\textsubscript{1}-R, MR, and K\textsubscript{ATP} subunits expression was normalized by the losartan and spironolactone treatments. The chronic nandrolone treatment impairs the exercise-induced cardioprotection against ischaemia/reperfusion injury by activating the cardiac renin-angiotensin-aldosterone system and downregulating K\textsubscript{ATP} channel expression [13561].

**Telmisartan**

Due to its PPAR-affecting properties, telmisartan (an angiotensin II receptor blocker, ARB) was suggested to be implemented into the class of hormone and metabolic modulators in accordance, for example, to GW1516. By its modus operandi, it would fit into the category of metabolic modulators; however, its structure is not related to any of the listed and thus
prohibited substances. Consequently, if evidence (rather than hypotheses) for performance-enhancing properties in healthy athletic individuals is given, an inclusion of the substance might follow [13012].

It has recently been shown that the angiotensin II receptor blocker telmisartan might induce similar biochemical, biological, and metabolic changes (e.g., mitochondrial biogenesis and changes in skeletal muscle fiber type) as those reported for the former call of substances. It was suspect that metabolic modulators abuse such as telmisartan might become a tangible threat in sports and should be thereby targeted as an important antidoping issue. The 2012 WADA prohibited list does not provide telmisartan for a potential doping drug, but arguments supporting the consideration to include them among "metabolic modulators" are at hand [12333].

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Experimental

It was evaluated the effects of swimming and anabolic steroids on ventricular function, collagen synthesis, and the local renin-angiotensin system in rats. Male Wistar rats were randomized into control (C), steroid (S; nandrolone decanoate; 5 mg/kg sc, 2x/wk), steroid + losartan (SL; 20 mg/kg/day), trained (T), trained + steroid (T+S), and trained + steroid + losartan (T+SL; n=14/group) groups. Swimming was performed 5 times/wk for 10 weeks. Serum testosterone increased in S and T+S. Resting heart rate was lower in T and T+S. Percent change in left ventricular (LV) weight-to-body weight ratio increased in S, T, and T+S. LV systolic pressure declined in S and T+S. LV contractility increased in T. LV relaxation increased in T. It was significantly lower in T+S compared with C. Collagen volumetric fraction (CVF) and hydroxyproline were higher in S and T+S than in C and T, and the CVF and LV hypertrophy were prevented by losartan treatment. LV-ANG I-converting enzyme activity increased (28 %) in the S group (33 %), and type III collagen synthesis increased (56 %) in T+S but not in T group. A positive correlation existed between LV-ANG I-converting enzyme activity and collagen type III expression. The ANG II and angiotensin type 1a receptor expression increased in the S and T+S groups but not in T group. Supraphysiological doses of AS exacerbated the cardiac hypertrophy in exercise-trained rats. Exercise training associated with AS induces maladaptive remodeling and further deterioration in cardiac performance. Exercise training associated with AS causes loss of the beneficial effects in LV function induced by exercising. These results suggest that aerobic exercise plus AS increases cardiac collagen content associated with activation of the local renin-angiotensin system [09290].
In sports, diuretics are used for two main reasons: to flush previously taken prohibited substances with forced diuresis and in sports where weight classes are involved to achieve acute weight loss. A common property observed for thiazides is hydrolysis in aqueous media resulting in the formation of the degradation product aminobenzenedisulphonamide. This degradation product can be observed for several thiazides. Because there is limited information regarding the effect of pH, temperature and light on the stability of thiazides, these parameters were investigated for chlorothiaizide, hydrochlorothiazide and altizide. For all three compounds the degradation product could be detected after incubation at pH 9.5 for 48h at 60 degrees C. At lower pH and temperature the degradation product could not be detected for all compounds. When samples were exposed to UV-light altizide and hydrochlorothiazide were photodegraded to chlorothiazide. When the degradation rate between the different compounds was compared for a given temperature and pH, altizide is the most unstable compound. This study confirms that thiazide degradation products can be formed in urine during transport [08356].

Diuretics are drugs that increase the rate of urine flow and sodium excretion to adjust the volume and composition of body fluids. There are several major categories of this drug class and the compounds vary greatly in structure, physicochemical properties, effects on urinary composition and renal haemodynamics, and site and mechanism of action. Diuretics are often abused by athletes to excrete water for rapid weight loss and to mask the presence of other banned substances. Because of their abuse by athletes, diuretics have been included on The World Anti-Doping Agency's (WADA) list of prohibited substances; the use of diuretics is banned both in competition and out of competition and diuretics are routinely screened for by anti-doping laboratories. One review provided an overview of the pharmacology and toxicology of diuretics and discusses their application in sports. The most common analytical strategies currently followed by the anti-doping laboratories accredited by the WADA are discussed along with the challenges laboratories face for the analysis of this diverse class of drugs [10383].

The variety of chemical structures of diuretic compounds has encouraged the development of new methods and techniques of analysis, especially as regards to acidic compounds. LC/MS has so grown to be the reference technique for this kind of analysis in forensic and anti-doping confirmation purposes. Multiple stage MS permits identification of single drugs with high selectivity, but some unexpected pathways could weaken the entire process. In one work it was explained some unusual fragmentation steps using high-resolution MSn. For example, in the case of amiloride an intense product ion in MS3 analysis generates an apparent loss of 10Da. Water adduct formation and successive carbon monoxide elimination can explain this uncommon behavior, which was studied using different ion traps. Bendroflumethiazide MSn spectra show instead three successive HF losses, in spite of the presence of a radical site in the parent structure. Homolytic cleavages with radical ion production occur also in the case of protonated positive ion of ethacrynic acid (loss of chlorine radical) showing that such fragmentation behavior is not so rare as generally reported. Different ionization modes were studied and a tentative correlation with acidic-base properties was done. Multiple stage high-resolution mass spectra of positive and negative ions may be discussed [08357].

In one paper, a sensitive, rapid and convenient analytical method for the determination of acidic (furosemide and bumetanide) and basic (triamterene) diuretics in human urine was developed by hollow fiber-based liquid-liquid-liquid microextraction (LLLME) coupled with
HPLC-UV. The LLLME conditions, such as the organic extraction solvent, the acidity and basicity of the donor- and acceptor-phases, stirring speed, extraction time and ionic strength, were studied in detail. Under the optimum conditions, the linear ranges of furosemide, bumetanide and triamterene were 1.2-250, 1.2-250 and 5.0-500 ng/mL, respectively. The detection limits were 0.5 ng/mL for furosemide, 1.2 ng/mL for bumetanide and 2.0 ng/mL for triamterene. The LLLME obtained a great improvement of the detection limits for all the analytes considered here, to the ng/mL level, which almost reaches the level of the LC-MS method. This new LLLME method provided very high enrichments: 117-fold for furosemide, 175-fold for bumetanide and 68-fold for triamterene. Since the hollow fiber membrane was sealed, it could be used for extracting the diuretics directly from "dirty" human urine samples without any clean-up procedures. The method was successfully applied to analyse the amounts of the three diuretics in real urine samples of volunteers after oral drug-taking [08358].

The reliability of ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) for high throughput screening in anti-doping control has been tested. A method to screen for the presence of diuretics and other doping agents in urine has been optimised and validated. The extraction procedure consisted of an alkaline extraction (pH 9.5) with ethyl acetate and salting-out effect (sodium chloride). The extracts were analysed by UPLC-MS/MS. Analysis of 34 forbidden drugs and metabolites was achieved in a total run time of 5 min, using a C18 column and a mobile phase containing deionised water and acetonitrile with formic acid, with gradient elution at a flow-rate of 0.6 mL/min. Identification of the compounds was performed by multiple reaction monitoring, using electrospray ionisation in positive- or negative-ion mode. Precursor and product ions were studied for each compound and cone voltage and collision energy were optimised. Due to the different chemical structure of the compounds under study, extraction recoveries varied from less than 10 to 100 percent depending on the analyte. The limits of detection ranged from 50 ng/mL to 200 ng/mL, and all the compounds comply with the requirements of quality established by the World Anti-doping Agency. Intra-assay precision was evaluated at two concentrations for each compound and, in most cases, a relative standard deviation of the signal ratio lower than 20 percent was obtained [08359].

Trace amounts of diuretics were determined in human urine by hollow fiber liquid-phase microextraction (LPME) combined with liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) in this study. Chromatography was performed on a C8 reversed-phase column. A 25 microL n-octanol was used to extract analytes in urine. Extraction was optimized using a pH 2 solution spiked with 0.15 g/mL NaCl for 40 min at 40 degrees C with 1010 rpm stirring. The limits of detection of diuretics in urine were 0.3-6.8 ng/mL, and linearity range was 1-1000 ng/mL. Recoveries of spiked 50 ng/mL diuretics were 98-103 percent. The diuretics concentration profiles in patient urine were also determined. The results of this study reveal the adequacy of LPME-LC-MS/MS method for analyzing diuretics in urine and quantification limits exceed WADA requirements [08360].

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analyzing diuretics in urine and quantification limits exceed World Anti-Doping Agency requirements [07199].

In sports, athletes abuse diuretics to reduce body weight so as to qualify themselves for a lower weight-class (such as boxing, weightlifting, and wrestling). Interestingly, diuretics are abused along with androgenic-anabolic steroids by bodybuilders to accentuate muscle definition and body tone as reported in this case. Practically, the most effective use of diuretics in sport doping is likely before an anti-doping test, as these agents reduce the urinary concentration of other prohibited substances and to avoid a positive doping result. Hence, use of all class of diuretics in sports is banned. Diuretics are also abused by models, ballet-dancers, or stewardesses for weight control purposes. Some of the challenges to diagnose diuretic abuse in the emergency department (ED) are related to an individual's behavior such as denial, intermittent use, or co-consumption with native plant materials and pharmacokinetics of these agents. It is important to note that the diuretics most abused by athletes have a short half-life and are therefore untraceable in urine if samples are not collected within 12 to 24 hours after the last administration. Furthermore, sustained exercise will decrease the renal and hepatic blood flow. Therefore, these substances are not always detected in urine samples collected post-competition or at the end of an intense training session. Despite diuretic misuse among sports persons, it is rarely mentioned in clinical teaching. A clinical picture of hypokalemic metabolic alkalosis is most often due to surreptitious vomiting, diuretic abuse, or Bartter's syndrome. These conditions can be differentiated by measuring urine chloride concentration. Low urinary chloride (<15 mEq/L) is seen in vomiting, whereas high urinary chloride is observed in the rest. For example urinary chloride level may vary between 20 and 70 mEq/L considerably in those persons using loop diuretics. Indeed, highly variable urinary chloride levels are not seen in any other conditions causing hypokalemic metabolic alkalosis. This has not received much attention in literature, although it provides an insight to diuretic abuse. As diuretic abuse often creates confusing clinical situations, more emphasis may be given to this easily obtained laboratory measurement as an indicator of diuretic abuse. A diurnal pattern of urinary chloride excretion may also be an adjunct to clinical diagnosis. Moreover laboratory aspects of diuretic misuse are not considered in busy ED, unless the emergency physicians are tuned their mind towards this entity [13801].

**Thiazides**

In sports, athletes abuse diuretics to reduce body weight so as to qualify themselves for a lower weight-class (such as boxing, weightlifting, and wrestling). Interestingly, diuretics are also abused along with androgenic-anabolic steroids by bodybuilders to accentuate muscle definition and body tone as reported in this case. Practically, the most effective use of diuretics in sport doping is likely before an antidoping test, as these agents reduce the urinary concentration of other prohibited substances and to avoid a positive doping result. Hence, use of all class of diuretics in sports is banned. Diuretics are also abused by models, ballet-dancers, or stewardesses for weight control purposes. Some of the challenges to diagnose diuretic abuse in the emergency department (ED) are related to an individual's behavior such as denial, intermittent use, or co-consumption with native plant materials and pharmacokinetics of these agents. It is important to note that the diuretics most abused by athletes have a short half-life and are therefore untraceable in urine if samples are not collected within 12 to 24 hours after the last administration. Furthermore, sustained exercise will decrease the renal and hepatic blood flow. Therefore, these substances are not always detected in urine samples collected post-competition or at the end of an intense training session. Despite diuretic misuse among sports persons, it is rarely mentioned in clinical
A clinical picture of hypokalemic metabolic alkalosis is most often due to surreptitious vomiting, diuretic abuse, or Bartter's syndrome. These conditions can be differentiated by measuring urine chloride concentration. Low urinary chloride (<15 mEq/L) is seen in vomiting, whereas high urinary chloride is observed in the rest. For example urinary chloride level may vary between 20 and 70 mEq/L considerably in those persons using loop diuretics. Indeed, highly variable urinary chloride levels are not seen in any other conditions causing hypokalemic metabolic alkalosis. This has not received much attention in literature, although it provides an insight to diuretic abuse. As diuretic abuse often creates confusing clinical situations, more emphasis may be given to this easily obtained laboratory measurement as an indicator of diuretic abuse. A diurnal pattern of urinary chloride excretion may also be an adjunct to clinical diagnosis. Moreover laboratory aspects of diuretic misuse are not considered in busy ED, unless the emergency physicians are tuned their mind towards this entity [12334].

In sports, thiazide diuretics are used to flush out previously taken prohibited substances with forced diuresis and in sports where weight classes are involved to achieve acute weight loss. Thiazide diuretics include compounds which are very unstable and hydrolyse in aqueous media. Because information regarding the urinary detection of the hydrolysis products is limited, urinary excretion profiles for the hydrolysis product 4-amino-6-chloro-1,3-benzenedisulphonamide were established in 6 healthy volunteers after oral administration of altizide (15 mg per tablet) and hydrochlorothiazide (25mg per tablet). Additionally, the excretion profile of chlorothiazide, a metabolite of altizide and hydrochlorothiazide, was also determined. A quantitative liquid-chromatographic tandem mass spectrometric method to detect the 4 substances was developed and validated. The result of this work shows that altizide is eliminated within 48 h in urine whereas hydrochlorothiazide was detectable after 120 h. Chlorothiazide was determined to be a minor metabolite of altizide and hydrochlorothiazide and could be detected up to 120 h. The hydrolysis product, 4-amino-6-chloro-1,3-benzenedisulphonamide, was detectable 120 h after administration, with concentrations at least 10 times higher than the parent drug. Concentrations ranged between 41-239 and 60-287 ng/mL after altizide and hydrochlorothiazide administration, respectively. The study shows that 4-amino-6-chloro-1,3-benzenedisulphonamide is an important target compound for the long time detection of thiazide diuretics in urine [09281].

Hydrochlorothiazide (HCTZ) has become by far the most commonly prescribed antihypertensive drug in the US. In 2008, 47.8 million prescriptions were written for HCTZ alone and 87.1 million prescriptions for HCTZ combinations. However, there is no evidence that HCTZ in its usual dose of 12.5-25 mg daily reduces myocardial infarction, stroke, or death. In a meta-analysis of 19 randomized trials with over 1400 patients, the 24-hour decrease in blood pressure with HCTZ was inferior to angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers, and calcium channel blockers. Even in combination with an angiotensin-converting enzyme inhibitor, HCTZ was found to reduce morbidity and mortality less well than a calcium channel blocker. As measured by the adherence rate, thiazides are less well tolerated than any other drug class. Because outcome data at the usual daily dose of 12.5-25 mg are lacking, antihypertensive efficacy is paltry, and adherence is poor, HCTZ is an inappropriate first-line drug in hypertension. If a "thiazide-type" diuretic is indicated, either chlorthalidone or indapamide should be selected [11512].

Rapid weight loss

One article briefly reviewed the weight loss processes in combat sports. It was aimed to discuss the most relevant aspects of rapid weight loss (RWL) in combat sports The review
was performed in the databases MedLine, Lilacs, PubMed and SciELO, and organized into sub-topics: prevalence, magnitude and procedures, psychological, physiological and performance effects, possible strategies to avoid decreased performance organizational strategies to avoid such practices. There was a high prevalence (50 %) of RWL, regardless the specific combat discipline. Methods used are harmful to performance and health, such as laxatives, diuretics, use of plastic or rubber suits, and sauna. RWL affects physical and cognitive capacities, and may increase the risk of death. Recommendations during different training phases, educational and organizational approaches were presented to deal with or to avoid RWL [12335].

**Mannitol**

The osmoïduretic mannitol can be potentially misused in sports, owing to its urine diluting effect and the possibility to decrease bodyweight. To reveal a doping offence, resulting urinary mannitol concentrations after a prohibited intravenous application and a permitted oral intake have to be differentiated. Therefore, a reliable gas chromatography-mass spectrometry (GC-MS) method was established based on peracetyl derivatives of the analytes. All possible hexitols (allitol, galactitol, iditol, altritols, sorbitol and mannitol) that can occur in human urine were separated and identified on a phenyl-methylpolysiloxane column (HP-5MS) within 10.75 min, and the method demonstrated its capability for quantification purposes. The lower limit of detection and lower limit of quantification were estimated at 0.9 microg/mL and 2.4 microg/mL, respectively, and the assay was validated for mannitol and sorbitol regarding the parameters specificity, linearity, intra- (<10 %) and inter-day precision (<15 %) and accuracy (92-102 %). To investigate urinary mannitol concentrations after oral intake the method was applied to an excretion study, providing a mean urinary excretion of mannitol of 20 percent. Comparison of theoretically expected urinary levels after a common therapeutic dose of mannitol and preliminary results on physiological urinary mannitol levels were promising, regarding a threshold level for mannitol that can be utilised for doping control purposes [08361].

**Laboratory techniques**

An analytical procedure was developed for the fast screening of 16 diuretics (acetazolamide, althiazide, amiloride, bendroflumethiazide, bumetanide, canrenonic acid, chlorthalidone, chlorthiazide, clopamide, ethacrynic acid, furosemide, hydrochlorothiazide, hydroflumethiazide, indapamide, triamterene, trichlormethiazide) and a masking agent (probenecid) in human urine. The whole method involves three analytical steps, including liquid/liquid extraction of the analytes from the matrix, their reaction with methyl iodide at 70 degrees C for 2 h to form methyl derivatives, and analysis of the resulting mixture by fast gas chromatography/electron impact mass spectrometry (fast GC/EI-MS). The analytical method was validated by determining selectivity, linearity, accuracy, intra and inter assay precision, extraction efficiencies and signal to noise ratio (S/N) at the lowest calibration level (LCL) for all candidate analytes. The analytical performances of three extraction procedures and five combination of derivatization parameters were compared in order to probe the conditions for speeding up the sample preparation step. Limits of detection (LOD) were evaluated in both EI-MS and ECNI-MS (electron capture negative ionization mass spectrometry) modes, indicating better sensitivity for most of the analytes using the latter ionization technique. The use of short columns and high carrier gas velocity in fast GC/MS produced efficient separation of the analytes in less than 4 min, resulting in a drastic reduction of the analysis time, while a resolution comparable to that obtained from classic GC conditions is
maintained. Fast quadrupole MS electronics allows high scan rates and effective data acquisition both in scan and selected ion monitoring modes [06216].
OTHER MASKING AGENTS

Masking agents that are forbidden in both in- and out-of-competition doping tests consist of a series of compounds that are misused in sports to mask the administration of other doping agents, and includes: diuretics, used to reduce the concentration in urine of other doping agents either by increasing the urinary volume or by reducing the excretion of basic doping agents by increasing the urinary pH; probenecid, used to reduce the concentration in urine of acidic compounds, such as glucuronoconjugates of some doping agents; 5alpha-reductase inhibitors, used to reduce the formation of 5alpha-reduced metabolites of anabolic androgenic steroids; plasma expanders, used to maintain the plasma volume after misuse of erythropoietin or red blood cells concentrates; and epitestosterone, used to mask the detection of the administration of testosterone. Diuretics may be also misused to achieve acute weight loss before competition in sports with weight categories [10199].

Epitestosterone

Epitestosterone (ET) has been used as a masking agent and prohibited by the World Anti-Doping Agency (WADA) because its administration will decrease the urinary testosterone/epitestosterone ratio, a marker of testosterone administration. In one study, an off-line immunoaffinity extraction coupled with high performance liquid chromatography (HPLC) was developed to quantify the endogenous steroid ET in human urine. The immunoaffinity column (IAC) was prepared by immobilizing the anti-ET monoclonal antibodies on CNBr-activated Sepharose 4B, which can remove the contaminations and non-target compounds from matrix to enrich the target analyte ET. The mobile phase was ammonium acetate at an isocratic flow of 1.0 mL/min and the UV absorbance detection wavelength was 244 nm for the detection of ET. The IAC showed good reliability and durability since it had been used for more than 100 runs in a year. The limit of quantification (LOQ) was 1 ng/mL. Satisfied repeatability and precision of the day-to-day and within-day were obtained with the RSD values less than 10 percent. Results of the recovery of the urine samples were ranged from 98 to 102 percent with repeatability less than 9 percent, indicating that the method developed can be used for the real urine sample analysis [10200].

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Proteases
Manipulation of urinary samples in sports drug testing with proteases is being used by athletes as masking agents to complicate or inhibit the detection of peptide hormones and proteins such as erythropoietin, human growth hormones or insulin. A great number of strategies have been suggested to prevent the use of proteases. The main problem in the use of proteases is that athletes have usually introduced proteases into the urethra before delivering a doping control urine sample. It was propose a viable, relatively simple, and inexpensive solution to prevent the use of proteases in sports [10202].

Desmopressin

Blood doping improves physical performance in sport. This is the reason why the antidoping authorities subject athletes to blood tests. Plasma volume expanders are prohibited agents used to reduce an artificial increase in hematological values using different illegal practices. The aim of our study was to test whether desmopressin (DDAVP)-induced hemodilution would alter the concentration of hematological parameters used to detect blood doping in sports. This was an intra-subject crossover study. Venous blood samples were obtained from eight physically active males on two occasions. On the first occasion the subjects ingested 1.5 L of mineral water and 4.3 microg/kg of DDAVP. On the second occasion the subjects ingested 1.5 L of mineral water. The samples were analyzed for hematocrit, hemoglobin, reticulocytes, OFF Hr-Score, glucose, albumin, creatinine and total proteins. After treatment with DDAVP it was found a significant decrease in the hematocrit, hemoglobin and in the OFF Hr-Score values. It was also found a significant decrease in glucose, albumin, creatinine and total proteins concentration; however, in this case, all the values were significantly below the physiological levels. Treatment with DDAVP has a very effective hemodilution effect [10204].

Desmopressin is a peptidic drug with antidiuretic properties, which can be administered either orally, intranasally, or intravenously. In 2011, a method allowing for the determination of 20 pg/mL of desmopressin in urine was presented, employing weak cation exchange SPE followed by liquid chromatography- ESI-QTOF-MS/MS. Applied to authentic elimination study urine samples, the drug was detected up to 22 h following intranasal or oral administration. A similar methodology for plasma was evaluated in 2012, where protein precipitation followed by weak cation exchange and subsequent LC-QqQ-MS/MS was used allowing for LODs of 50 pg/mL. Due to the comparably low concentration of desmopressin in plasma after therapeutic dosing, only intravenous applications resulted in detectable amounts of the banned substance in three different administration studies [12017].

The World Anti-Doping Agency (WADA) has recently added desmopressin, a synthetic analogue of the endogenous peptide hormone arginine vasopressin, to the Prohibited List, owing to the potential masking effects of this drug on hematic parameters useful to detect blood doping. A qualitative method for detection of desmopressin in human urine by high-performance liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) has been developed and validated. Desmopressin purification from urine was achieved by means of delipidation with a 60:40 di-isopropyl ether/n-butanol and solid-phase extraction with WCX cartridges. The lower limit of detection was 25 pg/mL. Extraction recovery was determined as 59 percent, and signal reduction owing to ion suppression was estimated to be 43 percent. The applicability of the method was proven by the analysis of real urine samples obtained after intravenous, oral and intranasal administration of desmopressin, achieving unambiguous detection of the peptide in all the cases [12339].
One work described a liquid chromatography-electrospray tandem mass spectrometry method for detection of desmopressin in human plasma in the low femtomolar range. Desmopressin is a synthetic analogue of the antidiuretic hormone arginine vasopressin and it might be used by athletes as a masking agent in the framework of blood passport controls. Therefore, it was recently added by the World Anti-Doping Agency to the list of prohibited substances in sport as a masking agent. Mass spectrometry characterization of desmopressin was performed with a high-resolution Orbitrap-based mass spectrometer. Detection of the peptide in the biological matrix was achieved using a triple-quadrupole instrument with an electrospray ionization interface after protein precipitation, weak cation solid-phase extraction and high performance liquid chromatography separation with an octadecyl reverse-phase column. Identification of desmopressin was performed using three product ions, m/z 328.0, m/z 120.0, and m/z 214.0, from the parent ion, m/z 535.5. The extraction efficiency of the method at the limit of detection was estimated as 40 percent (n=10), the ion suppression as 5 percent (n=10), and the limit of detection was 50 pg/mL (signal-to-noise ratio greater than 3). The selectivity of the method was verified against several endogenous and synthetic desmopressin-related peptides. The performance and the applicability of the method were tested by analysis of clinical samples after administration of desmopressin via intravenous, oral, and intranasal routes. Only after intravenous administration could desmopressin be successfully detected [12340].

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While diuretics are commonly included in multi-analyte screening procedures, two compounds (desmopressin and glycerol) potentially masking other doping measures necessitate more dedicated approaches. Desmopressin is a peptidic drug with antidiuretic properties, which can be administered either orally, intranasally, or intravenously. In 2011, a method allowing for the determination of 20 pg/ml of desmopressin in urine was presented, employing weak cation exchange SPE followed by liquid chromatography-ESI-QTOF-MS/MS. Applied to authentic elimination study urine samples, the drug was detected up to 22 h following intranasal or oral administration. A similar methodology for plasma was evaluated in 2012, where protein precipitation followed by weak cation exchange and subsequent LC-QqQ-MS/MS was used allowing for LODs of 50 pg/mL. Due to the comparably low concentration of desmopressin in plasma after therapeutic dosing, only intravenous applications resulted in detectable amounts of the banned substance in three different administration studies [13012].

Despite the facts that diuretics are readily detected with established analytical approaches in doping controls and severe side effects of diuretics abuse (such as temporary paralysis due to diuresis-induced hypokalemia) are sporadically reported, hundreds of AAFs were reported again in 2012. A more challenging situation is presented by masking agents such as the anti-diuretic drug desmopressin, a peptide hormone-based therapeutic that either necessitates a dedicated sample preparation and analysis or an implementation in a multi-analyte peptide
screening procedure. It was suggested an LLE-based delipidation of urine followed by weak cation exchange SPE to isolate desmopressin and its internal standard (deamino-Cys¹,Val⁴,D-Arg⁸-vasopressin) from 3 mL of urine. The analysis of the extract is subsequently conducted on a C-18 column (1 x 50 mm, particle size 3.5 microm) interfaced via ESI to a QqQ analyzer, allowing for detection limits for desmopressin of 25 pg/mL. The accomplished sensitivity is sufficient to determine the analyte in post-administration urine samples collected after intranasal, oral, and intravenous application of therapeutic doses [13009].

Other chemical and physical manipulation of doping tests

In 2009/2010, identical steroid profiles of supposedly eight different athletes (from different teams and collection sites) were found and DNA analyses requested, demonstrating that all eight urine specimens were provided from a single donor. This donor was eventually identified as the doping control officer and none of the athletes was actively involved in the sample manipulation. In another case of urine substitution, no natural endogenous steroid was observed in steroid profile analyses, triggering further investigations into the composition of the specimen. Based on findings of hordenine, trace amounts of alcohol, various saccharides and intact proteins including Serpin-Z4, the liquid was identified as non-alcoholic beer. This manipulation however entailed the suspension of the athlete [13012].
GLUCOCORTICOSTEROIDS

Overview

In addition to their therapeutic applications, glucocorticosteroids have been widely used and abused in the belief that these substances may enhance athletic performance. Analysis of athlete urine samples by antidoping laboratories around the world support this conclusion. It is commonly accepted in medical practice to use local glucocorticosteroid injections in the treatment of non-infectious local musculotendinous inflammatory conditions conveying symptom relief and often a speedier return to sporting activity. This practice is not to be considered illicit, but sports physicians must accept that such an intervention is not in itself an immediate cure and that an athlete will still require a period of recuperation before continuing sporting activity. How long such a period of recuperation should last is a matter of conjecture and there is little concrete data to support what is, or what is not, an acceptable period of inactivity. In the interest of athlete safety, we would propose to maintain systemic glucocorticosteroids on the World Anti-Doping Agency (WADA) list of prohibited substances, both in and out-of-competition as well as a mandatory period of 48 hours of rest from play after receiving a local glucocorticosteroid injection [12320].

Glucocorticoids are listed on the World Anti-Doping Agency (WADA) Prohibited List of substances. The detection of the administration of hydrocortisone and cortisone is complicated by the fact that the human body also produces these steroids naturally. Gas chromatography-combustion-isotope ratio mass spectrometry can be utilized to determine the use of endogenous glucocorticoids by measuring the carbon isotope ratio (CIR) of their resulting metabolites in human urine samples. A comprehensive sample preparation protocol for the analysis of endogenous glucocorticoid urinary metabolites was developed and validated, incorporating the use of high performance liquid chromatography (HPLC) for purification and chemical oxidation for derivatisation. Target compounds were tetrahydrocortisol and tetrahydrocortisone, and 11beta-hydroxyetiocholanolone, 11-oxoetiocholanolone and 11beta-hydroxyandrostosterone, while pregnanediol functioned as the endogenous reference compound. Urine samples from a population of 50 volunteers were analyzed to determine CIR reference limits. Excretion studies of the endogenous glucocorticoid preparation cortisone acetate (25 mg oral) and the dietary supplement adrenosterone (75 mg oral) were conducted with six male individuals. Variable changes in steroid metabolite isotopic composition were found across subjects after administration. The study also revealed that CIR analysis of the major glucocorticoid metabolites tetrahydrocortisol and tetrahydrocortisone is necessary to unambiguously distinguish administration of cortisone and adrenosterone, the former officially restricted to out-of-competition use by athletes, the latter not being restricted at the current time. Moreover, this study reaffirms that CIR methods for the doping control of endogenous steroids should not rely upon a single ERC, as the administration of an appropriate precursor to that ERC could cause complications during analysis [12321].

Systemic administration of glucocorticoids (GCs) is banned by the World Anti-Doping Agency (WADA) during competition. Few studies have examined the effects of GCs on exercise performance, but increasing evidence has shown that short-term GC intake enhances performance in animals and humans. However, there are many health risks associated with GC use. Based on the available evidence, as presented in one article, it was concluded that GCs are doping agents and should remain on the WADA's list of banned products. Because of the complexity of GCs, however, determining the boundaries between their medical use and abuse (eg, in sports) is a constant challenge for the WADA [10487].
Hair has long been used in toxicology, forensic science, doping control and other fields as a biological specimen for the detection of environmental agents, drugs, or toxins. Most recent evidence suggests that also hormones are incorporated and trapped inside the growing hair. This has led to the hypothesis that cortisol measurement of distinct hair segments could provide a retrospective calendar of cortisol production for the individual. In this first proof-of-concept study in humans, it was analyzed cortisol in hair donated by mothers with a neonate child (n-Mothers=103), mothers with toddlers 3-9 months of age (t-Mothers=19), and control women (n=20). It was cut hair strands from each women into at least three 3-cm segments, which, based on an average hair growth rate of 1 cm per month, would represent hair grown over the past three, six, and nine months, respectively. Since in the third trimester of pregnancy there is a well-documented increased production of cortisol, it was expected to see elevated levels of cortisol in the most proximal hair segment of women who had just given birth to a child (n-Mothers) compared with the control women. Likewise, it was expected to see elevated levels in the second, third, or fourth segment of mothers of 3-month olds, 6-months olds, and 9-months olds, respectively. These hair segments, cut at 4-12cm from the scalp, would represent hair grown throughout the third trimester of pregnancy. Results showed that there was a strong monotonic decline in cortisol concentration from the segment closest to the scalp to the most distal hair segment. Cortisol levels decreased by 30-40 percent from one segment to the next for the most recent four hair segments. Segments from hair older than one year had similarly, low levels of cortisol. Comparisons of cortisol levels in hair between n-Mothers and control women yielded the expected results: cortisol levels in the first 3-cm hair segment (i.e. closest to the scalp) of n-Mothers were two-fold higher than in controls, probably reflecting increased cortisol levels throughout the third trimester of pregnancy. No differences in cortisol content were apparent for the second or third 3-cm segments in n-Mothers. When hair from mothers with 6-9 months old toddlers was analyzed, the hair segment representing the third trimester period contained the same amount of cortisol as the hair grown more recently in mothers with 3-4 months old toddlers only. Age of the women, hair curvature, hair color, and frequency of hair washes per week were unrelated to cortisol levels. It was conclude that cortisol measured in human hair can be a valid reflection of increased cortisol production for a period of up to six months. Due to a rapid decline of cortisol levels in human adult hair, a retrospective calendar of cortisol exposure may be limited to the past six months [08363].

One paper described a liquid chromatographic/tandem mass spectrometric (LC/MS-MS) method specifically designed for the screening of synthetic glucocorticosteroids in human urine. The method is designed to recognize a common mass spectral fragment formed from the particular portion of the molecular structure that is common to all synthetic glucocorticosteroids and that is fundamental to their pharmacological activity. As such, the method is also suitable for detecting unknown substances, provided they contain the portion of the molecular structure selected as the analytical target. The effectiveness of this approach was evaluated on seventeen synthetic glucocorticosteroids. Urine samples, including blank urines spiked with one or more synthetic glucocorticosteroids, were treated according to a standard procedure (enzymatic hydrolysis, liquid/liquid extraction and evaporation to dryness) and analyzed using LC/MS-MS with electrospray ionization (ESI). MS-MS acquisition was carried out in a precursor ion scan, and the results were compared with those obtained by a previously developed reference technique based on acquisition in the multiple reaction monitoring (MRM) mode. All of the glucocorticosteroids considered in this study are clearly detectable in urine, with a limit of detection in the concentration range 5-20 ng/mL, depending on the glucocorticosteroid structure. The proposed method is therefore suitable for the detection of glucocorticosteroids in urine samples taken for "in competition" sport anti-doping control tests, matching the requirements of the World Anti-Doping Agency (WADA) for accredited anti-doping laboratories [08364].
Glucocorticoid hormones are important regulators of several physiological processes. Despite having been initially named based on their role in glucose metabolism, glucocorticoids are also fundamental in the regulation of developmental, metabolic, and neurobiological processes, as well as several other biological functions. Due to their involvement in a diverse array of biological pathways, its wide spectrum of action, it is predicted that a wide range of genes may have their expression regulated by the activated glucocorticoid receptor (GR). In fact, it has been demonstrated that in addition to the regulation of several effectors genes, the expression of the gene encoding for GR itself is regulated by physiological stimuli and fine-tuning mechanisms. Importantly, such generalized effector responses and fine-tuning responses seem to be largely mediated by mechanisms of gene regulation. Therefore, one review aimed to describe the mechanisms of gene regulation by glucocorticoid hormones, which are capable of regulating differential gene transcription, within a physiological context. From this discussion, it was hoped to shed light on how a single molecule that is capable of exerting such divergent effects is also capable of promoting such distinct responses in different target tissues [10377].

To examine variations in neuromuscular and hormonal status and their relationship to performance throughout a season of elite Australian Rules Football 15 elite players performed a single jump and 5 repeated countermovement jumps, and provided saliva samples for the analysis of cortisol and testosterone before the season commenced and during the 22-match season. It was concluded that the response of single jump flight time/contraction time suggests periods of neuromuscular fatigue. Change in testosterone to cortisol indicates subjects were unlikely to have been in a catabolic state during the season. Increase in cortison during the season compared with before had a small negative correlation with performance [09228].

Edge plane pyrolytic graphite electrode (EPPGE) modified with single-wall carbon nanotubes (SWNTs) has been used as a sensor to determine triamcinolone, abused by athletes for doping. A comparison of the voltammetric behavior between SWNTs modified EPPGE and fullerene - C(60)-modified EPPGE indicated that SWNTs modified EPPGE is more sensitive. The electrode exhibited an effective catalytic response with good reproducibility and stability. The effect of several parameters such as pH, square wave frequency and steroid concentration were studied. The square wave voltammetric response of the electrode to triamcinolone is linear in the range 0.1-25 nM with a detection limit and sensitivity of 8.9 x 10^7 to 2.06 microA per nM, respectively. The method was applied for the determination of triamcinolone in several commercially available pharmaceuticals and real urine samples obtained from patients undergoing pharmacological treatment with triamcinolone. A comparison of the observed results with HPLC analysis indicated a good agreement. The product obtained after reduction of triamcinolone was also characterized using 1H NMR and GC-MS and the site of reduction is found to be carbonyl group at position 20. The method described is rapid, simple and accurate and can be easily applied for detecting cases of doping [09229].

One study investigated whether short-term oral administration of glucocorticoid would modify performance and selected hormonal and metabolic parameters during submaximal exercise in healthy women. Nine recreational female athletes completed cycling trials at 70-75 percent VO2max until exhaustion after either placebo (Pla, gelatin) or oral prednisone (Cor, Cortancyl, 50 mg per day for 1 week) treatment, according to a double-blind and randomized protocol. Blood samples were collected at rest; after 10, 20, and 30 min of exercise; at exhaustion; and after 10 and 20 min of passive recovery for adrenocorticotropic hormone (ACTH), dehydroepiandrosterone (DHEA), prolactin (PRL), growth hormone (GH), insulin (Ins), blood glucose (Glu), and lactate (Lac) determination. Cycling time was significantly increased with
short-term prednisone intake. ACTH and DHEA remained completely blunted throughout the experiment with prednisone versus placebo whereas GH and PRL were significantly decreased with prednisone after, respectively, 20 and 30 min of exercise. No significant difference in insulin or blood glucose values was found between the two treatments but lactate concentrations were significantly increased with prednisone versus placebo between 10 and 30 min of exercise. These data indicate that short-term glucocorticoid intake improved endurance performance in women, but further investigation is needed to determine whether these results are applicable to elite female athletes and, if so, current WADA legislation needs to be changed [09230].

Certain international sports federations are requesting that glucocorticoids (GCs) be removed from the World Antidoping Agency's list of banned products. Their arguments are based on the fact that GCs are in widespread use in sports medicine and have no demonstrated ergogenic activity. However, there is scientific evidence that GCs mediate ergogenic effects in animals and humans. Moreover, the health risks of using GCs are well characterized. GCs are doping agents and should remain on the World Antidoping Agency's list of banned products [10161].

Doping control samples are normally collected under non-sterile conditions and sometimes, storage and transportation are influenced by parameters such as the temperature. Therefore, microbial contamination and subsequent alteration of a sample’s composition are possible. Studies regarding sample collection in cattle breeding have already shown enzymatic transformation of endogenous testosterone to boldenone causing false-positive findings. The aim of one study was to investigate whether positive doping cases with the synthetic corticosteroids prednisolone and prednisone may result from microbial transformation of the endogenous corticosteroids cortisol and cortisone, respectively. A method comprising parameters such as pH values and screening results for synthetic glucocorticosteroids as well as incubation experiments followed by liquid chromatographic and mass spectrometric analysis was employed to test for contaminating germs with delta-1-dehydrogenase activity. Over 700 urine samples comprising inpatient and doping control specimens were investigated. In none of them, 1,2-dehydrogenating activity was confirmed. These findings are in accordance with other studies. However, the problem of microbial alteration of doping control specimens with special respect to 1,2-dehydrogenation must not be underestimated [10162].

Glucocorticoid (GC) is an adrenal steroid hormone that controls a variety of physiological processes such as metabolism, immune response, cardiovascular activity, and brain function. In addition to GC induction in response to stress, even in relatively undisturbed states its circulating level is subjected to a robust daily variation with a peak around the onset of the active period of the day. It has long been believed that the synthesis and secretion of GC are primarily regulated by the hypothalamus-pituitary-adrenal (HPA) neuroendocrine axis. However, recent chronobiological research strongly supports the idea that multiple regulatory mechanisms along with the classical HPA neuroendocrine axis underlie the diurnal rhythm of circulating GC. Most notably, recent studies demonstrate that the molecular circadian clockwork is heavily involved in the daily GC rhythm at multiple levels. The daily GC rhythm is implicated in various human diseases accompanied by abnormal GC levels. Patients with such diseases frequently show a blunted GC rhythmicity and, more importantly, circadian rhythm-related symptoms. In one review, it was focused on recent advances in the understanding of the circadian regulation of adrenal GC and its implications in human health and disease [11175].

Corticosteroid-binding globulin (CBG) binds cortisol with high affinity and facilitates its transport in the blood. A recent discovery suggests that CBG may have a role beyond that of
a simple transport carrier protein. CBG functions as a protein thermocouple that is exquisitely sensitive to temperature change, releasing cortisol in response to increasing temperatures within the human physiological range. It is also expressed in the human hypothalamus and cerebrospinal fluid, while in the rodent it is also found in other intracellular neuronal locations, suggesting a role in regulating access of glucocorticoids to their receptors in the CNS. Genetic variants of CBG have been detected in man and have been associated with fatigue-pain syndromes and hypotension, again suggesting a potential effect of CBG on the access of cortisol to brain glucocorticoid receptors. These new findings provide the basis for a novel concept of the mechanisms through which the body regulates access of glucocorticoids to the brain and other tissues of the body [11176].

Changes in glucocorticoid (GC) receptor sensitivity can be categorized in three different types. First, generalized GC resistance syndrome is a hereditary disease. Patients present with signs and symptoms of increased androgen and/or mineralocorticoid action, combined with biochemical hypercortisolism, but lack of cushingoid features. Second, at a tissue level, transient changes in GC sensitivity are present during disease. Transient changes in GC sensitivity of leukocytes during infectious diseases like sepsis have been found, but also acquired forms or GC resistance occur, in particular in some types of neoplasms, major depression, AIDS, and several autoimmune diseases. Third, at the level of the general population, the diversity in GC sensitivity has a wide interindividual variation which in part can be explained by genetic variation of the GC receptor gene. Several single nucleotide polymorphisms of the gene have been associated with changes in GC sensitivity and its clinical phenotype. Four genetic variants are described of which two (rs6198 and rs6189/6190) are associated with a relative GC resistance and two (rs1695 and rs41423247) are associated with a relative GC hypersensitivity [11177].

One review considers the problem of the serious concomitant side effects of powerful anti-inflammatory drugs modelled upon the principal human glucocorticoid hormone, cortisol. The very nature of the original bio-assays to validate their cortisol-like hormonal and anti-inflammatory activities ensured that pleiotropic toxins were selected for clinical studies. Other complicating factors have been (1) considerable reliance on bio-assays conducted in laboratory animals that primarily secrete corticosterone, not cortisol, as their principal anti-inflammatory adrenal hormone; (2) some differences in the binding of xenobiotic cortisol analogues (vis à vis cortisol) to transport proteins, detoxifying enzymes and even some intracellular receptors; (3) the "rogue" properties of these hormonal xenobiotics, acting independently of—but still able to suppress—hormonal mechanisms regulating endogenous cortisol; and (4) problems of intrinsic/acquired "steroid resistance", diminishing their clinical efficacy, but not necessarily all their toxicities. The rather gloomy conclusion is that devising new drugs to reproduce the effect of multi-potent hormones may be a recipe for disaster, in contexts other than simply remedying an endocrine deficiency. Promising new developments include "designed" combination therapies that allow some reduction in total steroid doses (and hopefully their side effects); sharpening strategies to limit the actual duration of steroid administration; and resurgent interest in searching for more selective analogues (both steroidal and non-steroid) with less harmful side effects. Some oversights and neglected areas of research are also considered. Overall, it now seems timely to engage in some drastic rethinking about (retaining?) these "licensed toxins" as fundamental therapies for chronic inflammation [11178].

Similar to discussions as to whether cannabinoids should be banned in elite sport, glucocorticosteroids have also caused numerous controversies. In a recent editorial, the importance of controlling the use and misuse of glucocorticosteroids was presented from a sports medicine viewpoint and issues associated particularly with long-term/high-dose abuse were broached. Concerning analytical challenges, the possibility of microbial transformation
of cortisol to prednisolone was investigated. A series of micro-organisms possess delta-
steroid dehydrogenase activity and, thus, could potentially convert endogenous (urinary) cortisol into the prohibited substance prednisolone. Using in vitro methodologies, the conversion of deuterated cortisol to deuterated prednisolone was unambiguously demonstrated; however, so far no urine sample was found to contain the currently known germs allowing for the transformation of cortisol to prednisolone [13012].

The risk of infection is increased in patients treated with glucocorticoids, especially in those taking long-term and high dosage treatment. However, there is little valid practice for the prevention of infections in this patient population. The risk of reactivation or worsening of a latent infection (e.g. hepatitis B, tuberculosis, strongyloidiasis) is proved and individual reflection should be conducted in at-risk patients. Preventions of Pneumocystis jiroveci or upper urinary tract infections are considered differently according to practitioners’ habits and their specialties. Adequate prevention should be prescribed in glucocorticoid-treated patients who have been in contact with varicella zoster or measles virus. Many vaccines could be prescribed in those people but live vaccines should be avoided. A consultation of travel medicine should be systematically proposed before a travel in intertropical zone. Anti-inflammatory and stimulant properties of glucocorticoids are frequently misused in order to improve sport performances. All glucocorticoids are considered as performance-enhancing drugs. Their prescription should therefore be adapted to the laws in force in the sport. By reducing vomiting and pain, glucocorticoids may be beneficial in patients undergoing surgery. However, in people prescribed long-term glucocorticoid therapy, the risk of postoperative adrenal insufficiency has to be considered, even though very few data are available on this topic. Oral contraceptives or intra-uterine devices are effective contraceptives methods in patients treated with systemic glucocorticoids [13550].

The use of glucocorticosteroids is manifold in different fields of medicine, and their therapeutic potential has been utilized as well as abused also in elite sport. In order to prevent inadvertent doping and to ensure best practice in asthma care and other health conditions to athletes, guidelines concerning the use and declaration of glucocorticosteroids have been established by WADA. These have been shown to substantially influence, for example, asthma management in sport. For doping control laboratories, the main challenge concerning this class of compounds resides in the differentiation of natural/endogenous corticosteroids and their synthetic preparations, especially regarding cortisol and, as recently corroborated, the natural or artifact-derived occurrence of prednisolone in human urine [13009].

An additional issue with glucocorticosteroid analysis in doping controls is the fact that systemic administrations are prohibited while topical applications are not, and viable analytical means to differentiate these routes of administration are desirable. In this context, it was therefore conducted elimination studies with methylprednisolone and studied the relative and absolute amount of the intact drug as well as its main urinary metabolites. Comparing the metabolic profiles, two analyte candidates were suggested to support distinguishing systemic (oral) and topical use of the drug, namely 16beta,17alpha,21-trishydroxy-6alpha-methylpregna-1,4-diene-3,11,20-trione and 17alpha,20alpha,21-trishydroxy-6alpha-methylpregna-1,4-diene-3,11-dione. These metabolites were predominantly observed after oral application and only at trace amount level following topical administration [13009].

Stimulation of myocytes
Muscle wasting is associated with poor prognosis in chronic obstructive pulmonary disease (COPD). Exercise stimulates muscle recovery but its efficacy is variable depending on the clinical condition and medical treatment. Systemic glucocorticoids, commonly administered in high-doses during acute disease exacerbations or as maintenance treatment in end-stage disease are known to contribute to muscle wasting. As muscle mass recovery involves IGF-I signaling, which can be stimulated by anabolic steroids, the impact of glucocorticoids and the effect of simultaneous IGF-I stimulation by anabolic steroids on muscle recovery and growth were investigated. The effects of and interactions between glucocorticoid and IGF-I signaling on skeletal muscle growth were assessed in differentiating C2C12 myocytes. As proof-of-principle, it was performed a post-hoc analysis stratifying patients by glucocorticoid use, of a clinical trial investigating the efficacy of anabolic steroid supplementation on muscle recovery in muscle wasted patients with COPD. Glucocorticoids strongly impaired protein synthesis signaling, myotube formation and muscle-specific protein expression. In contrast, in presence of glucocorticoids, IGF-I synergistically stimulated myotube fusion and myofibrillar protein expression, which corresponded with restored protein synthesis signaling by IGF-I and increased transcriptional activation of muscle-specific genes by glucocorticoids. In COPD patients on maintenance glucocorticoid treatment, the clinical trial also revealed an enhanced effect of anabolic steroids on muscle mass and respiratory muscle strength. In conclusion, synergistic effects of anabolic steroids and glucocorticoids on muscle recovery may be caused by relief of the glucocorticoid-imposed blockade on protein synthesis allowing effective translation of glucocorticoid-induced accumulation of muscle-specific gene transcripts [12322].

Effects on exercise performance

To study the effects of a therapeutical dose of corticosteroid alone or associated with beta-2 agonist on performance and substrate response during intense submaximal exercise, seven healthy moderately trained male volunteers participated in the double-blind randomized cross-over study. An intense endurance exercise test to exhaustion was performed after ingestion of placebo (Pla), 20 mg prednisolone (Pred), and 20 mg prednisolone plus 4 mg salbutamol (Pred-Sal). Blood samples were collected at rest, after 5, 10 min of exercise, at exhaustion, and after 5 (r5), 10 (r10), and 20 (r20) min of passive recovery for ACTH, growth hormone, insulin, blood glucose, and lactate measurements. There were no significant differences in exercise time to exhaustion between the three treatments. ACTH was significantly lowered after Pred and Pred-Sal vs. Pla from the start of exercise to the end of the experiment. Pred and Pred-Sal increased resting and recovery (r10 and r20) significantly but not exercise blood glucose values. There were no significant differences in growth hormone concentrations between the three treatments whereas insulin was significantly higher at rest, during exercise, and at r20 after Pred-Sal administration versus Pred and Pla. Pred and Pred-Sal showed no significant effect on blood lactate compared with Pla treatment. These preliminary results do not support the hypothesis that acute oral therapeutic corticosteroid intake alone or associated with beta-2 mimetic improves performance during intense submaximal exercise, but further studies are necessary with tests of longer duration [06210].

To examine whether acute glucocorticoid (GC) intake alters performance and selected hormonal and metabolic variables during submaximal exercise in total, 14 recreational male athletes completed two cycling trials at 70-75 percent maximum O$_2$ uptake starting 3 h after an ingestion of either a lactose placebo or oral GC (20 mg of prednisolone) and continuing until exhaustion, according to a double-blind randomised protocol. Blood samples were collected at rest, after 10, 20, 30 minutes, and at exhaustion and recovery for measurement
of growth hormone (GH), adrenocorticotropic hormone (ACTH), dehydroepiandrosterone (DHEA), prolactin, insulin, blood glucose, lactate and interleukin (IL) -6 determination. Cycling duration was not significantly changed after GC or placebo administration. A significant decrease in ACTH and DHEA was observed with GC during all of the experiments and in IL-6 after exhaustion. No change in basal, exercise or recovery GH, prolactin, insulin or lactate was found between the two treatments but blood glucose was significantly higher with GC at any time point. From these data, acute systemic GC administration does seem to alter some metabolic markers but did not influence performance during submaximal exercise [07181].

To examine the prednisolone's ergogenic and metabolic effects during submaximal exercise ten recreational male athletes completed two cycling trials at 70-75 percent peak O2 consumption until exhaustion after either placebo (Pla, lactose) or oral prednisolone (Pred, 60 mg/day for 1 week) treatment, according to a double-blind and randomized protocol. Blood samples were collected at rest and during exercise and recovery to determine ACTH, growth hormone (GH), prolactin (PRL), DHEA, insulin, blood glucose, and blood lactate values. Time of cycling was significantly increased after chronic Pred treatment (Pred: 75; Pla: 46). Pred intake significantly lowered basal, exercise, and recovery ACTH, DHEA, and PRL concentrations, whereas GH concentrations were significantly lowered by Pred after 30 min of exercise. Blood glucose and insulin were significantly increased by Pred during the whole experiment and until 30 min of exercise. Blood lactate concentrations were higher after Pred versus Pla at 10 min of exercise until 10 min of recovery. From these data, short-term Pred intake did seem to significantly improve performance during submaximal exercise, with concomitant alterations in hormonal and metabolic responses. Further studies will be necessary to elucidate the mechanisms of these hormonal and metabolic changes, and to determine whether the changes may be associated with the marked performance improvement obtained [07182].

**Adrenal insufficiency after use of corticosteroids**

The frequent use of glucocorticoids by athletes necessitates testing for adrenal insufficiency because of the risk of death in cases of associated severe stress (trauma, infection). During the 2001 and 2002 sporting seasons, we assessed the value of measuring baseline serum cortisol concentrations and the frequency of corticosteroid use during compulsory medical tests carried out by the French Cycling Federation on 659 elite cyclists (585 men and 74 women); the risk of adrenal insufficiency is negatively correlated with the basal serum cortisol level. Adrenal insufficiency was suspected in 34 cyclists (5%; 22 in 2001 and 12 in 2002) on the basis of below normal cortisol concentrations and in three cyclists (in 2001) because they had received corticosteroid treatment. In 2001, 10 of the 25 cyclists convoked underwent baseline follow-up serum cortisol determinations and 15 underwent dynamic exploration of adrenal function with the short ACTH test. Adrenal function was found to be deficient in four of these cyclists, at the limits of the normal range in four and normal in seven. Based on these results, the FFC sent a questionnaire in 2002 to all the cyclists to assess the use of corticosteroid in this population. This survey revealed that 85 of 538 cyclists (16%) had received corticosteroid treatment in the previous 3 months. Moreover, 11 of the 12 cyclists (92%) with low basal serum cortisol concentrations had received corticosteroid therapy. These results show that basal serum cortisol is relevant to detect adrenal insufficiency in sportsmen, in particular in cases of values below the normal range. The high frequency of corticosteroid use among elite cyclists, and in particular road cyclists who are at risk of trauma and infection, justifies screening tests to detect adrenal insufficiency [06211].

*Corticosteroid-induced adrenal insufficiency in elite cyclists*
The frequent use of glucocorticoids by athletes necessitates testing for adrenal insufficiency because of the risk of death in cases of associated severe stress (trauma, infection). During the 2001 and 2002 sporting seasons, we assessed the value of measuring baseline serum cortisol concentrations and the frequency of corticosteroid use during compulsory medical tests carried out by the French Cycling Federation on 659 elite cyclists (585 men and 74 women); the risk of adrenal insufficiency is negatively correlated with the basal serum cortisol level. Adrenal insufficiency was suspected in 34 cyclists (5 %; 22 in 2001 and 12 in 2002) on the basis of below normal cortisol concentrations and in three cyclists (in 2001) because they had received corticosteroid treatment. In 2001, 10 of the 25 cyclists convoked underwent baseline follow-up serum cortisol determinations and 15 underwent dynamic exploration of adrenal function with the short ACTH test. Adrenal function was found to be deficient in four of these cyclists, at the limits of the normal range in four and normal in seven. Based on these results, the FFC sent a questionnaire in 2002 to all the cyclists to assess the use of corticosteroid in this population. This survey revealed that 85 of 538 cyclists (16 %) had received corticosteroid treatment in the previous 3 months. Moreover, 11 of the 12 cyclists (92 %) with low basal serum cortisol concentrations had received corticosteroid therapy. These results show that basal serum cortisol is relevant to detect adrenal insufficiency in sportsmen, in particular in cases of values below the normal range. The high frequency of corticosteroid use among elite cyclists, and in particular road cyclists who are at risk of trauma and infection, justifies screening tests to detect adrenal insufficiency [07183].

ACTH

To investigate the effects of short-term prednisolone ingestion combined with intense training on exercise performance, hormonal (adrenocorticotropic hormone (ACTH), prolactin, luteinising hormone (LH), growth hormone (GH), thyroid-stimulating hormone (TSH), dehydroepiandrosterone (DHEA), testosterone, insulin) and metabolic parameters (blood glucose, lactate, bicarbonate, pH). Eight male recreational athletes completed four cycling trials at 70-75 percent peak O\textsubscript{2} consumption until exhaustion just before and after either oral placebo or prednisolone (60 mg/day for 1 week) treatment coupled with standardised physical training (2 hours/day), according to a double-blind and randomised protocol. Blood samples were collected at rest, during exercise and passive recovery for the hormonal and metabolic determinations. Time of cycling was not significantly changed after placebo but significantly increased after prednisolone administration (50 min for placebo 1, 64 min for placebo 2, 56 min for prednisolone 1 and 107 min for prednisolone 2). There was no significant difference in any measured parameters after the week of training with placebo but a decrease in ACTH, DHEA, PRL, GH, TSH and testosterone was seen with prednisolone treatment during the experiment. No significant change in basal, exercise or recovery LH, insulin, lactate, pH or bicarbonate was found between the two treatment, but blood glucose was significantly higher under prednisolone at all time points. It was concluded that short-term glucocorticoid administration induced a marked improvement in endurance performance [07184].

Synacthen®

Despite the controversy regarding the performance enhancing properties of corticotrophins, informed “street talk” has frequently indicated that substances such as Synacthen have been misused in sports. As shown also in earlier studies, Synacthen proved extremely unstable in plasma if the specimen is not stored frozen immediately after sampling [12016].
Synacthen is a synthetic analogue to human adrenocorticotropic hormone, which plays an important physiological role by stimulating production of cortisol. In sports, corticosteroids as well as releasing factors (corticotropins) are prohibited according to the regulations of the World Anti-Doping Agency, and the misuse of Synacthen has been reported several times. Hence, an assay enabling the detection of Synacthen in doping control samples has been developed using immunoaffinity chromatographic isolation of Synacthen from human plasma combined with a concentration of collected fractions using solid-phase extraction. Unambiguous determination of the target analyte was accomplished using microbore liquid chromatography/electrospray ionization tandem mass spectrometry. Diagnostic product ions such as m/z 223 were characterized using high-resolution/high-accuracy Orbitrap mass spectrometry and employed for triple quadrupole MS/MS analysis. The established assay requiring 2 mL of plasma allowed a lower limit of detection (LLD) at 100 fmol/mL, a recovery of 97 percent and a precision at the LLD < 20 percent. Authentic plasma samples obtained from a patient undergoing a standard short Synacthen test were used to prove the applicability of the developed procedure [06213].

A peptide hormone termed synacthen, which belongs to the class of corticotrophins, has also been determined in human plasma using immunoaffinity purification and LC-MS/MS analysis. The peptide consists of 24 amino acids and mimics the adrenocorticotropic hormone ACTH, which triggers the secretion of endogenously produced cortisone. Due to its relatively small size, no further enzymatic hydrolysis was required and unambiguous information on its presence was obtained by multiple reaction monitoring experiments, including those monitoring the quadruply charged precursor ion and the diagnostic product ion a2 at m/z 223. Using administration study specimens as well as spiked blank plasma samples, detection limits of 0.3 ng/mL have been obtained, while sampling and storage have been identified as key factors regarding the stability of the target analyte. Only specimens stored at −20 °C were stable for up to three months, which demonstrates that blood sampling requires different conditions than the collection of urine specimens in doping controls [07050].

Tetracosactide (Synacthen®), a synthetic analogue of adrenocorticotropic hormone (ACTH), can be used as a doping agent to increase the secretion of glucocorticoids by adrenal glands. The only published method for anti-doping control of this drug in plasma relies on purification by immunoaffinity chromatography and LC/MS/MS analysis. Its limit of detection is 300 pg/mL, which corresponds to the peak value observed 12 h after 1 mg Synacthen IM administration. It was reported here a more sensitive method based on preparation of plasma by cation exchange chromatography and solid-phase extraction and analysis by LC/MS/MS with positive-mode electrospray ionization using 7-38 ACTH as internal standard. Identification of Synacthen was performed using two product ions. The recovery was estimated at 70 percent. A linear calibration curve was obtained from 25 to 600 pg/mL (R² = 0.99). The lower limit of detection was 8 pg/mL (S/N > 3). The lower limit of quantification was 15 pg/mL (S/N > 10; CV % < 20 %). The performance of the method was illustrated by an 8-h kinetic analysis of plasma samples from nine subjects submitted to IM injections of either Synacthen® (five subjects) or Synacthen® Depot, the slow-release form of the drug (four subjects). Concentrations of Synacthen between 16 and 310 pg/mL were observed. A sensitive method for quantitation of Synacthen in plasma is proposed for anti-doping control analyses [11179].

Doping control analysis of performance-enhancing peptides in urine represents a challenging requirement in modern sports drug testing. Low dosing, effective metabolism and short half-life lead to target concentrations in the low fmol/mL range in urine. Synthetic adrenocorticotropic hormone (1-24, Syn-ACTH-en) shares all these characteristics and improved analytical performance is required for its sufficient determination by means of liquid
chromatography/tandem mass spectrometry (LC/MS/MS). The desired effects for cheating sportsmen are mainly due to enhanced release of corticosteroids as well as androgenic steroids into the circulation after systemic administration of the drug. Immunoaffinity purification with coated magnetic beads and subsequent liquid chromatography with nano-ultra-performance liquid chromatography (UPLC) coupled to tandem mass spectrometry (high resolution/high mass accuracy) of Synacthen from urinary specimens is described in the present study. The general proof of principle was obtained by analysis of excretion study urine samples and validation was performed with focus on the limit of detection (3 pg/mL), linearity, precision (<20 %), recovery (approximately 30 %), robustness, specificity and stability. For all experiments, the ACTH fragment 1-17 was used as the internal standard [09231].

Budesonide

Budesonide, a corticosteroid frequently used in the treatment of asthma, is most often administered via inhalation. Its use in sports is allowed when medically necessary. A fast, sensitive and accurate LC-MS method was developed and validated for the quantification of budesonide and its major metabolite 16alpha-hydroxyprednisolone in urine samples after inhalation of a metered dose (Pulmicort-Turbohaler 200). Sample preparation consists of an alkaline liquid-liquid extraction with ethyl acetate. Analysis was performed using liquid chromatography-tandem mass spectrometry with electrospray ionization (ESI). The method was linear in the range of 5-100 and 0.5-10ng/mL for 16alpha-hydroxyprednisolone and budesonide, respectively. The limits of quantification were 5ng/ml for 16alpha-hydroxyprednisolone and 0.5ng/mL for budesonide. The accuracy ranged from 2.2 to 3.5% for 16alpha-hydroxyprednisolone and from 0.8 to 16.4 percent for budesonide. After administration of 200microg of budesonide to five healthy volunteers budesonide could not be detected in any urine sample whereas 16alpha-hydroxyprednisolone was detectable up to 12 h post-administration [06212].

Budesonide (BUD) is a glucocorticoid widely used for the treatment of asthma, rhinitis, and inflammatory bowel disease. Its use in sport competitions is prohibited when administered by oral, intravenous, intramuscular, or rectal routes. However, topical preparations are not prohibited. Strategies to discriminate between legal and forbidden administrations have to be developed by doping control laboratories. For this reason, metabolism of BUD has been re-evaluated using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with different scan methods. Urine samples obtained after oral administration of 3 mg of BUD to two healthy volunteers have been analyzed for metabolite detection in free and glucuronide metabolic fractions. Structures of the metabolites have been studied by LC-MS/MS using collision induced dissociation and gas chromatography-mass spectrometry (GC/MS) in full scan mode with electron ionization. Combination of all structural information allowed the proposition of the most comprehensive picture for BUD metabolism in humans to this date. Overall, 16 metabolites including ten previously unreported compounds have been detected. The main metabolite is 16alpha-hydroxy-prednisolone resulting from the cleavage of the acetal group. Other metabolites without the acetal group have been identified such as those resulting from reduction of C20 carbonyl group, oxidation of the C11 hydroxyl group and reduction of the A ring. Metabolites maintaining the acetal group have also been identified, resulting from 6-hydroxylation (6alpha and 6beta-hydroxy-budesonide), 23-hydroxylation, reduction of C6-C7, oxidation of the C11 hydroxyl group, and reduction of the C20 carbonyl group. Metabolites were mainly excreted in the free fraction. All of them were excreted in urine during the first 24 h after administration, and seven of them were still detected up to 48 h after administration for both volunteers [12323].
**Methylprednisolone**

Methylprednisolone (MP) is prohibited in sports competitions when administered by systemic routes; however its use by topical administration is allowed. Therefore, analytical approaches to distinguish between these different administration pathways are required. A reporting level of 30 ng/mL was established for this purpose. However, the suitability of that reporting level for MP is not known. In the present work, excretion profiles of MP and different metabolites after oral and topical administrations have been compared. A method for the quantification of MP and the qualitative detection of fifteen previously reported metabolites has been validated. The method involved an enzymatic hydrolysis, liquid-liquid extraction and analysis by liquid chromatography coupled to tandem mass spectrometry. The method was found to be linear, selective, precise and accurate. The high sensitivity (limit of detection 0.1 ng/mL) and linear range (0.1-250 ng/mL) achieved allowed for the quantification of MP at both the low concentrations present after topical administration and the high concentrations detected after oral intake. The method was applied to samples collected after oral (4 or 40 mg) and topical administration (10 mg of MP aceponate/day for 5 consecutive days) to healthy volunteers. After oral administration, MP and all metabolites were detected in urines collected up to at least 36 h. Only MP and five metabolites were detected in samples obtained after topical administration. As expected, concentrations of MP after topical administration were well below current reporting level (30 ng/mL), however 3 out of 4 samples in range 8-24 h after the low oral dose (4 mg) were also below that concentration. Taking into account metabolites detected after both administration routes, metabolites 16beta,17alpha,21-trihydroxy-6alpha-methylpregna-1,4-diene-3,11,20-trione (M8) and 17alpha,20alpha,21-trihydroxy-6alpha-methylpregna-1,4-diene-3,11-dione (M11) are best markers to differentiate between topical and oral administrations. Their signals after topical administration were lower than those obtained in the first 48 h after all oral doses [13551].

**Dexamethasone**

The illegal administration of hormones, steroids, beta-agonists and other anabolic agents to productive livestock in the European Union continues, despite the long-term ban on their use and despite the measures provided under the directives to monitor certain substances and residues thereof in the interest of protecting consumer health and animal wellbeing. Often administered in low doses in the form of a drug cocktail, these compounds escape detection by common analytical techniques. The aim of one study was to determine whether low-dose dexamethasone administration (0.4 mg orally per day, for 20 days) in white-meat calves produced variations in blood coagulation, as measured by thromboelastometry. A second aim was to determine whether such variations could be valid in detecting illicit low-dose dexamethasone treatment. The study population was 42 Friesian calves kept under controlled conditions until 6 months of age. The calves were subdivided into 2 groups: a control group (group A, n=28) and a group treated with dexamethasone (group B, n=14) for 20 days beginning at 5 months of age. When compared against the age-matched control group, the dexamethasone-treated calves showed a significant increase in alpha angle, maximum clot firmness and a significant decrease in clot formation time on all thromboelastometric profiles. The clotting time was significantly decreased on the in-TEM® profile but increased on the ex-TEM® and fib-TEM® profiles. The Receiver Operating Characteristic curves, plotted for the Maximum Clot Elasticity (MCE), had a cut-off value ≥ 488.23 mm for in-TEM® and a cut-off value ≥ 63.94 mm for fib-TEM®. In order to increase the sensitivity of the test two parameters (in-TEM® and fib-TEM® MCE) were used as two
parallel tests; subsequently, the sensitivity rose to a point value of 99 percent. It was concluded that thromboelastometry identified a state of hypercoagulability in the dexamethasone-treated subjects. Furthermore, the results of this preliminary study suggest that TEM may be useful in the detection of illicit low-dose dexamethasone treatment [13552].

**Cortisol value during an exercise season**

The purpose of this study was to track creatine kinase (CK) and serum cortisol over an American college football season starting with the preseason practice. A secondary purpose was to observe changes in basic clinical chemistries. Twenty-two National Collegiate Athletic Association Division I football players (age: 20 years) volunteered to participate in the study. Each of the players had participated in the summer strength and conditioning supervised program. Resting blood samples were obtained just before the start of preseason practice (T-1), 2 weeks later (T-2), and the day after game 2 (T-3), game 4 (T-4), game 6 (T-5), and game 9 (T-6) of a 12-game season. Creatine kinase, a panel of clinical chemistries, cortisol, and testosterone were assayed at each time point. No significant changes in CK concentrations were observed over the season with peak values of each range ≤1,070.0 IU/L, but the largest range was observed at T-6 after game 9 (119–2,834 IU/L). The analysis of covariance analysis demonstrated that the number of plays in the ninth game (T-6) explained the magnitude of the changes in CK. No changes in serum cortisol concentrations were observed yet, again large variations existed with peak values of each range ≤465.0 nmol/L. Clinical chemistries showed various significant changes from T-1, but none were considered clinically relevant changes for any player over the time course of the study. In conclusion, the strength and conditioning program before preseason camp or the structure of summer camp practices and the in-season strength and conditioning appeared to mute muscle damage and the stress response of cortisol. Such data demonstrate that changes in muscle damage and adrenal cortical stress over the season are minimal, yet large individual variations can be observed. Management of these variables appears to be related to optimal strength and conditioning and sports medicine programs. Thus, the greater concerns for student-athlete safety in the sport of American football are related to preventing sudden death, traumatic injury, and managing concussion syndromes [13553].

**Prednisolone in human urine**

The possibility of an endogenous presence of the glucocorticoid prednisolone has already been demonstrated in bovine and horse urine, with the aim of clarifying its origin in this matrix, which is used by official agencies for the control of illicit treatments. From this point of view, the endogenous nature of prednisolone could be a major topic in doping control of both amateur and professional human athletes. A study was therefore made on 34 human volunteers (13 males and 21 females; aged 22–62) to detect the presence of prednisolone in their urine by HPLC-MS(3). One of the volunteers underwent vernal allergy treatment with betamethasone for two subsequent years. An investigation was carried out with the aim of verifying if the suppression, and the circadian rhythm, of cortisol urinary levels could also apply to prednisolone. The results of the study show that prednisolone was present in the urine of all 34 volunteers, with a concentration very close to 100-times lower that of cortisol, with no dependence on gender. The same ratio (1/100) was observed in the prednisolone and cortisol levels detected during the 24h together with the suppression of prednisolone by betamethasone treatment. These data demonstrate the endogenous nature of low concentrations of prednisolone in human urine, and motivate further studies about the
biosynthetic pathways of this corticosteroid and its relationship with stress in humans, as already described in cows [13554].

**Topical administration of prednisolone**

Methylprednisolone (MP) is prohibited in sports competitions when administered by systemic routes; however its use by topical administration is allowed. Therefore, analytical approaches to distinguish between these different administration pathways are required. A reporting level of 30 ng/mL was established for this purpose. However, the suitability of that reporting level for MP is not known. In one work, excretion profiles of MP and different metabolites after oral and topical administrations have been compared. A method for the quantification of MP and the qualitative detection of fifteen previously reported metabolites has been validated. The method involved an enzymatic hydrolysis, liquid-liquid extraction and analysis by liquid chromatography coupled to tandem mass spectrometry. The method was found to be linear, selective, precise and accurate. The high sensitivity (limit of detection 0.1ng/mL) and linear range (0.1-250 ng/mL) achieved allowed for the quantification of MP at both the low concentrations present after topical administration and the high concentrations detected after oral intake. The method was applied to samples collected after oral (4 or 40mg) and topical administration (10 mg of MP acepionate/day for 5 consecutive days) to healthy volunteers. After oral administration, MP and all metabolites were detected in urines collected up to at least 36 h. Only MP and five metabolites were detected in samples obtained after topical treatment. As expected, concentrations of MP after topical administration were well below current reporting level (30ng/mL), however 3 out of 4 samples in range 8-24 h after the low oral dose (4 mg) were also below that concentration. Taking into account metabolites detected after both administration routes, metabolites 16beta,17alpha,21-trihydroxy-6alpha-methylpregna-1,4-diene-3,11,20-trione (M8) and 17alpha,20alpha,21-trihydroxy-6alpha-methylpregna-1,4-diene-3,11-dione (M11) are best markers to differentiate between topical and oral administrations. Their signals after topical administration were lower than those obtained in the first 48 h after all oral doses [13555].

**Effect on muscles**

Muscle wasting is associated with poor prognosis in chronic obstructive pulmonary disease (COPD). Exercise stimulates muscle recovery, but its efficacy is variable, depending on the clinical condition and medical treatment. Systemic glucocorticoids, commonly administered in high doses during acute disease exacerbations or as maintenance treatment in end-stage disease, are known to contribute to muscle wasting. As muscle mass recovery involves insulin-like growth factor (IGF)-I signaling, which can be stimulated by anabolic steroids, the impact of glucocorticoids and the effect of simultaneous IGF-I stimulation by anabolic steroids on muscle recovery and growth were investigated. The effects of, and interactions between, glucocorticoid and IGF-I signaling on skeletal muscle growth were assessed in differentiating C2C12 myocytes. As proof of principle, we performed a post hoc analysis stratifying patients by glucocorticoid use of a clinical trial investigating the efficacy of anabolic steroid supplementation on muscle recovery in muscle-wasted patients with COPD. Glucocorticoids strongly impaired protein synthesis signaling, myotube formation, and muscle-specific protein expression. In contrast, in the presence of glucocorticoids, IGF-I synergistically stimulated myotube fusion and myofibrillar protein expression, which corresponded with restored protein synthesis signaling by IGF-I and increased transcriptional activation of muscle-specific genes by glucocorticoids. In COPD patients on maintenance glucocorticoid treatment, the clinical trial also revealed an enhanced effect of anabolic
steroids on muscle mass and respiratory muscle strength. In conclusion, synergistic effects of anabolic steroids and glucocorticoids on muscle recovery may be caused by relief of the glucocorticoid-imposed blockade on protein synthesis signaling, allowing effective translation of glucocorticoid-induced accumulation of muscle-specific gene transcripts [13556].

Glucocorticoid-induced skeletal muscle atrophy

Many pathological states characterized by muscle atrophy (e.g. sepsis, cachexia, starvation, metabolic acidosis and severe insulinopenia) are associated with an increase in circulating glucocorticoids (GC) levels, suggesting that GC could trigger the muscle atrophy observed in these conditions. GC-induced muscle atrophy is characterized by fast-twitch, glycolytic muscles atrophy illustrated by decreased fiber cross-sectional area and reduced myofibrillar protein content. GC-induced muscle atrophy results from increased protein breakdown and decreased protein synthesis. Increased muscle proteolysis, in particular through the activation of the ubiquitin proteasome and the lysosomal systems, is considered to play a major role in the catabolic action of GC. The stimulation by GC of these two proteolytic systems is mediated through the increased expression of several Atrogenes (“genes involved in atrophy”), such as FOXO, Atrogin-1, and MuRF-1. The inhibitory effect of GC on muscle protein synthesis is thought to result mainly from the inhibition of the mTOR/S6 kinase 1 pathway. These changes in muscle protein turnover could be explained by changes in the muscle production of two growth factors, namely Insulin-like Growth Factor (IGF)-I, a muscle anabolic growth factor and Myostatin, a muscle catabolic growth factor. This review will discuss the recent progress made in the understanding of the mechanisms involved in GC-induced muscle atrophy and consider the implications of these advancements in the development of new therapeutic approaches for treating GC-induced myopathy [13557].

Transfer factor

Inhaled glucocorticoids are the most effective and potent drugs used to control the inflammatory bronchial reaction in patients with asthma. There are several research projects evaluating the use of immune modulators in the treatment of the asthma related inflammatory process. To evaluate the effect of transfer factor in the treatment of pediatric patients with moderate persistent allergic asthma in terms of inhaled glucocorticoid dosing and time of using a randomized, double blind, placebo controlled pilot clinical trial in a cohort of pediatric patients (6-17 years old) with moderate persistent allergic asthma was performed. Two groups were formed. Group one received transfer factor and group two was given placebo. Both groups received conventional therapy with inhaled budesonide and formoterol. Daily respiratory symptoms (cough during day, or at night, and wheezing episodes) were recorded in a personal diary. Spirometric evaluations were performed before enrolling patients, and at 1, 3 and 6 months after. Eleven patients were enrolled in each group. Patients in the transfer factor group showed a statistical significant reduction in the inhaled glucocorticoid doping since month 3, and this difference was maintained until the end of study. Patients on transfer factor group showed also a non statistical significant improvement in spirometrical findings and also showed a better asthma control. Transfer factor helps to reduce inhaled glucocorticoids dose in patients with allergic rhinitis; however, studies with a larger number of patients should be done in order to obtain better results [09232].

Use in football
Glucocorticosteroids are widely used in medicine and have shown unchallenged therapeutic potential in several chronic inflammatory and other diseases. They are also widely used in sports medicine for the treatment of conditions such as asthma and acute injuries. In fact, as banned substances, most requests for therapeutic use exemption concern glucocorticosteroids. Nevertheless, their beneficial effect in certain conditions in sports, where inflammation is only a secondary reaction, remains to be validated. One paper aimed to provide a comprehensive review of the literature covering the therapeutic use of glucocorticosteroids since 1977 in conditions ranging from chronic rheumatic illness to peritendinous or intra-articular injection in acute injuries. Search of the medical literature published between 1977 and 2006 using PubMed. Articles relevant to the question "When and if at all is the use of glucocorticosteroids justified in football?" were selected and analysed. The findings clearly point out that, despite the common use of glucocorticosteroids in acute injuries in sports, there is actually limited evidence of the true benefits of such a practice. Physicians must take the possible adverse effects into consideration. In an athlete with clinically verified asthma, inhalational glucocorticosteroids remain first line therapy. Finally, for the purposes of education and prevention of misuse, it should be stressed that a measurable performance enhancing effect of glucocorticoids could not be proved on the basis of the results of the scientific studies to date [06214].

Endogenous prednisolone

The possibility of an endogenous presence of the glucocorticoid prednisolone has already been demonstrated in bovine and horse urine, with the aim of clarifying its origin in this matrix, which is used by official agencies for the control of illicit treatments. From this point of view, the endogenous nature of prednisolone could be a major topic in doping control of both amateur and professional human athletes. A study was therefore made on 34 human volunteers (13 males and 21 females; aged 22-62) to detect the presence of prednisolone in their urine by HPLC-MS(3). One of the volunteers underwent vernal allergy treatment with betamethasone for two subsequent years. An investigation was carried out with the aim of verifying if the suppression, and the circadian rhythm, of cortisol urinary levels could also apply to prednisolone. The results of the study show that prednisolone was present in the urine of all 34 volunteers, with a concentration very close to 100-times lower that of cortisol, with no dependence on gender. The same ratio (1/100) was observed in the prednisolone and cortisol levels detected during the 24h together with the suppression of prednisolone by betamethasone treatment. These data demonstrate the endogenous nature of low concentrations of prednisolone in human urine, and motivate further studies about the biosynthetic pathways of this corticosteroid and its relationship with stress in humans, as already described in cows [12324].

Microbial transformation of cortisol to prednisolone

Doping control samples are normally collected under non-sterile conditions and sometimes, storage and transportation are influenced by parameters such as the temperature. Therefore, microbial contamination and subsequent alteration of a sample's composition are possible. Studies regarding sample collection in cattle breeding have already shown enzymatic transformation of endogenous testosterone to boldenone causing false-positive findings. The aim of the present study was to investigate whether positive doping cases with the synthetic corticosteroids prednisolone and prednisone may result from microbial transformation of the endogenous corticosteroids cortisol and cortisone, respectively. A method comprising parameters such as pH values and screening results for synthetic glucocorticosteroids as
well as incubation experiments followed by liquid chromatographic and mass spectrometric analysis was employed to test for contaminating germs with \( \Delta_1 \)-dehydrogenase activity. Over 700 urine samples comprising inpatient and doping control specimens were investigated. In none of them, 1,2-dehydrogenating activity was confirmed. These findings are in accordance with other studies. However, the problem of microbial alteration of doping control specimens with special respect to 1,2-dehydrogenation must not be underestimated [12325].

**Ophtalmological aspects**

Prescribing for athletes requires an up-to-date knowledge of the World Anti-Doping Agency's list of prohibited substances. As the London 2012 Olympic Games attract athletes from around the world, we review the current guidelines with respect to all medications licensed for ophthalmic use in the United Kingdom. It was described the process that an ophthalmologist can use to check for permissible medications and also highlight treatments that are contraindicated. It was systematically reviewed all 77 drugs listed in Section 11 of the British National Formulary (Issue 63) for use in the treatment of ophthalmic conditions, and referenced these against the 2012 Prohibited List published by the World Anti-Doping Agency. The majority of ophthalmic preparations are suitable for use in- and out-of-competition. Some preparations, such as glucocorticoids, are prohibited when administered systemically but permitted for topical administration. Beta-blockers are prohibited in-competition and oral carbonic anhydrase inhibitors are prohibited in- and out-of competition. Thus, the 2012 Prohibited List has important implications for the pharmacological treatment of ophthalmic conditions in athletes. Clinicians prescribing for athletes have a duty to familiarize themselves with the list in order to avoid causing significant damage to their patient's career and reputation [12326].

**Side effects**

**Heart**

Glucocorticoids are classified as doping substances and so their use in any form for therapeutic reasons must be declared. An indirect side effect that concerns the cardiovascular system is the disturbance of lipid metabolism, leading to an increase in total cholesterol, triglycerides, and LDL cholesterol. The main direct complication is arterial hypertension, which is attributable to fluid retention, an increase in peripheral vascular resistances, and an increase in myocardial contractility [12126].

**Laboratory technique**

A liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) method was developed using the latest high-resolution LC column technology, the ultra performance liquid chromatography (UPLC), and electrospray ionization (ESI) in the positive ion mode. Gradient UPLC separation conditions were optimized for a group of 22 analytes comprising 17 glucocorticosteroids, specific designer steroids such as tetrahydrogestrinone (THG) and specific beta2-agonists such as formoterol. The UPLC/TOFMS separation obtained required 5.5 min only for all the substances tested. Even the critical pair of dexamethasone and betamethasone isomers was almost completely resolved. Thanks to the over 10,000 full-width at half maximum (FWHM) mass resolution and high mass accuracy features of TOFMS.
50 mDa window accurate mass chromatograms could be reconstructed for the individual analytes. Sensitive screening in human and calf urine samples fortified at the glucocorticosteroids minimum required performance limit (MRPL) of 30 microg/L (human urine, sports doping) and 2 microg/L (calf urine, veterinary control) could be obtained. The potential of UPLC/TOFMS for confirmatory analysis was shown by determining the accurate mass of all compounds and fragment ions upon in-source collision-induced dissociation (CID) at different energies. The exact mass measurement errors for all glucocorticosteroids were found to be within 6 ppm. Considering veterinary control, limits of detection (LOD) and limits of quantification (LOQ) were determined for most of the analytes in calf urine and found to range from 0.1 to 3.3 and from 0.4 to 4.4 microg/L, respectively. The method can be easily extended with other banned substances of interest, as demonstrated by the addition of 21 beta2-agonists to the original analyte mixture in urine, without causing any interferences [06215].

One study presents a fast multi-analyte screening method specifically developed for the detection of xenobiotics in urine. The proposed method allows the screening of several classes of substance in a single chromatographic method with a run-time of 11 min, inclusive of post-run and reconditioning times. Chromatographic separation is achieved in 7.2 min using a reversed-phase 2.7 µm fused-core particle column, generating a back-pressure not exceeding 400 bar and therefore enabling the use of traditional high performance liquid chromatography (HPLC) instruments. The effectiveness of this approach was evaluated, by liquid-chromatography tandem mass spectrometry (LC-MS/MS) in positive electrospray ionization, using 20 blank urine samples spiked with 45 compounds prohibited in sport: 11 diuretics, 16 glucocorticoids, 9 stimulants, 5 anti-oestrogens, as well as formoterol, carboxyfinasteride (previously prohibited by the World Anti-Doping Agency (WADA) in 2008), gestrinone and tetrahydrogestrinone. Qualitative validation shows the proposed method to be specific with no significant interference. All of the analytes considered in this study were clearly distinguishable in urine, with limits of detection ranging from 5 ng/mL to 350 ng/mL, significantly below the Minimum Required Performance Levels (MRPL) set by WADA for the accredited sports anti-doping laboratories. All compounds of interest were separated, including synthetic and endogenous glucocorticoids with similar retention times and fragmentation patterns [10378].

The detection of corticosteroids and sex steroids in samples with no content indication, which are confiscated for forensic investigation, is a challenge in doping analysis. A screening method based on the identification of androgens, estrogens, gestagens, and their esters by means of a mass spectral library, along with a fast ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) method, was recently developed in a laboratory for the analysis of dietary supplements. However, for forensic investigations, it is important to extend the scope of the method to corticosteroids in various matrices. Therefore, 36 corticosteroids were added to the mass spectral library, and the sample preparation step was modified so that androgens, gestagens, corticosteroids, and their esters could be analyzed with only one injection with the UPLC-MS method. A complementary tool to the existing library identification was found in the extraction of common fragment ions out of the full scan data obtained for the library search. The fragment ion with m/z 147 was found to be a good marker for the detection of steroids. Extra confirmation was obtained from the fragment ions with m/z 135 (for all steroids) and 237 (specific for corticosteroids) or from the fragment ions with m/z 77, 91, and 105. The effectiveness of this approach was evaluated on some samples previously screened for forensic investigation with thin-layer chromatography and confirmed with a targeted gas chromatography-mass spectrometry method. The study shows that the combination of the library identification and the common fragment ions approach can be a valuable tool in the detection of steroids without defining any target at the start of the analysis [11366].
Glucocorticoids are listed on the World Anti-Doping Agency (WADA) Prohibited List of substances. The detection of the administration of hydrocortisone and cortisone is complicated by the fact that the human body also produces these steroids naturally. Gas chromatography-combustion-isotope ratio mass spectrometry can be utilized to determine the use of endogenous glucocorticoids by measuring the carbon isotope ratio (CIR) of their resulting metabolites in human urine samples. A comprehensive sample preparation protocol for the analysis of endogenous glucocorticoid urinary metabolites was developed and validated, incorporating the use of high performance liquid chromatography (HPLC) for purification and chemical oxidation for derivatisation. Target compounds were tetrahydrocortisol and tetrahydrocortisone, and 11beta-hydroxyetiocholanolone, 11-oxoetiocholanolone and 11beta-hydroxyandrosterone, while pregnanediol functioned as the endogenous reference compound. Urine samples from a population of 50 volunteers were analyzed to determine CIR reference limits. Excretion studies of the endogenous glucocorticoid preparation cortisone acetate (25 mg oral) and the dietary supplement adrenosterone (75 mg oral) were conducted with six male individuals. Variable changes in steroid metabolite isotopic composition were found across subjects after administration. The study also revealed that CIR analysis of the major glucocorticoid metabolites tetrahydrocortisol and tetrahydrocortisone is necessary to unambiguously distinguish administration of cortisone and adrenosterone, the former officially restricted to out-of-competition use by athletes, the latter not being restricted at the current time. Moreover, this study reaffirms that CIR methods for the doping control of endogenous steroids should not rely upon a single ERC, as the administration of an appropriate precursor to that ERC could cause complications during analysis [12327].

The use of prednisolone and prednisone is prohibited by the World Anti-Doping Agency (WADA) due to their performance-enhancing effect. The purpose of one work was to explore the possibility of identification and detection of various metabolites of prednisolone by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in excretion study samples. Ten metabolites of prednisolone could be identified namely prednisone (11-oxo metabolite) [M-1], 6-beta-OH-prednisolone [M-2], 20-beta-OH-prednisolone [M-3], 20-alpha-OH-prednisolone [M-4], 20-alpha-OH-prednisone [M-5], 20-beta-OH-prednisolone [M-6], 2 tetrahydro epimers of 20-beta-OH-prednisolone [M-7], 2 tetrahydro epimers of 20-alpha-OH-prednisolone [M-8], 2 tetrahydro epimers of 20-beta-OH-prednisone [M-9], and 2 tetrahydro epimers of 20-alpha-OH-prednisone [M-10]. Prednisolone was administered in 10, 20, and 40 mg dosage to healthy volunteers to study detection of various metabolites. The parent, M-1, M-2, and M-3 could be detected up to 72 h while rest of the metabolites were detectable up to 24 h after drug administration. The detection of newer metabolites of the drug can further be used for confirmatory purposes [12328].

**Measurement in hair**

Endogenous corticosteroids and endocannabinoids are both known to be involved in stress adaption and anti-inflammatory and immuneregulatory effects. The application of hair as retrospective specimen for long-term recording of corticosteroids and its association with stress-induced biochemical alterations was intensively examined. To evaluate the stability and correlation of various parameters of the endocannabinoid and corticosteroid systems, a prospective study was carried out. Hair samples were collected monthly over a pregnancy cycle (sixth week of pregnancy to 9 weeks postpartum). By comparison of hair concentrations in particular segments (ie, grown in the same time span but collected at different times), an examination of analyte stability in hair was achieved. Additionally, the comparison of proximal segments provided on biochemical information that is independent of
alteration due to physical instability. The detection limits of a validated procedure using solid-phase extraction cleanup and liquid chromatography-mass spectrometry proved to be suitable to identify the endogenous levels of cortisol (limits of detection = 1.6 pg/mg), cortisone (2.1 pg/mg), anandamide (AEA, 0.3 pg/mg), and 2-arachidonoylglycerol (15 pg/mg). Corticosteroid concentrations in corresponding hair segments were found to be reduced with increasing hair age; an average decline of cortisol and cortisone by 50% in 4 months was estimated. Independently, an increase of cortisol and cortisone in proximal segments collected during pregnancy was confirmed, which is assumed to be stress related. Endocannabinoids proved to be by far more stable, as demonstrated by subsequent monthly collection of corresponding segments and there was hardly any washout of AEA detectable. Elevated hair concentrations of AEA and 2-arachidonoylglycerol were detected in the first-second trimester of pregnancy, which corresponds to negative correlations between AEA, cortisol, and cortisone [13558].

**Experimental**

Although exercise is a common and potent activator of the hypothalamic-pituitary adrenal (HPA) axis, the effects of exercise on the acute stress response are not well understood. Here, we investigated the effects of short- (2 wk) and long-term (8 wk) voluntary wheel running on adrenal sensitivity to ACTH stimulation and the acute stress response to restraint in male rats. Diurnal glucocorticoid patterns were measured on days 7 (all groups) and 35 (8-wk groups). Rats were subjected to 20 min of restraint stress on either week 1 or on week 7 of treatment to assess HPA activation. One week later, exogenous ACTH (75 ng/kg) was administered to assess adrenal sensitivity to ACTH. Following this, adrenals were collected and analyzed for key proteins involved in corticosterone synthesis. The results show that volitional wheel running initially causes hyperactivation of the HPA axis, due to enhanced adrenal sensitivity to ACTH, but that these alterations in HPA activity are completely restored by 8 wk of training [09233].

Overload tendon injuries are frequent in recreational and elite sports. The optimal treatment strategy remains unknown, but local administration of corticosteroids is one common treatment option. The direct effects of the corticosteroid administration on the tissue are not fully understood. One study examined the biomechanical effects of intratendinous corticosteroid injections on healthy rat-tail tendon collagen fascicles. A total of 24 Wistar male rats were divided into (A) a corticosteroid group where the animals were injected in the tail tendon with methylprednisolone acetate, 1.0 mL of 40 mg/mL mixed with 1.0 mL 9 percent saline (n=12), and (B) a control group that was injected with 9 percent saline (n=12). Three days after the injections, the animals were sacrificed and single individual collagen fascicles were collected and underwent displacement to failure. Corticosteroid administration significantly reduced tensile fascicle yield strength, while the strain properties were unaffected. Peak stress was similar between the two groups. There was no difference in fascicle diameter between the two groups [09234].
MELATONIN

Melatonin (N-acetyl-5-methoxytryptamine) is a pineal gland hormone with effects upon circadian rhythms, sleep onset and reproductive systems. Blood levels of melatonin are generally undetectable during daytime, but rise sharply during darkness. Light–dark sensations at the retina are relayed to the suprachiasmatic nucleus of the hypothalamus. Fibres from the hypothalamus descend to the spinal cord and ultimately project to the superior cervical ganglia, from which sympathetic postganglionic neurons ascend back to the pineal gland. Melatonin peak amplitudes decrease with age, which may explain the flattening of the circadian rhythm also associated with ageing. Melatonin is well known as an antioxidant, immunomodulator and anticancer agent. Melatonin is of interest to sports performance due to its direct actions on body temperature and improved management of jet lag. It may potentially be of interest in cases of amenorrhoea. A meta-analysis of seven studies has shown little evidence of adverse effects. Mild heat loss initiation by peripheral vasodilation, modulated by melatonin secretion, may be the mechanism that initiates sleep. Supplemental melatonin could allow planned induction of this heat loss and potentially be advantageous to exercise performance. Studies have investigated supplementation of melatonin prior to exercise in the heat without finding reductions in core body temperature or improved performance: however, these have limited general applicability given only low-intensity exercise in clothing that did not allow environmental heat loss. A further study looked at aspects of both physical and mental performance following daytime supplementation with melatonin and found decreased intra-aural temperature but without any change to short-duration athletic performance. Notably, decrements in mental performance were identified. With increasing levels of international travel, optimising recovery from jet lag may offer a significant performance advantage. Jet lag has been defined as occurring in response to a minimum of a three-zone change at a rate faster than one zone per day and has been suggested to cause decreased performance and increased injury risk though direct evidence is limited. Supplemental melatonin appears to reduce both subjective and objective symptoms of jet lag. It is unclear at this point whether the main action is hypnotic, that is, inducing sleepiness, or chronobiotic, helping to “reset” the body clock to the new time zone. There does not appear to be a performance hangover from supplementation taken the night before exercise. Melatonin inhibits the secretion of luteinizing hormone and follicle-stimulating hormone from the anterior pituitary. As an apparent result of the loss of this inhibition, female long-term nightworkers have a higher risk of breast cancer risk than women who do not work at night. Disturbances in melatonin production through continuous changes in time zone may also be linked to the frequency of amenorrhoea found in flight attendants. There also appears to be a relationship between melatonin levels, exercise and amenorrhoea in female athletes that requires further investigation [11359].

Low overnight urinary melatonin metabolite concentrations have been associated with increased risk for breast cancer among postmenopausal women. The Postmenopausal Women's Alcohol Study was a controlled feeding study to test the effects of low to moderate alcohol intake on potential risk factors for breast cancer including serum and urinary levels of hormones and other biomarkers. Previously, it was observed significant increases in concentrations of serum estrone sulfate and dehydroepiandrosterone sulfate in participants after consumption of 15 or 30 g (one or two drinks) of alcohol per day. In one analysis, it was evaluated the relationship of alcohol consumption with 24-h urinary 6-sulfatoxymelatonin (6-SMT) concentration (micrograms per 24 h). Healthy postmenopausal women (n=51) consumed a controlled diet plus each of three treatments (a nonalcoholic placebo beverage or 15 or 30 g alcohol/d) during three 8-weeks periods in random order under conditions of weight maintenance. 6-SMT was measured in 24-h urine samples that were collected at entry into the study (baseline) and at the midpoint (4 wk) and end (8 wk) of each of the three
diet periods. Concentration of 6-SMT was not significantly modified by the alcohol treatment after adjustment for body mass index, hours of sleep, daylight hours, and baseline level of 6-SMT. These results suggest that low to moderate daily alcohol consumption does not significantly affect 24-h urinary levels of melatonin among healthy postmenopausal women [11511].

As marker for training

The objectives of the study were to evaluate the influence of a whole training season on 6-sulfatoxymelatonin (alphaMT6s) and citrate excretion in 12 elite swimmers. Urine samples were obtained (before bedtime and after waking up) at the beginning of the season, basic training, macrocycle I, tapering and macrocycle II stages. For alphaMT6s, at basic training, mainly with aerobic training, the evening concentrations were significantly lower than at the beginning, tapering and macrocycle II stages. At macrocycle II stage, with the maximal training workload, the total sum (evening plus morning) was significantly higher than at the beginning, basic training and macrocycle I stages. The ratio (morning/evening) that represents the capacity to produce melatonin at night depending on the evening values at the basic training stage and the nocturnal increment at the macrocycle II stage were significantly higher than at all other stages. Citrate morning values at basic training and tapering stages were significantly lower than in the evening indicating that a metabolic recuperation took place. The total sum significantly decreased as the aerobic training progressed from the beginning to macrocycle I. The basic training ratio (morning/evening) was significantly lower compared to the beginning and macrocycle II stages, and the nocturnal increment was significantly higher compared to the beginning. Melatonin and citrate represent complementary markers that could be used to evaluate the assimilation of the training workload by noninvasive methods [10529].

Effect of performance

One study investigated the effects of a heavy resistance exercise session (RES) with the oral daytime ingestion of melatonin on the physiological responses and acute performance. In a randomized and double-blind controlled study 10 healthy male subjects undertook an 80 min intensive hypertrophic RES for major muscles of the lower and upper extremities. The subjects were studied on two occasions receiving either melatonin (6 mg) or placebo (6 mg) in random order 60 min before each RES. Blood samples were taken from an antecubital vein both in fasting conditions in the morning and before RES (pre 60 min, pre 0 min), during RES (middle) and after RES (post 0 min, post 15 min, post 30 min, post 60 min). Maximal jumping ability and maximal strength in bench press and squat were measured before and immediately after RES in which there were no differences between the melatonin and placebo groups. The serum melatonin concentration increased significantly in the melatonin group following oral ingestion of melatonin and was elevated at every time point after that. The concentration reached a peak value of 1171 ± 235 pg/mL in 60 min at pre 0. Serum melatonin increased slightly but significantly also in the placebo group just before RES, in the middle of RES and after RES (post 0, post 15). There were large differences in the serum melatonin concentration between the groups at all time points. There were no differences in the growth hormone (GH), testosterone and cortisol peak concentrations at any time points between the groups but the area under the curve of GH during RES and during the 60 min after RES was lower in the melatonin condition. In conclusion, the present findings give evidence that oral ingestion of melatonin (6 mg) during daytime with heavy resistance exercise may slightly decrease GH concentrations. On the other hand, it seems that
Effects on strenuous exercise

Strenuous exercise induces inflammatory reactions together with high production of free radicals and subsequent muscle damage. One study was designed to investigate for the first time and simultaneously whether over-expression of inflammatory mediators, oxidative stress, and alterations in biochemical parameters induced by acute exercise could be prevented by melatonin. This indoleamine is a potent, endogenously produced free radical scavenger and a broad-spectrum antioxidant; consequently, it might have positive effects on the recovery following an exercise session. The participants were classified into two groups: melatonin-treated men (MG) and placebo-treated individuals (controls group, CG). The physical test consisted in a constant run that combined several degrees of high effort (mountain run and ultra-endurance). The total distance of the run was 50 km with almost 2800 m of ramp in permanent climbing and very changeable climatic conditions. Exercise was associated with a significant increase in TNF-alpha, IL-6, IL-1ra (in blood), and also an increase in 8-hydroxy-2'-deoxyguanosine (8-OHdG) and isoprostane levels (in urine), and indicated the degree of oxidative stress and inflammation induced. Oral supplementation of melatonin during high-intensity exercise proved efficient in reducing the degree of oxidative stress (lower levels of lipid peroxidation, with a significant increase in antioxidative enzyme activities); this would lead to the maintenance of the cellular integrity and reduce secondary tissue damage. Data obtained also indicate that melatonin has potent protective effects, by preventing over-expression of pro-inflammatory mediators and inhibiting the effects of several pro-inflammatory cytokines. In summary, melatonin supplementation before strenuous exercise reduced muscle damage through modulation of oxidative stress and inflammation signaling associated with this physical challenge [11228].

Experimental

One study aimed to determine the changes in plasma melatonin levels of rats performing acute swimming exercise, immediately following the exercise and after 24 and 48 hours. The study included 40 Spraque Dawley species adult male rats divided in to 4 groups as follows: group 1: general control group, group 2: swimming group A, the animals were decapitated after performing 30-minute acute swimming exercise, group 3: Swimming group B, the animals were decapitated 24 hours after performing 30-minute acute swimming exercise and group 4: swimming group C, the animals were decapitated 48 hours after performing 30-minute acute swimming exercise. Blood samples were collected from all experimental animals by decapitation method and plasma melatonin levels were determined according to RIA method. The comparison of plasma melatonin levels among groups revealed that group 3 had the highest plasma melatonin levels. The levels in group 1 (control) and group 4 were not different. The lowest plasma melatonin levels were found in group 2. The results of our study demonstrate that plasma melatonin levels that decrease immediately after acute swimming exercise increase significantly after 24 hours and restore to resting levels after 48 hours [12331].

Acute sport exercise leads to a strong stimulation of muscle tissue and a change in the organism energy demands. This study was designed to investigate the effect of oral melatonin supplementation on human physiological functions associated with acute exercise. Immune, endocrine and metabolic parameters were measured in 16 young male football players, who were divided into two groups, an experimental group (supplementation with 6
mg of melatonin administered 30 min prior to exercise) and a control group (placebo without melatonin). They performed a continuous exercise of high intensity (135 beats/min). Samples were collected 30 min before the exercise and 3, 15 and 60 min during the exercise. The results indicated that the acute sport training presented increased lipid peroxidation products (MDA) in both groups, control and experimental, with levels significantly decreased in the group treated with melatonin after 15 and 60 min of high-intensity exercise, and the total antioxidant activity (TAS) was lower in the control group than in the experimental, the latter showing significant differences at 60 min of high-intensity exercise. Moreover, the lipid profile of subjects in the experimental group showed lower triglyceride levels than the control group after 15 and 60 min of high-intensity exercise, and immunological studies only showed, in the experimental group, an increase in IgA levels at 60 min after the exercise, and finally there were no significant differences between the groups for any of the other variables. In conclusion these results indicated that treatment with melatonin in acute sports exercise reversed oxidative stress, improved defenses and lipid metabolism, which would result in an improvement in fitness [12332].

Jet lag

Jet lag has potentially serious deleterious effects on performance in athletes following transmeridian travel, where time zones are crossed eastwards or westwards; as such, travel causes specific effects related to desynchronization of the athlete's internal body clock or circadian clock. Athletes are particularly sensitive to the effects of jet lag, as many intrinsic aspects of sporting performance show a circadian rhythm, and optimum competitive results require all aspects of the athlete's mind and body to be working in tandem at their peak efficiency. International competition often requires transmeridian travel, and competition timings cannot be adjusted to suit individual athletes. It is therefore in the interest of the individual athlete and team to understand the effects of jet lag and the potential adaptation strategies that can be adopted. In this review, we describe the underlying genetic and physiological mechanisms controlling the circadian clock and its inherent ability to adapt to external conditions on a daily basis. It was then examine the fundamentals of the various adaptation stimuli, such as light, chronobiotics (e.g. melatonin), exercise, and diet and meal timing, with particular emphasis on their suitability as strategies for competing athletes on the international circuit. These stimuli can be artificially manipulated to produce phase shifts in the circadian rhythm to promote adaptation in the optimum direction, but care must be taken to apply them at the correct time and dose, as the effects produced on the circadian rhythm follow a phase-response curve, with pronounced shifts in direction at different times. Light is the strongest realigning stimulus and careful timing of light exposure and avoidance can promote adjustment. Chronobiotics such as melatonin can also be used to realign the circadian clock but, as well as timing and dosage issues, there are also concerns as to its legal status in different countries and with the World Anti-Doping Agency. Experimental data concerning the effects of food intake and exercise timing on jet lag is limited to date in humans, and more research is required before firm guidelines can be stated. All these stimuli can also be used in pre-flight adaptation strategies to promote adjustment in the required direction, and implementation of these is described. In addition, the effects of individual variability at the behavioural and genetic levels are also discussed, along with the current limitations in assessment of these factors, and we then put forward three case studies, as examples of practical applications of these strategies, focusing on adaptations to travel involving competition in the 2016 Summer Olympics in Rio de Janeiro, Brazil [12330].

To perform at the highest level of international competition, athletes need to maximize rest during long travel, and expeditiously overcome the detrimental effects of "jet lag" (JL). The
negative effects of JL may be alleviated by adopting a multimodality approach, including the judicious use of melatonin and other pharmacologic agents to aid re-entrainment and improve sleep characteristics. Strict compliance with anti-doping policy is pivotal before and during competition. There have been several recent updates regarding the use of selected medications, which mandate constant vigilance by sports medicine personnel to both evaluate drug efficacy and judiciously prescribe approved medications. It is critical that medical staff maintain familiarity and awareness on a continual basis to effectively educate athletes and support staff [11227].

Salivary

Salivary melatonin concentration is an established marker of human circadian rhythmicity. It is thought that melatonin is relatively robust to the masking effects of exercise. Nevertheless, the extent and even the direction of exercise-related change is unclear, possibly due to between-study differences in the time of day exercise is completed. Therefore, it was aimed to compare melatonin responses between morning and afternoon exercise, and explore the relationships between exercise-related changes in melatonin and heart rate. At 08:00 and 17:00 hours, seven male subjects (27 ± 5 years) completed 30 min of cycling at 70 percent peak oxygen uptake followed by 30 min of rest. Light intensity was maintained at about 150 lux. Salivary melatonin (ELISA) and heart rate were measured at baseline, 15 min during exercise, immediately post-exercise and following 30 min recovery. Melatonin was significantly higher in the morning trials compared with the afternoon. The exercise-related increase in melatonin was more pronounced in the morning than in the afternoon. The slope of the heart rate-melatonin relationship was significantly steeper in the morning than in the afternoon. In conclusion, it was reported for the first time that the masking effect of moderate-intensity exercise on melatonin is approximately twice as high in the morning than the afternoon. The much steeper relationship between heart rate and melatonin changes in the morning raises the possibility that time of day alters the relationships between exercise-mediated sympathetic nervous activity and melatonin secretion [11229].

Experimental

Influence on plasma glucose

The aim of one study was to examine how melatonin supplementation affects plasma glucose and liver glycogen levels in rats subjected to acute swimming exercise. Sprague-Dawley species thirty adult male rats were allocated to 3 groups with equal number of animals: general control group which was not subjected to any procedure (Group 1), the group subjected to a 30-minute acute swimming exercise (Group 2), and the group subjected to a 30-minute acute swimming exercise after intraperitoneal (i.p.) melatonin supplementation (3 mg/kg/day) for 4 weeks (Group 3). Blood samples collected from the experimental animals by decapitation method were analyzed in terms of plasma glucose, and glycogen levels were determined in liver tissue samples by histological method. The highest plasma glucose levels were obtained in group 2. Plasma glucose levels in groups 1 and 3 were not different. Mean liver glycogen level in group 3 was significantly higher than those in the other groups, while there was no significant difference between group 1 and group 2 in terms of this parameter. Results of the study demonstrate that melatonin supplementation can have a protective effect on liver glycogen reserves in rats subjected to acute swimming exercise [10417].
**Combination with imipramine**

Although there are tools to treat depressive patients, a considerable amount of the cases remains to be untreated. These drug-resistant patients need new drugs or drug combinations to overcome this problem. Thus, the potential synergistic effect of melatonin on a classical drug, imipramine was evaluated in the present study. To test this hypothesis, porsolt swim test, a test predictive of antidepressant-like action, was conducted in mice. Imipramine at doses of 20 and 40 mg/kg caused no alteration and statistically significant reduction in the duration of immobility in forced swim test, respectively. While 5 mg/kg melatonin had no effect, 10 mg/kg melatonin slightly reduced the duration of immobility. When sub-effective doses of imipramine and melatonin (20 and 5 mg/kg, respectively) were co-administered, there was no alteration in responses compared with those of each drug alone. Likewise, the effective dose of melatonin (10 mg/kg) did not cause any increase in responses to 20 mg/kg imipramine. Although combination of imipramine (40 mg/kg) and melatonin (5 mg/kg) did not exert an antidepressant effect above that of imipramine alone, co-administration of the effective doses (10 and 40 mg/kg for melatonin and imipramine, respectively) displayed an additive effect. There were no significant differences between groups in relation with locomotor activity test. The results show that co-administration of imipramine and melatonin exhibits an additive effect and that there seems to be no interaction between the drugs [08407].
SILDENAFIL (VIAGRA®)

Viagra (sildenafil citrate) has surfaced as the latest everyday-product-turned-performance-booster. Athletes are said to be popping the anti-impotence pill because it increases blood flow to the lungs, thereby boosting cardiovascular capacity. One study found that in some cases the pill improved cyclists' times on a 6-kilometre course by up to 15 percent [08322].

The discovery of the vasodilator role of nitric oxide (NO) has led to a revolution in pharmacology over the past two decades which has brought considerable innovations in NO-related therapy. Apart from helping to elucidate the mode of action of well established treatments such as nitroglycerine, the contribution of advances in NO research have mainly exerted an effect in the clinic through advances in the understanding and application of nitrite, a precursor to NO. Just over a decade ago, the efficiency of NO production by the metallo-enzyme xanthine oxidoreductase was demonstrated. In vitro and under hypoxia, this enzyme is considerably more effective than nitric oxide synthase at generating NO. More recently, this phenomenon was observed for deoxyhaemoglobin, leading to the recent demonstration that nitrite has considerable protective effects in a range of cardiovascular conditions, including myocardial infarctions. It is not surprising that many athletes have looked at vasodilators to embellish their performances on the playing field. Reports of reliance on vasodilator drugs used for sexual dysfunction are common, even at the national team level. One report identifies the distributions of Viagra® to a national soccer team playing at high altitude, supposedly without the players' knowledge. This use has also been recognised by sport governing bodies as the World Anti Doping Agency (WADA) currently sponsor a study of the performance enhancing effects of sildenafil (Viagra®) at mild altitude. Reports suggesting the use of prescription vasodilators to enhance athletic performance by professional athletes, may lead to an increased interest in prescription vasodilators in the sub-elite level of athletes leading to wider public health concerns. Furthermore, use of prescription vasodilators, whether obtained by prescription or not, may lead to the adoption of non-prescription nitrite supplements. For these reasons, it is timely to study the observed interest in these distinct classes of vasodilators in order to compare and contrast trends in interest by athletes. One study aimed to explore potential misuse of vasodilators by the athletes, and to highlight the growing concern over these agents. Retrospective analyses of anonymous inquiries recorded in the Drug Information Database (DID) between January 2006 and June 2008 (inclusive). In this 30-month period, the DID recorded 198,023 inquiries, of which 118,724 were UK Licensed Pharmaceutical products with a further 79,299 inquiries made for substance not found in the database. Phosphodiesterase type 5 (PDE-5) inhibitors, dominated by Viagra®, ranked 16th among the substance groups. The proportion of the inquiries made regarding PDE-5 inhibitors, especially in comparison to antibiotics, painkillers or alcohol, appears to be above the level that would normally be expected from medical need. No significant change in the months leading up to the Beijing Olympics was observed. On the contrary, the Nitric/Nitrate group showed a notable increase between 2006-2007 and 2008, suggesting a potential increase in interest in using nitric oxide among athletes. It was concluded that with patents recently filed for the use of agents containing sodium nitrite/nitrate to enhance blood flow for performance enhancement in sport, coupled with anecdotal evidence from internet athlete forums and media, there is a concern that athletes may endanger their health by using vasodilators to enhance athletic performance. PDE-5 inhibitors or chemicals in the nitrate/nitrate group are currently not prohibited or tested for by the doping control agencies but some are highly dangerous to health and can lead to cardiovascular collapse, coma and death. Its promotion among athletes as a performance enhancing supplement is ethically and medically questionable. This report demonstrates that, in contrast to interest in prescription vasodilators, athletes exhibited an increasing interest in
"nitric-oxide precursor" vasodilators as observed in the DID records. There was a marked increase in inquiries made about these supplements leading up to the Beijing Olympics. Without medical supervision, use of vasodilators, especially (sodium) nitrite is potentially very serious and the adverse effects should be publicised [10214].

The market success of the three approved synthetic phosphodiesterase type-5 (PDE-5) inhibitors for the treatment of erectile dysfunction has led to an explosion in counterfeit versions of these drugs. In parallel a large market has developed for herbal products claimed to be natural alternatives to these synthetic drugs. The herbal products are heavily advertised on the internet and are freely available to purchase without prescription. Furthermore, adulteration of these supposed natural medicines is a very common and serious phenomenon. Recent reports have shown that the adulteration has extended to the analogues of the three approved synthetic PDE-5 inhibitors. An Atmospheric Solids Analysis Probe (ASAP) was used for the direct analysis of the counterfeit pharmaceuticals and herbal products. Using the ASAP combined with time-of-flight mass spectrometry (TOF MS) it was possible to detect fraudulent counterfeit tablets. The physical appearance of the pills resembled the pills from the original manufacturer but contained the wrong active pharmaceutical ingredient (API). Detecting adulteration for five herbal supplements marketed as natural alternatives to PDE-5 inhibitors was also possible using the ASAP. Three types of adulteration were found in the five samples: adulteration with tadalafil or sildenafil, mixed adulteration (tadalafil and sildenafil), and adulteration with analogues of these drugs [10215].

The presence of a sildenafil derivative, the thiosildenafil, in an herbal product has been evidenced first by HPTLC and later determined by isolation and analysis of spectroscopic data. The analyzed product is nowadays marketed as dietary supplement containing herbal extracts and claimed for male and female sexual improvement. One report is noteworthy since it is clear that adulterated materials can cause serious health problems if they are consumed as herbal "natural" products, generally considered deprived of toxicity by the consumers. The use of a simple and reliable method, based on HPTLC, to determine synthetic adulterations is reported in the paper [11368].

Particularly in healthy males, 20 mg of tadalafil administered 8 h before exercise increased salivary cortisol response to acute aerobic exercise and blood lactate concentrations after anaerobic power exercise, with respect to placebo. In addition, it was found that a two-day administration of tadalafil reduced the hypothalamus–pituitary–adrenal axis responses to exercise test until exhaustion in vivo, and that in skeletal muscle cells tadalafil influences aerobic and anaerobic energy metabolisms depending on its doses and duration of exposure [12094].

Moderate exercise training improves energetic metabolism, tissue perfusion and induces cardiac and skeletal muscle remodeling. Sildenafil, a potent phosphodiesterase-5 inhibitor used to treat erectile dysfunction, reduces infarct size and increases tissue oxygenation in experimental models of cardiovascular disease. We have evaluated the effects of prolonged moderate exercise training and a repeat administration of sildenafil on the rat gastrocnemius and cardiac muscles. Animals were divided into two groups: sedentary and trained. Each group was subdivided into animals treated with vehicle or with two doses of sildenafil (10 or 15 mg/kg/day) during the last week of training. Physical exercise did not induce cardiac hypertrophy, whereas it increased mRNA levels of the PGC-1alpha, HIF-1alpha and VEGF genes, which are involved in mitochondrial biogenesis and angiogenesis, and reduced mRNA levels of FoxO3a, MuRF-1 and Atrogin-1. Sildenafil dose-dependently promoted both angiogenesis, as shown by increased capillary density, and muscle atrophy, as shown by muscle fibre size. These effects were more pronounced in trained animals. The data confirm
the beneficial effects of a moderate and prolonged training on cardiovascular and skeletal systems and document the positive and negative effects of sildenafil on these tissues at doses higher than those used in clinical practice. This report may impact on the use of sildenafil as a substance able to influence sports performance [13565].

Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease with pulmonary vasculopathy. The purpose of one study was to determine whether sildenafil improves 6-min walk distance (6MWD) in subjects with IPF and right ventricular dysfunction. The IPFnet, a network of IPF research centers in the United States, conducted a randomized trial examining the effect of sildenafil on 6MWD in patients with advanced IPF, defined by carbon monoxide diffusing capacity; 35 percent predicted. A substudy examined 119 of 180 randomized subjects where echocardiograms were available for independent review by two cardiologists. Right ventricular (RV) hypertrophy (RVH), right ventricular systolic dysfunction (RVSD), and right ventricular systolic pressure (RVSP) were assessed. Multivariable linear regression models estimated the relationship between RV abnormality, sildenafil treatment, and changes in 6MWD, St. George's Respiratory Questionnaire (SGRQ), the EuroQol instrument, and SF-36 Health Survey (SF-36) from enrollment to 12 weeks. The prevalence of RVH and RVSD were 13 percent and 19 percent, respectively. RVSP was measurable in 71 of 119 (60 %) subjects; mean RVSP was 42.5 mm Hg. In the subgroup of subjects with RVSD, subjects treated with sildenafil experienced less decrement in 6MWD and greater improvement in SGRQ and EuroQol visual analog scores than subjects receiving placebo. In the subgroup with RVH, sildenafil was not associated with change in 6MWD, but was associated with greater relative improvement in SGRQ versus subjects receiving placebo. Sildenafil treatment in those with RVSD and RVH was not associated with change in SF-36. It was concluded that sildenafil treatment in IPF with RVSD results in better preservation of exercise capacity as compared with placebo. Sildenafil also improves quality of life in subjects with RVH and RVSD [13566].
A selective and sensitive screening method for the detection of prohibited narcotic and stimulating agents in doping control is described and validated. This method is suitable for the detection of all narcotic agents mentioned on the World Anti-Doping Agency (WADA) doping list in addition to numerous stimulants. The analytes are extracted from urine by a combined extraction procedure using CH$_2$Cl$_2$/MeOH (9/1, v/v) and t-butylmethyl ether as extraction solvents at pH 9.5 and 14, respectively. Prior to GC-MS analysis the obtained residues are combined and derivatised with MSTFA. The mass spectrometer is operated in the full scan mode in the range between m/z 40 and 550. The obtained limits of detection (LOD) for all components included in this extensive screening method are in the range 20-500 ng/ml, which is in compliance with the requirements set by WADA. Besides narcotic and stimulating agents, this method is also capable of detecting several agents with anti-estrogenic activity and some beta-agonists. As an example, a positive identification of hydroxyl-methoxy-tamoxifen is shown [07201].

A hydrophilic interaction liquid chromatography-time-of-flight mass spectrometry (HILIC-TOFMS) method for the quantification and confirmation of morphine (M), codeine (C), morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G) and codeine-6-glucuronide (C6G) is presented. The method was validated in terms of specificity, selectivity, extraction recovery, accuracy, repeatability, linearity and matrix effect. After a straightforward sample preparation by solid phase extraction (SPE) the compounds were analyzed directly without the need for hydrolysis, solvent transfer, evaporation or reconstitution. The HILIC technique provided good chromatographic separation which was critical for isomers M3G and M6G. The analytes were detected after electrospray ionization (ESI) in positive mode with mass accuracies below 2 mDa using a 5-mDa window. A measurement range of 50-5000 ng/mL was applied for calibration using deuterated analogs as internal standards. The precision of the method was 5.7% and 10.2 percent (RSD) within and between days, respectively. The applicability of the method was demonstrated with authentic urine samples known to contain codeine and/or morphine and their intact glucuronide conjugates. Identification of the analytes was based on in-source collision induced dissociation (ISCID), applying three diagnostic ions with accurate mass [10379].

A rapid method has been developed to analyse morphine, codeine, morphine-3-glucuronide, 6-monoacetylmorphine, cocaine, benzylegonine, buprenorphine, dihydrocodeine, cocaethylene, 3,4-methylenedioxymethamphetamine, ketamine, 3,4-methylenedioxymethamphetamine, pseudoephedrine, lignocaine, benzylpiperazine, methamphetamine, amphetamine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine and methadone in human urine. Urine samples were diluted with methanol:water (1:1, v/v) and sample aliquots were analysed by hybrid linear ion trap-triple quadrupole mass spectrometry with a runtime of 12.5 min. Multiple reaction monitoring (MRM) as survey scan and an enhanced product ion (EPI) scan as dependent scan were performed in an information-dependent acquisition (IDA) experiment. Finally, drug identification and confirmation was carried out by library search with a developed in-house MS/MS library based on EPI spectra at a collision energy spread of 35 ± 15 in positive mode and MRM ratios. The method was validated in urine, according to the criteria defined in Commission Decision 2002/657/EC. At least two MRM transitions for each substance were monitored in addition to EPI spectra and deuterated analytes were used as internal standards for quantitation. The reporting level was 0.05 µg/mL for the range of analytes tested. The regression coefficients in the study were ≥0.98. The method proved to be simple and time efficient and was implemented as an analytical strategy for the illicit drug monitoring of opioids, cocaines and amphetamines in criminal samples from crime offenders, abusers or victims in the Republic of Ireland. To the best of our knowledge there are no
hybrid LC-MS applications using MRM mode and product ion spectra in the linear ion trap mode for opioids, cocaines or amphetamines with validation data in urine [10493].

A rapid method has been developed to analyse morphine, codeine, 6-monoacetylmorphine, cocaine, benzoylcgonine, dihydrocodeine, cocaethylene, 3,4-methylenedioxyamphetamine, ketamine, 3,4-methylenedioxymethamphetamine, pseudoephedrine, lignocaine, benzylpiperazine, methamphetamine, methadone, phenylethylamine and levamisole in human blood. Blood samples were cleaned up using mixed mode solid phase extraction using Evolute™ CX solid phase extraction cartridges and the sample aliquots were analysed by hybrid triple quadrupole linear ion trap (QTRAP) mass spectrometry with a runtime of 12.5 min. Multiple reaction monitoring (MRM) as survey scan and an enhanced product ion (EPI) scan as dependent scan were performed in an information-dependent acquisition (IDA) experiment. Finally, drug identification and confirmation was carried out by library search with a developed in-house MS/MS library based on EPI spectra at a collision energy spread of 35 ± 15 in positive mode and MRM ratios. The method was validated in blood, according to the criteria defined in Commission Decision 2002/657/EC. At least two MRM transitions for each substance were monitored in addition to EPI spectra. Deuterated analogues of analytes were used as internal standards for quantitation where possible. The method proved to be simple and time efficient and was implemented as an analytical strategy for the illicit drug monitoring of opioids, cocaines, amphetamines and adulterants in forensic cases of crime offenders, abusers or victims in the Republic of Ireland [11222].

**Decreased sensitivity after exercise**

Numerous studies have shown a decreased analgesic potency of morphine in exercise trained animals. Since G-protein subunits have a critical role in opioid effects at the cellular level and the exact mechanism(s) of exercise-induced morphine insensitivity has not been fully clarified, the present study was designed to determine the changes in the levels of Ga\(_i\) and G\(\beta\) proteins gene expression involved in this phenomenon. All experiments were carried out on male Wistar rats. Nociceptive thermal threshold was determined by the tail-flick method. Physical training was performed using treadmill apparatus. Semiquantitative RT-PCR and Western blot techniques were used to estimate Galphai and Gbeta mRNA and protein levels, respectively and normalized to an internal standard (beta-actin). The antinociceptive effect of intrathecal morphine (5, 10 and 15 microg) was significantly suppressed in trained rats. Following physical training, a significant decrease in the mRNA and protein levels of Galphai and Gbeta were observed in the dorsal portion of the lumbar spinal cord. In conclusion, the results reveal that the expression patterns of the opioid receptor signaling elements change in exercise training animals. This may be, at least partly, responsible for exercise-induced morphine insensitivity [13518].

Electromembrane extraction (EME) coupled with electrochemical detection on screen-printed carbon electrode has been developed for the quantification of morphine in urine samples. Charged morphine molecules were extracted from an aqueous sample by applying an electrical potential through a thin supported liquid membrane (SLM) into an acidic aqueous acceptor solution (20 microL) placed inside the lumen of a hollow fiber. Then, the acceptor solution was mixed with 20 microL of NaOH solution (0.1M) and analyzed using screen printed electrochemical strip. Differential pulse voltammetry (DPV) peak current at 0.18V was selected as the signal and the influences of experimental parameters were investigated and optimized using Box-behnken design and also one-variable-at-a-time methodology as follows: adsorptive accumulation time, 40s; SLM, 2-nitrophenyl octyl ether+10 percent tris-(2-ethylhexyl) phosphate+10% di-(2-ethylhexyl) phosphate; pH of the sample solution, 6.0; pH
of the acceptor solution, 1.0; EME time, 24 min; EME potential, 90 V and stirring rate, 1000rpm. The calibration curve which was plotted by the variation of DPV currents as a function of morphine concentration was linear within the range of 0.005-2.0 microg/mL. The limit of detection and the limit of quantification were 0.0015 (S/N=3) and 0.005 microg/mL, respectively. Finally, the proposed method was able to determine morphine simply and effectively at concentration levels encountered in toxicology and doping [13519].

**Dermorphine**

Dermorphine and HYP₆-dermorphin are hepta-peptides and natural opioids originally isolated from the skin of South American frogs. They are more potent than morphine but less likely to produce drug tolerance and addiction. These properties make them ideal candidates for the doping of racehorses to enhance performance during competition. Dermorphin was recently classified as a Class I drug by Racing Commissioners International, indicating that it is a banned substance in equine athletes. To enforce this ban, a fast and sensitive method was developed for dermorphin and HYP₆-dermorphin analysis in equine plasma. Equine plasma (2 ml) was extracted on a mixed mode cation exchange solid-phase column. After extraction, dermorphin and HYP₆-dermorphin were separated and detected using a liquid chromatography (LC) triple quadrupole linear ion trap mass spectrometry in positive multiple-reaction-monitoring (MRM) mode. Each analysis was 3.5 min. Four MRM transitions were used for identification of each compound. The extraction procedure was efficient and the limits of detection (LOD) were 2 pg/ml and 10 pg/ml for dermorphin and HYP₆-dermorphin, respectively. The method has good selectivity and precision. Results of stability studies showed that both analytes were stable at low temperature. This is the first report of dermorphin and HYP₆-dermorphin analysis in equine plasma, which could be adopted as a regular screening or confirmation method for controlling the abuse of these compounds in equine sports [13514].

Dermorphin is a unique opioid peptide that is 30-40 times more potent than morphine. It was misused and went undetected in horse racing until 2011 when intelligence obtained from a few North American race tracks suggested its use. To prevent such misuse, a reliable analytical method became necessary for detection and identification of dermorphin in post-race horse samples. This paper describes the first liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for such a purpose. Equine plasma and urine samples were pre-treated with ethylenediamine tetra-acetic acid and urea prior to solid-phase extraction (SPE) on Oasis MCX cartridges. Resulting eluates were dried under vacuum and analyzed by LC-MS/MS for dermorphin. The matrix effect, SPE efficiency, intra-day and inter-day accuracy and precision, and stability of the analyte were assessed. The limit of detection was 10 pg/mL in plasma and 20 pg/mL in urine, and the limit of confirmation was 20 pg/mL in plasma and 50 pg/mL in urine. Dermorphin in plasma is stable at ambient temperature, but its diastereomer is unstable. With isotopically labeled dermorphin as an internal standard, the quantification range was 20-10,000 pg/mL in plasma and 50-20,000 pg/mL in urine. The intra-day and inter-day accuracy was from 91 % to 100 % for the low, intermediate, and high concentrations. The intra-day and inter-day coefficients of variation were less than 12 %. The method differentiates dermorphin from its diastereomer. This method is very specific for identification of dermorphin in equine plasma and urine, as assessed by BLAST search and targeted SEQUEST search, and by MS/MS spectrum library search. The method has been successfully applied to analysis of samples collected following dermorphin administration to research horses and of official post-race samples [13515].
The use of thin-film solid-phase microextraction (SPME) as the sampling preparation step before direct analysis in real time (DART) was evaluated for the determination of two prohibited doping substances, cocaine and methadone, in urine samples. Results showed that thin-film SPME improves the detectability of these compounds: signal-to-blank ratios of 5 (cocaine) and 13 (methadone) were obtained in the analysis of 0.5 ng/mL in human urine. Thin-film SPME also provides efficient sample cleanup, avoiding contamination of the ion source by salt residues from the urine samples. Extraction time was established in 10 min, thus providing relatively short analysis time and high throughput when combined with a 96-well shaker and coupled with DART technique [13516].
Cannabis

Overviews

Since 2004, cannabis has been prohibited by the World Anti-Doping Agency for all sports competitions. In the years since then, about half of all positive doping cases in Switzerland have been related to cannabis consumption. In doping urine analysis, the target analyte is 11-nor-9-carboxy-delta9-tetrahydrocannabinol (THC-COOH), the cutoff being 15 ng/mL. However, the wide urinary detection window of the long-term metabolite of delta9-tetrahydrocannabinol (THC) does not allow a conclusion to be drawn regarding the time of consumption or the impact on the physical performance. The purpose of one study on light cannabis smokers was to evaluate target analytes with shorter urinary excretion times. Twelve male volunteers smoked a cannabis cigarette standardized to 70 mg THC per cigarette. Plasma and urine were collected up to 8 h and 11 days, respectively. Total THC, 11-hydroxy-delta9-tetrahydrocannabinol (THC-OH), and THC-COOH were determined after hydrolysis followed by solid-phase extraction and gas chromatography/mass spectrometry. The limits of quantitation were 0.1-1.0 ng/mL. Eight puffs delivered a mean THC dose of 45 mg. Plasma levels of total THC, THC-OH, and THC-COOH were measured in the ranges 0.2-59.1, 0.1-3.9, and 0.4-16.4 ng/mL, respectively. Peak concentrations were observed at 5, 5-20, and 20-180 min. Urine levels were measured in the ranges 0.1-1.3, 0.1-14.4, and 0.5-38.2 ng/mL, peaking at 2, 2, and 6-24 h, respectively. The times of the last detectable levels were 2-8, 6-96, and 48-120 h. Besides high to very high THC-COOH levels (245 ± 1,111 ng/mL), THC (3 ± 8 ng/mL) and THC-OH (51 ± 246 ng/mL) were found in 65 and 98 percent of cannabis-positive athletes' urine samples, respectively. In conclusion, in addition to THC-COOH, the pharmacologically active THC and THC-OH should be used as target analytes for doping urine analysis. In the case of light cannabis use, this may allow the estimation of more recent consumption, probably influencing performance during competitions. However, it is not possible to discriminate the intention of cannabis use, i.e. for recreational or doping purposes. Additionally, pharmacokinetic data of female volunteers are needed to interpret cannabis-positive doping cases of female athletes [10193].

The aim of one study was to examine prospective memory impairments associated with cannabis use in young adults. An independent measures design utilising pre-existing groups of users and non-users was employed in which an opportunity sample of 90 undergraduates studying at universities in the north east of England participated. The number of prospective memory failures reported on the Prospective Memory Questionnaire and the number of location-action combinations correctly recalled during a video-based prospective memory task were measured. The number of strategies used to assist memory, level of anxiety and depression, and use of alcohol, nicotine and any other recreational drugs in addition to cannabis were also measured and controlled during the analysis. Analysis revealed no significant differences in the number of self-reported prospective memory failures; however, cannabis users recalled significantly fewer location-action combinations than non-users in the video-based prospective memory task. The findings from the present study suggest that cannabis use has a detrimental effect on prospective memory ability in young adults but users may not be aware of these deficits [10194].

Referred to as "spice", several new drugs, advertised as herbal blends, have appeared on the market in the last few years, in which the synthetic cannabinoids JWH-018 and a C(8) homologue of CP 47,497 were identified as major active ingredients. Due to their reported
cannabis-like effects, many European countries have banned these substances. The World Anti-Doping Agency has also explicitly prohibited synthetic cannabinoids in elite sport competition. Since urine specimens have been the preferred doping control samples, the elucidation of the metabolic pathways of these substances is of particular importance to implement them in sports drug testing programmes. In a recent report, an in vitro phase-I metabolism study of JWH-018 was presented yielding mainly hydroxylated and N-dealkylated metabolites. Due to these findings, a urine sample of a healthy man declaring to have smoked a “spice” product was screened for potential phase-I and -II metabolites by high-resolution/high-accuracy mass spectrometry in the present report. The majority of the phase-I metabolites observed in earlier in vitro studies of JWH-018 were detected in this urine specimen and furthermore most of their respective monoglucuronides. As no intact JWH-018 was detectable, the monohydroxylated metabolite being the most abundant one was chosen as a target analyte for sports drug testing purposes; a detection method was subsequently developed and validated in accordance to conventional screening protocols based on enzymatic hydrolysis, liquid-liquid extraction, and liquid chromatography/electrospray tandem mass spectrometry analysis. The method was applied to approximately 7500 urine doping control samples yielding two JWH-018 findings and demonstrated its capability for a sensitive and selective identification of JWH-018 and its metabolites in human urine [10380].

In one study, it was investigated the effect of delta(9)-tetrahydrocannabinol (THC), the principal psychoactive component of marijuana, on immobility time during the forced swim test. THC (2 and 6 mg/kg, i.p.) significantly prolonged the immobility time. In addition, THC at the same doses did not significantly affect locomotor activity in the open-field test. The selective cannabinoid CB(1) receptor antagonist rimonabant (3 mg/kg, i.p.) significantly reduced the enhancement of immobility by THC (6 mg/kg). Similarly, the selective serotonin (5-HT) reuptake inhibitor (SSRI) citalopram (10 mg/kg, i.p.) and 5-HT(1A/7) receptor agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT, 0.3 mg/kg, i.p.) significantly reduced this THC-induced effect. These findings suggest that the 5-HT(1A) receptors are involved in THC-induced enhancement of immobility [08365].

As recently reported, the synthetic cannabinoid JWH-018 is the subject of extensive phase I and II metabolic reactions in vivo. Since these studies were based on LC-MS/MS and/or GC-MS identification and characterisation of analytes, the explicit structural assignment of the metabolites was only of preliminary nature, if possible at all. It was reported the chemical synthesis of five potential in vivo metabolites of JWH-018 derivatives featuring an alkylcarboxy (M1), a terminal alkylhydroxy (M2), a 5-indolehydroxy (M3), an N-dealkylated 5-indolehydroxy (M4) and a 2'-naphthylhydroxy (5) analogue, respectively, and their characterisation by nuclear magnetic resonance spectroscopy. The collision-induced dissociation (CID) patterns of the protonated compounds were studied by high-resolution/high-accuracy tandem mass spectrometry (MSn) applying an LTQ Orbitrap with direct infusion and electrospray ionisation of target analytes. An unusual dissociation behaviour including a reversible ion-molecule reaction between a naphthalene cation (m/z 127) and water in the gas phase of the MS was shown to be responsible for nominal neutral losses of 10 u in the course of the CID pathway. LC-MS/MS-supported comparison of synthesised reference standards with an authentic urine sample using an API 4000 QTrap mass spectrometer identified the synthetic JWH-018 analogues M1-M4 as true in vivo metabolites, presuming a chromatographic separation of potentially present regioisomeric analogues. Existing doping control methods were expanded and validated according to international guidelines in order to allow for the detection of the carboxy and the alkylhydroxy metabolites, respectively, as urinary markers for the illegal intake of the synthetic cannabinoid JWH-018. Both metabolites were quantified in authentic doping control urine samples that had been suspicious of JWH-018 abuse after routine screening procedures,
and a stable isotope-labelled $^{13}$C$_6$-$^{15}$N-carboxy metabolite was synthesised for future analytical applications [11223].

To estimate the prevalence of youth who use cannabis but have never been tobacco smokers and to assess the characteristics that differentiate them from those using both substances or neither substance a total of 5263 students (2439 females) aged 16 to 20 years divided into cannabis-only smokers ($n=455$), cannabis and tobacco smokers ($n=1703$), and abstainers ($n=3105$) were investigated regarding regular tobacco and cannabis use; and personal, family, academic, and substance use characteristics. Compared with those using both substances, cannabis-only youth were younger (adjusted odds ratio, AOR 1.33), to be students (AOR 2.56), and to have good grades (AOR 1.57) and less likely to have been drunk (AOR 0.55), to have started using cannabis before the age of 15 years (AOR 0.71), to have used cannabis more than once or twice in the previous month (AOR 0.64), and to perceive their pubertal timing as early (AOR 0.59). Compared with abstainers, they were more likely to be male (AOR 2.10), to have a good relationship with friends (AOR 1.62), to be sensation seeking (AOR 1.32), and to practice sports (AO, 1.37) and less likely to have a good relationship with their parents (AOR 0.59). They were more likely to attend high school (AOR 1.43), to skip class (AOR 2.28), and to have been drunk (AOR 2.54) or to have used illicit drugs (AOR 2.28). It was concluded that cannabis-only adolescents show better functioning than those who also use tobacco. Compared with abstainers, they are more socially driven and do not seem to have psychosocial problems at a higher rate [07204].

If the doors of perception were cleansed, everything would appear to man as it is, infinite. Ingestion of perception-altering substances dates back millennia, when they may have been used routinely as a dietary source of neurotransmitters, which are energetically expensive to synthesize. It has been suggested that there is a “deep-time” relationship (co-evolution) between hominids and psychotropic plants, their premise based on the synthesis by plants of defensive chemicals that mimic mammalian neurotransmitters, and the ability of mammals to metabolize and/or utilize these phytochemicals to their advantage. Ingestion of these compounds as a routine component of early hominid diets, even if this is no longer necessary, may explain in part the current propensity for the widespread use/abuse of these compounds. Almost all recreational drugs (cocaine, nicotine, amphetamines, tetrahydrocannabinol – THC) are plant toxins that induce changes in dopaminergic circuitry. Marijuana is widely recognized as the most used recreational drug, at least in Europe and North America; however, this is a generalization when dealing with specific countries, regions, or neighborhoods. For instance, members of the Los Angeles and San Francisco Police Department Narcotics Divisions confirmed that in many neighbourhoods in those cities the impact of cannabis is minimal compared with crack, methamphetamine, and/or heroin. This is undoubtedly true for many neighborhoods in major metropolitan areas in the USA and elsewhere. It is generally accepted that the addiction potential of the psychoactive components (primarily THC) of cannabis is limited to psychological habituation, although heavy users may experience slight to moderate short-term physical withdrawal symptoms. There are no documented instances of mortality due solely to cannabis poisoning. Most adverse health effects involve respiratory problems due to ingestion of particulates and other components associated with heavy smoking of any substance, psychoactive or not; nevertheless, legal and illegal use of THC and analogs has and will continue to have a significant impact on society. Even with current restrictions in the USA, the medicinal marijuana market is estimated to be 1.7 billion dollars in 2011, 92 percent accounted for by California and Colorado. This is dwarfed by estimates of the illicit market, for example, a commonly quoted figure from the US Drug Enforcement Agency (DEA) is USD 35 billion. Statistics from the NHTSA/FARS (US National Highway Traffic Safety Administration/Fatality
Analysis Reporting System) from 2009 state that 32% of all traffic fatalities in the USA (33,808) could be directly attributed to alcohol impairment, while those involving use of marijuana are essentially identical to those in which no drug use is suspected. This is not an anomaly. This is not a defense of using cannabinoids and their mimetics, or a denigration of current laws regulating their use; rather, it is to emphasize the need for a rational approach to drug enforcement policies which can be abetted in part by increased understanding of the physiological and psychological bases of their effects on human behaviour. There is a grey area when dealing with many recreational drugs including cannabinoids, steroids, opiates, and tropane alkaloids (scopolamine, atropine), since they have legitimate medicinal uses. One spotlight article explored the current knowledge of THC receptors, computational approaches to development of THC receptor analogues and other designer drugs for medicinal and recreational use, and the unexpected synergistic effects of tropane (belladonna) alkaloids on smoking cannabinoids [11555].

Despite a history of being used for thousands of years, and the subject of numerous chemical investigations dating back to the 1800s, it was not until 1964 that the correct structure for THC was identified. THC is the major psychoactive component of about 85 estimated cannabinoids synthesized by Cannabis sativa. Like the majority of popular recreational drugs – including nicotine, cocaine, amphetamines, and opiates – THC is a plant neurotoxin that affects dopaminergic circuitry primarily through G protein coupled receptors (GPCR). Receptors for cannabinoids were difficult to identify due to the highly lipophilic nature of THC. During the 1980s, Pfizer embarked on a major effort to synthesize analogues based on the THC structure, one of which, CP55,940, a high affinity CB1 ligand, led to identification of two receptors for THC in the early 1990s. CB1 is primarily localized in the central nervous system, and CB2 in cells associated with the immune system; however, there are exceptions, and tentative evidence for additional receptors. Identification of these receptors renewed efforts to synthesize THC analogues, to date numbering over 3000, with the goals of enhancing activity and/or specifically targeting either CB1 or CB2 [11555].

Since over 3000 compounds that bind to CB1 and/or CB2 have been synthesized, it is not surprising that some of these have been exploited for recreational drug use. Some strains of Cannabis have a significant muscle relaxing effect (CB2-mediated), while others are almost entirely euphoric (CB1-mediated). Since THC has similar binding affinities for CB1 and CB2, synergistic compounds have to be invoked to explain these differences. There are four main classes of cannabinoid mimetics: analogues of endogenous ligands related to anandamide (N-arachidonylethanolamine), especially oleamide; THC analogues based on the dibenzopyran ring, including HU-210 with reported 100 times the potency of THC; the cyclohexylphenol series; and the JWH series named after the eponymous John W. Huffman, a structurally diverse group that includes indenes, indoles, and pyrenes. Cannabinoid analogs first became a significant market in Europe sometime in the mid-2000s (and in the USA several years later) with the emergence of Spice, K2, Green Buddha and similar products marketed as “exotic incense blends”. Spice and its spin-offs consist of mixtures of up to 10 or 12 dried herbs (onlabel) and (apparently) one or more designer drugs (including compounds from the four groups listed previously) added for the psychoactive effects. Sometime between 2006 and 2008, Spice and its cousins triggered notice from the Psychonaut Web Mapping Project and the Early Warning System (EWS), which are coordinated by the European Monitoring Centre for Drugs and Drug Addiction. EWS links police, customs, regulatory agencies, and other illegal drug specialists, while Psychonaut (now defunct) trawled the Web for novel recreational drugs. Specific mimetics have subsequently been banned or otherwise regulated in European and Scandinavian countries beginning in 2009 [11555].

Since the World Anti-Doping Agency (WADA) was conceived in 1999 by the sport movement and governments of the world to fight against doping in sport in all its forms, the prohibition of
cannabis in sport has been one of the controversial issues debated by the scientific and political anti-doping authorities. Prior to 2004 and the establishment of the World Anti-Doping Code by WADA, cannabinoids were prohibited only in certain sports. The decision was left to the governing international sport federation as to whether cannabinoids were prohibited in their discipline(s) and whether anti-doping tests were conducted. Consequently, a limited population of athletes was tested and sanctioned for cannabis anti-doping rule violations. In 2004, WADA assumed responsibility for establishing the list of prohibited substances and methods in sport (the Prohibited List). In the first Prohibited List, published under the auspices of WADA that same year, prohibition of cannabinoids was extended to all sports in competition. At the second World Conference on Doping in Sport, held in Copenhagen in March 2003, extension of the cannabinoids ban to all sports was one of the most controversial issues. Some delegates strongly argued that cannabinoids should not be included in sport regulations because consumption of cannabis is not performance enhancing in sports and therefore it should remain a social issue. In this context, it is discussed the potential of cannabis to enhance sports performance, the risk it poses to the athlete's health and its violation of the spirit of sport. Although these compounds are prohibited in-competition only, it may explain why the pharmacokinetics of their main psychoactive compound, delta(9)-tetrahydrocannabinol, may complicate the results management of adverse analytical findings. Passive inhalation does not appear to be a plausible explanation for a positive test [11584].

Cannabis contains over 400 different chemical compounds, including at least 61 cannabinoids. During smoking, more than 2000 compounds may be produced by pyrolysis. Eighteen different classes of chemicals, including nitrogenous compounds, amino acids, hydrocarbons, sugars, terpenes, and simple and fatty acids, contribute to the known pharmacological and toxicological properties of cannabis. The main psychoactive drug in cannabis is delta9-tetrahydrocannabinol (THC), but other cannabinoids also contribute to its pharmacological effects. Cannabidiol (CBD) lacks psychoactivity but possesses anxiolytic, antipsychotic and alerting properties, and it is the basis of pharmacotherapies with multiple indications. CBD content in cannabis is believed to modulate the effects of THC. Other cannabinoids include cannabinol, which is approximately 10 percent as psychoactive as THC, and cannabigerol, cannabichromene and multiple minor cannabinoid components. Over the last 20 years, the knowledge of cannabinoid pharmacology has increased tremendously. Discoveries include identification of CB1 and CB2 cannabinoid receptors, multiple endogenous neurotransmitters (e.g. N-arachidonoyl ethanolamine (anandamide), 2-arachidonoylglycerol, 2-arachidonoyl glyceryl ether,N-arachidonoyldopamine and virodhamine); synthetic pathways, enzymes for neurotransmitter inactivation (fatty acid amide hydrolase and monoacylglycerol lipase), and transport across cell membranes. CB1 receptors are primarily located in the CNS, in high density in the cerebral cortex, hippocampus, amygdala, striatum and cerebellum and functional areas associated with the most prominent behavioural effects of cannabinoids [11584].

The most common neurocognitive deficit observed during acute intoxication is short-term memory impairment. Deficits in motor inhibition, decision making and inhibitory control are also prominent. Less consistent results are available for risk taking after cannabis use. After chronic cannabis exposure, it appears that cannabinoid receptors are desensitized and internalized. The distinct CB2 cannabinoid receptor, primarily located in the periphery, has a critical role in immunomodulation. The endogenous cannabinoid system is highly conserved throughout evolution; it modulates important functions including locomotion, emotional behaviour, cognition, cardiovascular response, pain, feeding behaviour and drug dependence. The stimulating effects of THC on the brain reward system are characteristic of drugs with abuse liability and are similar to other drugs of abuse such as heroin, cocaine, methamphetamine and nicotine. Cannabis produces substantial changes in human
behaviour as well as physiological and biochemical changes. The behavioural and subjective effects of cannabis are highly dose-dependent and include euphoria, enhancement of sensory perception, sedation, relaxation, altered perceptions of time, lack of concentration, impairment of learning/memory, mood changes, panic reaction, paranoia and impaired psychomotor activity. Well described physiological effects include tachycardia, conjunctival injection, dry mouth and throat, increased appetite, vasodilation, bronchodilation, increased sleep and analgesia. This spectrum of behavioural effects is unique, preventing classification of the drug as a stimulant, sedative, tranquilizer or hallucinogen. Subjective and physiological effects of cannabis appear after the first puff of a THC-containing product. Although cannabis smoking produces rapid changes in heart rate, pronounced hypotension and dizziness is observed in approximately 25 percent of individuals approximately 10 minutes after smoking. Cannabis does not produce death directly, as there are few cannabinoid receptors in the brain stem, other than the vomiting centre, limiting cannabis' effects on respiratory function. Around-the-clock, high-dose THC is needed to produce tolerance to the physiological and behavioural effects of cannabinoids. Controversy exists as to whether long-term exposure produces irreversible changes in brain function. Abstinence may permit elimination of cannabinoids from the brain and a return to baseline performance. There are conflicting reports in the literature on the chronic toxic effects of cannabis. Impaired health including lung damage, behavioural changes and reproductive, cardiovascular and immunological effects are associated with cannabis use [11584]

Acute effects of cannabis include increased heart rate, followed in many individuals by hypotension, dizziness and disorientation, increased subjective feelings of euphoria or being “high” and a state of intoxication or being “stoned”, and sometimes psychosis, panic reactions and paranoia. Additional effects that could harm the athlete during competition are loss of vigilance, increased reaction times and short-term memory loss. A different spectrum of effects occurs with chronic daily cannabis use. Multiple studies report decreased cognitive performance after long-term cannabis exposure. Other chronic effects include pulmonary toxicity following smoking and cannabis smoke may induce bronchial irritation, chronic cough and wheeze. Cardiovascular damage, liver steatosis and negative reproductive effects are all associated with chronic cannabis exposure. The exacerbation of symptoms of schizophrenia and the early initiation of the disease has been noted by several investigators. The cannabis withdrawal syndrome is characterized by psychological rather than physical symptoms, including restlessness, anxiety, insomnia, muscle tremor and increased aggression. THC increases dopamine release in the nucleus accumbens and prefrontal cortex similar to other reinforcing drugs of abuse such as cocaine and amphetamines. Synthetic cannabinoid agonists and antagonists at CB1 and CB2 cannabinoid receptors are approved or under development as pharmacotherapies. Cannabinoid agonists are approved for the treatment of nausea and vomiting from cancer chemotherapy and as an appetite stimulant in patients with HIV/AIDS wasting disease [11584].

Cannabis is often portrayed as a substance that has detrimental effects on performance. Cannabis decreases coordination, distorts spatial perception and alters perception and awareness of the passage of time. It has been found that cannabis smoking did not increase vital capacity or grip strength, and maximal exercise performance in 12 cyclists reduced from 16 to 15 minutes at 10 minutes after smoking a THC 1.7 percentcigarette. However, in this study vasodilation and bronchodilation were increased, suggesting that cannabis could also improve oxygenation to the tissues. Cannabis is presented as a drug that has significant positive effects in sports, such as improvement of vision for goalkeepers and muscle relaxation. Smoked cannabis can decrease anxiety, fear, depression and tension. THC is anxiolytic at low doses, the doses reportedly consumed by athletes. It has been suggested that cannabis smoking reduces anxiety, allowing athletes to better perform under pressure and to alleviate stress experienced before and during competition. Furthermore,
cannabinoids play a major role in the extinction of fear memories by interfering with learned aversive behaviors. The endocannabinoid system is also involved in the modulation of mood. Animal studies demonstrate antidepressant-like effects in models based on inescapable or chronic stress. In adolescents and young adults, cannabis also helps in coping with negative mood and emotional distress. Clearly, cannabis induces euphoria, improves self-confidence, induces relaxation and steadiness and relieves the stress of competition. Cannabis improves sleep and recovery after an event, reduces anxiety and fear and aids the forgetting of negative events such as bad falls and so forth. Cannabis increases risk taking and this perhaps improves training and performance, yielding a competitive edge [11584].

In urine, the presence of THCCOOH equal to or greater than the threshold value of 15 ng/mL is reported by WADA-accredited laboratories as an adverse analytical finding (AAF). THC, the primary psychoactive component of cannabis, is rapidly absorbed into the bloodstream following inhalation and is extensively metabolized in the liver into multiple metabolites. The equipotent metabolite 11-hydroxy-THC (11-OH-THC) of THC is further oxidized to THCCOOH and THCCOOH-glucuronide and sulphate. THC is extensively metabolized to multiple other alcohols and acids, but THCCOOH was selected as the analyte monitored in urine for virtually all drug-testing programs. After alkaline hydrolysis of urine to free THCCOOH from its conjugates, THCCOOH is the most abundant urinary marker of cannabis use. THC is distributed initially to the highly perfused organs including the brain, heart, liver and kidneys, with secondary distribution into adipose tissue, because of its high lipophilicity. With chronic daily THC exposure, the THC body burden in fat is large; the rate-limiting step in THC elimination is the slow release of stored drug from the tissues. Cannabinoid concentrations in body fluids depend upon the cannabis potency, smoking topography, frequency of cannabis use and time since last use. In plasma, for example, THC was detected for 6–27 hours after smoking a single cannabis joint containing approximately THC 34 mg (gas chromatography/mass spectrometry limit of quantification 0.5 ng/mL) in individuals who smoked less frequently than daily. Mean (range) peak plasma THC concentrations were 162 (76–267) ng/mL. The mean detection time for THCCOOH in plasma was longer, from 3 to 7 days at the same LOQ. Recently, nondaily cannabis users smoked THC 69 mg and achieved similar mean peak serum THC concentrations of 190 – 106 ng/mL, despite twice the available dose. This is consistent with other reports of cannabis smokers who titrate their dose to the desired level of intoxication and tolerable cardiovascular response. Mean peak urinary THCCOOH concentrations were 90 → 32 ng/mL and 153 → 49 ng/mL approximately 8 and 14 hours after smoking cigarettes containing THC 16 mg and 34 mg, respectively. All urine specimens were collected and individually analysed. THCCOOH was detected in urine at a concentration of 15 ng/mL for 34 → 9 hours (range 8 to 69 hours) and 89 → 10 hours (range 57–122 hours) after these doses. When subjects smoked a cannabis cigarette containing THC 20-25 mg; mean detection time for the last positive urine specimen was 58 – 6 hours (range 16–72 hours). Thus, in cannabis users smoking less frequently than daily, a positive urine cannabinoid test would likely be positive for <4 days. However, when an individual smokes cannabis daily for an extended period, it is possible to have a positive urine specimen for at least 4 weeks. When cannabis is smoked daily, the body burden of cannabinoids is high. It was reported cannabinoid excretion data based on creatinine-normalized urine concentrations from 60 cannabis smokers who resided on a closed research unit under 24-hour monitoring for up to 30 days. All urine specimens were collected and individually analysed for THCCOOH. When urinary cannabinoid excretion data are normalized to urinary creatinine, the individual’s state of hydration is taken into account and the excretion curve is smoothed. Cannabinoid excretion data were divided into three groups based on the initial cannabinoid to creatinine ratio in ng/mg. The three groups were <50 ng/mg, 51-150 ng/mg and >150 ng/mg. In the <50 ng/mg group, normalized cannabinoid/creatinine concentrations on admission ranged from 0 to 47 ng/mg. These
individuals reported smoking cannabis from 2 to 30 days per month for up to 25 years. The first negative urine specimen (<50 ng/mL by immunoassay screen) in this group occurred from 0 to 2.2 days after admission, and the last positive specimen occurred up to 8.6 days after admission, except for one individual who was positive 21.8 days later. The latter individual reported smoking daily for 5 years. In the >150 ng/mg group, normalized cannabinoid/creatinine concentrations on admission ranged from 155 to 1165 ng/mg. These individuals reported cannabis use from 12 to 30 days per month for up to 28 years. There were statistically significant correlations between groups and number of days until first negative and last positive urine specimens; mean number of days were 0.6 and 4.3, 3.2 and 9.7, and 4.7 and 15.4 days, respectively, for the three groups. In individuals generally smoking cannabis on a daily basis, creatinine normalized urine specimens would on average be negative after 15.4 days; however, the urine specimens of many cannabis smokers remained positive for up to 30 days after long-term use. Thus, for athletes who are occasional cannabis smokers, abstinence for 1 week prior to competition should result in negative cannabinoid urine tests and no AAFs, while chronic cannabis use would require abstinence of 1 month or longer. Based on the documented cognitive impairment and toxicity that occurs following chronic use, it is not expected that a significant number of elite athletes would be chronic daily cannabis smokers [11584].

When chronic cannabis users test positive, it is not possible to differentiate new cannabis use from residual cannabinoid excretion from a single urine specimen. Models were developed to predict whether new cannabis use has occurred between two urine specimens collected up to 21 days apart from cannabis users smoking less frequently than daily. These models require the creatinine-normalized cannabinoid concentrations and the time between specimen collections. Minimum, median and maximum ratios between the later urine specimen normalized concentration to that of the first specimen are provided to guide interpretation of whether or not new cannabis use has occurred between the two urine collection dates. Others suggested that measurement of the psychoactive components of cannabis, THC and/or 11-OH-THC, in urine could also indicate recent cannabis use, even in chronic cannabis smokers. Following Escherichia coli b-glucuronidase hydrolysis of urine, THC and 11-OH-THC could be found for up to 8 hours after cannabis smoking. It was evaluated cannabinoid excretion in blood, plasma and urine from the heaviest chronic daily cannabis users encountered in more than 15 years of research. Cannabis smokers resided in a closed research unit for up to 30 days of continuously monitored abstinence. Surprisingly, THC was quantified in blood and plasma from some chronic users for at least 7 days and in the urine for up to 24 days after initiation of abstinence with LOQs of 0.25, 0.25 and 2.5 ng/mL, respectively. Interestingly, 11-OH-THC was present in urine for as long as THCCOOH was throughout the 30-day monitoring period. Thus, neither THC nor 11-OH-THC in urine identify recent cannabis smoking. Another approach to identifying recent cannabis smoking is to monitor drug biomarkers in an alternative matrix other than urine. Oral fluid (saliva) testing offers a simple, fully observed specimen collection method, thereby reducing the potential for adulteration, and it does not require clinical personnel or same-sex doping control officers. THC appears immediately in oral fluids in high concentrations after cannabis smoking, because of the exposure of the oral mucosa to the drug in the cannabis smoke. It has been found that approximately 30-45 minutes after the end of smoking, THC concentrations in oral fluid decreased considerably, correlating temporally with blood concentrations. To date, there are no data on the window of THC detection in oral fluid after chronic cannabis smoking, and oral fluid is not an approved matrix for anti-doping testing. It has been argued that passive inhalation of cannabinoids can yield urinary THCCOOH concentrations >15 ng/mL. Under realistic exposure conditions, passive inhalation of cannabis or hashish smoke does not produce detectable levels of urinary cannabinoids. However, under extreme conditions in laboratory settings with high cannabis smoke concentrations, measurable THCCOOH is possible. Thus, passive inhalation is used as a
line of defence following positive drug tests in the workplace. Scientific data document that THCCOOH concentrations following passive THC inhalation under less than extreme experimental conditions do not exceed the threshold value of THCCOOH 15 ng/mL by GCMS. More recently, another experiment proved the low probability of detecting THCCOOH in the urine of subjects exposed to passive inhalation. The scientific evidence on passive inhalation was reviewed and it was noted that only when the air volume was extremely low and the THC smoke amount unrealistically high, bordering on intolerable discomfort, was it possible to detect the presence of cannabinoids in the urine of participants smoking passively for more than 24 hours. Based on the review of all published articles on the topic, they concluded that ignorant passive cannabis smoking can be excluded with high certitude as a cause of positive samples. Most studies on passive inhalation of cannabis smoke were, however, conducted more than 20 years ago. THC cannabis content increased from 2.8 to as high as 40 percent in some preparations sold in Dutch coffee shops. It could, therefore, be argued that since THC content is higher, there is a greater chance of testing positive if passively exposed to high-content cannabis. However, the average THC content in most street cannabis is 6-7 percent and the passive exposure conditions required to produce positive urine tests are unrealistic. When ingested, peak concentrations are much lower and peak later than after smoking. Less euphoria is experienced and exposure to the more toxic ingredients produced from burning cannabis is avoided [11584].

The consumption of substances derived from cannabis, such as hashish (resin) and marijuana ("grass", "pot"), particularly in the form of joints, is widespread. The relatively high incidence of cannabinoids detection in urine reflects the high prevalence of cannabis use among young adults. Various studies carried out in Europe and elsewhere have shown an impressive increase in the frequency and quantity of consumption, essentially in the younger population, with an earlier onset of use. In a report by the European Monitoring Center for Drugs and Drug Addiction, 1.2–16 percent of young people aged 15–24 from 18 European countries indicated cannabis consumption during the last month whereas 16 percent of young US adults aged 18–25 reported past month use of cannabis. As cannabis smoking impairs cognition, and psychomotor and exercise performance, it is considered to be an ergolytic drug. It has been shown that marijuana smoking reduce maximal exercise performance; when 12 healthy young adults cycled to exhaustion 10 minutes after smoking, exercise duration decreased from 16 to 15 minutes. Driving and piloting skills are also negatively affected, and point to the dangers of cannabis exposure when high level of alertness and quick reflexes are required, such as in automobile sports. Thus it can be inferred from the psychological effects of marijuana that cannabis is effective only in allowing an athlete to relax and to escape from social pressures. However, cannabis is on the list of prohibited substances in the practice of sport, although its performance enhancing effect has not yet been proved. Its popularity among the younger generations as a social drug puts cannabis at the top of the list of compounds detected by the anti-doping laboratories accredited by the World Anti-Doping Agency worldwide. The management of the results of urine analysis is quite difficult for the medical and disciplinary committees not only because of the social use of the substance, but also because of the interpretation of the analytical data from urine samples. One paper gave an overview of what is presently known about cannabis in relation with the practice of sport [06226].

Cannabis species has been used for a variety of purposes for thousands of years, and has been the subject of chemical investigations since the 1800s; however, it was not until 1964 that the correct structure for tetrahydrocannabinol (THC) was characterized. THC is the major psychoactive component of about 85 cannabinoids estimated to be synthesized by Cannabis sativa. Receptors for cannabinoids were difficult to identify because of their highly lipophilic nature. During the 1980s, Pfizer embarked on a major effort to synthesize analogues based on the THC structure, one of which, CP55,940, a high affinity CB1 ligand,
led to identification of two receptors for THC in the early 1990s. CB1 is primarily localized in the central nervous system, and CB2 in cells associated with the immune system. Today many studies state that the palliative benefits of marijuana outweigh the adverse effects of the drug, and recommend that marijuana be administered to patients who have failed to respond to other therapies. Differentiation between direct agonists (including THC), which directly bind to and stimulate cannabinoid receptors, and indirect agonists, which indirectly stimulate these receptors by increasing levels of endogenous CB transmitters, is central. The literature reveals that behavioural animal models yield inconsistent and often contradictory results; however, all drugs of abuse increase dopamine levels in the nucleus accumbens; a convincing argument is made that the degree of abuse potential of a very diverse group of compounds acting on completely different targets via independent mechanisms can reliably be predicted by monitoring dopamine release [12349].

A well-documented, rational case is that while cannabinoid intoxication can have some effects (decreased performance in critical tracking and divided attention tasks, reduced speed, and decreased reaction time), these are minimal compared to alcohol and benzodiazepines, especially among chronic, heavy users of THC and analogues. The average marijuana potency has progressively and substantially increased over the last four decades in the United States. A number of reasons for this trend have been presented, including a shift from foreign to domestic cultivation; advances in cultivation techniques; and cultivars with enhanced tetrahydrocannabinol (THC) levels, especially the switch to higher quality sinsemilla products. Broken down by decade, the 1970s were characterized by poor quality, and low potency kilobrick marijuana; the 1980s and 1990s by a mid-quality mix of kilobrick products; and the 2000s with a dramatic increase in market share by high quality, high potency sinsemilla products. This has led to the widespread belief that potency has increased by a factor of 10 during the last four decades; however, when adjusted for artefacts introduced by substantially reduced seizure-to-analysis times and modified analytical techniques with increased sensitivity, the actual increase is a factor of 6-7 [12349].

The discussion regarding whether cannabinoids such as delta^9-tetrahydrocannabinol (THC) and the synthetic cannabimimetics (e.g. JWH-018, HU-210, etc.) necessitate consideration by anti-doping authorities has been debated for years. Based on the growing knowledge concerning cannabinoid pharmacology, the reasoning for the prohibition of this class of compounds was revisited and reviewed in comprehensive contributions recently. One of the cannabimimetics explicitly mentioned in the WADA Prohibited List is HU-210, the in vitro metabolism of which was studied by means of LC-MS/MS. Using human liver microsomal preparations, 24 phase-I metabolites were obtained, resulting predominantly from oxygenation, hydroxylation, and a combination of both. Although assigning the modifications to either the tricyclic nucleus or the alkyl side chain of HU-210 was accomplished by diagnostic product ions and high resolution/high accuracy mass spectrometry, unambiguous characterization e.g. by chemical synthesis, complementary analytical strategies including for instance (selective) derivatization, GC-MS or NMR was not obtained [0012].

The synthetic cannabinoid JWH-200 (1-[2-(4-morpholinyl)ethyl]-3-(1-naphthoyl)-indole) appeared on the market around 2009. In order to identify markers for misuse of this compound and allow for the development of adequate routine methods, the metabolism of this compound was investigated using two models. In vitro and in vivo (both with and without enzymatic hydrolysis) samples were purified by solid-phase extraction and analyzed using liquid chromatography. Electrospray ionization high-resolution Orbitrap mass spectrometry was used for the identification of the metabolites. To confirm the results in vivo, triple-quadrupole mass spectrometry was employed. In the in vitro model, using human liver microsomes, 22 metabolites were detected which could be divided into 11 metabolite classes. By using the chimeric mouse model with humanized liver, most of these metabolites
were confirmed in vivo. It was found that all metabolites are excreted in urine as conjugates, mostly as glucuronides with varying conjugation rates. The metabolite formed by consecutive morpholine cleavage and oxidation of the remaining side chain to a carboxylic group was detected in the highest amounts with the longest detection time. Therefore, it is the best candidate metabolite to detect JWH-200 abuse in urine [13488].

Recent debate and cases involving elite athletes raised the question whether or not Cannabis sativa (cannabis) should be considered doping in sports. Results from a 2010 report in the United States showed that cannabis is the most used illicit drug, with 17.4 million users smoking cannabis and 6.9 million users smoking cannabis on a daily or near daily basis. The World Anti-Doping Agency (WADA) included cannabis in its Prohibited List in 2004, claiming that cannabis may improve performance in some sports and is an illegal drug in most countries; however, the inclusion of a substance in the Code is complex, requiring intense debate among delegates and the fulfillment of specific criteria. For instance, Section 4 of the Code establishes that a substance be considered for inclusion in the Prohibited List if it is a masking agent or meets two of the three following criteria [13489]:

- potential to enhance performance in sports – smoked cannabis affects cognition and performance, causes memory loss, executive function, and motor impairment, among other undesirable effects. Cannabis smoking may be helpful for some activities such as extreme sports, as it improves muscle relaxation, reduces anxiety, and extinguishes fear memories (e.g. negative experiences) leading to enhanced performance. It is also worthwhile to note that cannabis smoking improves sleep time and recovery, which may favor performance when an athlete is facing multiple competitions in a short period of time. In light of these positive effects, one can assume cannabis is a doping substance that relaxes the mind and improves recovery.
- potential or actual health risk – cannabis’ cognitive effects in chronic users are still unclear, but it may downregulate CB1 receptors, affect executive functions, and cause motor impairment, reversed only after weeks of abstinence. It seems unlikely that athletes are chronic cannabis smokers due to the detrimental effects of chronic use including inconsistent performance, concentration, and motivation. Cyclists who smoked cannabis had a 1-min decrease in maximal exercise performance at 10 min after smoking. These negative effects on cognition and performance can impair critical skills (e.g. decision making, vigilance, alertness) required in high-risk sports to avoid accidents and/or injuries.
- violation of the spirit of sport – doping is essentially contrary to the spirit of sport, which is the principle of Olympism, characterized by several values, such as ethics, fair play and honesty, health, respect for rules and laws, and respect for self and other participants.

The performance of the previously validated LUCIO(®)-Direct-enzyme linked immunosorbent assay (direct ELISA) screening tests according to forensic guidelines is compared to that of cloned enzyme donor immunoassays (CEDIA) test for drugs of abuse in urine as defined in the new re-licensing German medical and psychological assessment (MPA) guidelines. The MPA screening cut-offs correspond to 10 ng/ml 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), 50 ng/ml amphetamine and designer amphetamines, 25 ng/ml morphine, codeine and dihydrocodeine, 30 ng/ml benzylecgonine, 50 ng/ml methadone metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and metabolites of diazepam, oxazepam, bromazepam, alprazolam, flunitrazepam and lorazepam at 50 ng/ml. Average relative sensitivities and relative specificities were 99.7 and 98.4 percent for direct ELISA and 66 and 91 percent for CEDIA, respectively [13490].
Cannabis (Cannabis sativa) is known for its widespread use worldwide. In total, more than 400 different compounds, distributed by 18 chemical groups, including its most active substance delta⁹-THC have been detected in different species of cannabis plants. Consumption of THC-containing cannabis products, such as marijuana (herbal cannabis) and hashish (resinous cannabis) are commonly consumed in the form of cigarettes or even in small pipes. In addition, dronabinol, a THC synthetic product, has been approved in many countries to treat medical conditions such as HIV and cancer. The widespread popularity of use of substances derived from cannabis, such as marijuana among young athletes has led to its high detection frequency. In 2012, 7.6 million individuals 12 years of age or older used marijuana on 20 or more days in the past month [13008].

Long-term use

Cannabis is the most widely used illicit drug in the US, and the number of illicit and licit users is rising. Lasting neurocognitive changes or deficits as a result of use are frequently noted despite a lack of clarity in the scientific literature. In an effort to resolve inconsistencies in the evidence of lasting residual effects of cannabis use, we conducted two meta-analyses. First, it was updated a previous meta-analysis on broad nonacute cognitive effects of cannabis use through inclusion of newer studies. In a second meta-analysis, it was focused on evidence for lasting residual effects by including only studies that tested users after at least 25 days of abstinence. In the first meta-analysis, 33 studies met inclusion criteria. Results indicated a small negative effect for global neurocognitive performance as well for most cognitive domains assessed. Unfortunately, methodological limitations of these studies prevented the exclusion of withdrawal symptoms as an explanation for observed effects. In the second meta-analysis, 13 of the original 33 studies met inclusion criteria. Results indicate no significant effect of cannabis use on global neurocognitive performance or any effect on the eight assessed domains. Overall, these meta-analyses demonstrate that any negative residual effects on neurocognitive performance attributable to either cannabis residue or withdrawal symptoms are limited to the first 25 days of abstinence. Furthermore, there was no evidence for enduring negative effects of cannabis use [12350].

Epidemiology of use of cannabis

In 2010, an estimated 22.6 million Americans age 12 or older self-identified as current (past month) illicit drug consumers. A majority of these respondents, some 17.4 million, acknowledged having consumed cannabis. Year 2010 survey data further estimates that some 10.6 million persons, or 4.2 percent of the population age 12 or older, reported driving under the influence of an illicit drug during the past year. Those respondents who were age 18 to 25 were most likely to have reported engaging in this behavior. A previous US government analysis estimated that among those who acknowledge having driven following the consumption of an illicit substance, a majority of respondents, 70 percent, affirm having done so following the ingestion of cannabis. While this total is far from negligible, it is far fewer than the number of respondents who acknowledge having driven while under the influence of alcohol [12351].

Mechanism of action

The structure of THC was described long before its receptors, CB₁ and CB₂ were discovered. CB₁, which has been primarily found in the CNS, likely explains the central psychotropic effects of marijuana. CB₂ receptors, on the other hand, are mainly found in sensory tissue mediating an analgesic effect. Anandamide, an endogenous ligand binds to these receptors. Anandemide, however, appears to also signal via other receptors than CB₁ and CB₂. Future
studies should investigate the effects of exercise on the cannabinoid receptor system and how this is modulated by marijuana use. Numerous cannabimimetics (cannabis receptor agonists) are being developed that have similar pharmacologic effects but limited negative side effects. However, illicit cannabimimetics such as Spice and K2 (synthetic cannabis) with dangerous side effects are on the rise, which is a concerning issue [13008].

**Synthetic cannabinoids**

A number of synthetic cannabinoids such as JWH-018 and JWH-073 have been incorporated into “spice” products. Despite having labels warning against human consumption, the products are smoked for their cannabinoid-like effects and the extent of their use by athletes has not been adequately described. Urine samples collected from 5,956 athletes were analyzed by high-performance liquid chromatography-tandem mass spectrometry for the presence of JWH-018, JWH-073, and their metabolites. Metabolites of JWH-018 and/or JWH-073 were detected in 4.5 percent of the samples. Metabolites of JWH-018 and JWH-073, only JWH-018, and only JWH-073 were detected in 50 percent, 49 percent, and approximately 1 percent of positive samples, respectively. In total, JWH-018 metabolites were detected in 99 percent (50 % + 49 %) and JWH-073 metabolites were detected in approximately 50 percent (49 % + 1 %) of the positive samples. Parent JWH-018, JWH-018-2-OH-indole, and JWH-018-4-OH-indole were not detected in any of the samples. All samples in which JWH-073 metabolites were detected contained JWH-073-N-butanoic acid. Parent JWH-073 and its N-(4-OH-butyl), 4-OH-indole, 5-OH-indole, and 7-OH-indole metabolites were not detected. Given the number of synthetic cannabinoids that have been synthesized, their limited regulation, and the prevalence of JWH-018 and JWH-073 metabolites detected in the athletes, these compounds should remain a priority for anti-doping programs [12352].

**Medical use**

In the first half of the twentieth century, a number of pharmaceutical companies marked cannabis for indications such as asthma and pain, but since then its use has sharply declined, mainly due to its unpredictable effects, but also for socio-political issues. Recently, great attention has been directed to the medical properties of phytocannabinoids present in the cannabis plant alongside the main constituent delta9-tetrahydrocannabinol (THC); these include cannabinoids such as cannabidiol (CBD), cannabigerol (CBG), and tetrahydrocannabivarin (THCV). Evidence suggests an association between cannabis and schizophrenia: schizophrenics show a higher use of marijuana as compared to the healthy population. Additionally, the use of marijuana can trigger psychotic episodes in schizophrenic patients, and this has been ascribed to THC [12353].

**Duration of detection in blood**

The presence of THC may be detectable in the blood of occasional cannabis consumers for several hours after past use. In more chronic users, THC may be present at relatively low blood levels for a period of days after past use, long after any performance impairing effects have dissipated. Carboxy THC may be present for far longer periods of time. In some cases, the presence of carboxy THC has been identified in the urine of chronic cannabis consumers for periods of 30 to 100 days postabstinence [12351].

**Effects**
The main feature of recreational use of cannabis is that it produces a feeling of euphoria with decreased anxiety and increased sociability, which may alleviate the stress generated by competition. However, cannabis can also produce dysphoric reactions, including severe anxiety and panic disorders, paranoia, and psychosis. These undesirable reactions are commoner in naïve, anxious subjects and psychologically vulnerable individuals and occur more frequently after oral use than after smoking. Because cannabis diminishes alertness, and has relaxing and sedative properties, it may be used to improve sleeping time and sleep quality. Lorente and coworkers reported that relaxing, pleasure, and improved sleeping were the main motives to use cannabis as indicated by students from French sport science universities. A good sleep before competition should improve performance. Athletes engaged in “X-treme” sports are more likely to use cannabis. A relation between drug use and “sensation seeking” behaviour has been often reported. Competition level must be also considered as an increased risk factor for cannabis use for coping with stress and anxiety.

There has been an increase in the use of cannabis and cannabis derivatives in some countries, both in society in general and also among members of football clubs, even those at top professional level. However, the issue is not restricted to football; it affects all sports. This has led to increasing concern and uncertainty among young players, parents, coaches, and managers. Sportspeople who consume cannabis derivatives usually restrict themselves to low doses. Consumption usually takes place outside sports facilities and so is beyond the control of team coaches and/or doctors. The lack of control is accentuated by the fact that testing for the substance is only done during competitions. Data gathered from WADA-accredited laboratories show that cannabis is easily the commonest drug leading to positive results, in particular, it is ahead of testosterone and nandrolone. Positive results only relate to competition testing. They do not take into account any positive results found in urine samples taken out of competition; these are not reported by the laboratories. With development of cultivation methods it has become possible to grow cannabis plants that have significantly higher levels of THC than before. These high THC levels may reinforce and change the immediate effects of consumption. The effects of high levels of THC in terms of long term damage to health have not been well documented, although any activity requiring concentration and energy will be affected by its use. The effects of cannabis derivatives are varied with physical and psychological repercussions as well as influencing a player’s social behaviour. Isolated or infrequent consumption can lead to:

- mild intoxication
- sedative effect on behaviour
- slower reaction times
- memory problems
- tendency towards drowsiness

In terms of the effects on the body, although heightened sensory perception can be expected, THC also engenders a certain heaviness, marked relaxation, and excessive fatigue of the limbs. As the dose increases, the user may experience hallucinations, an alteration of the perception of reality, and a marked reduction in concentration. Furthermore, as these products are generally smoked, this can only have a negative effect on sporting performance and the player’s health (detrimental effects on the lungs, oral cavity, and upper respiratory tract). As regards psychological and social behaviour, cannabis accentuates the mood. So a user may become carefree, happy, and relaxed, but also risks becoming stressed, depressed, or paranoid. Other effects on a user include reduced inhibition and developing a certain indifference. Regular consumption leads to psychological dependence, a chronic sedative effect, and even social detachment.
Exercise increases THC levels

The major psychoactive ingredient of cannabis, delta(9)-tetrahydrocannabinol (THC) accumulates in fat tissue from where it slowly diffuses back into blood. THC pre-treated rats can show elevated plasma cannabinoid levels when subjected to conditions that promote fat utilization, such as fasting. Here we examine whether fasting and exercise increase plasma THC concentrations in regular cannabis users. Fourteen regular cannabis users completed 35 min of exercise on a stationary bicycle in either a fed or overnight fasted state. Plasma cannabinoid levels were assessed prior to exercise, immediately post-exercise and 2 h post-exercise. Plasma samples were also analyzed for indices of lipolysis (free fatty acids (FFA) and glycerol). Exercise induced a small, statistically significant increase in plasma THC levels accompanied by increased plasma FFA and glycerol levels. Exercise-induced increases in plasma THC concentrations were positively correlated with body mass index. Fasting induced a significant increase in plasma FFA levels, and a lowering of blood glucose, but did not significantly alter plasma cannabinoid levels. Thus it was demonstrated that exercise enhances plasma THC levels in regular cannabis users. The lack of a fasting effect may reflect the modest duration of fasting used which was associated with only a modest increase in fat utilization relative to exercise. Overall, these results suggest that exercise may elevate blood THC levels by releasing dormant THC from fat stores. These data suggest the interpretation of blood THC levels in roadside and workplace tests might be complicated by recent exercise [13497].

Influence on psychomotor performance

In closed course and driving simulator studies, cannabis’s acute effects on psychomotor performance may include increased break latency, variation in lateral positioning (weaving), increased headway (leaving greater distance between the subject’s vehicle and the car in front of them), decreased performance in critical tracking and divided attention tasks, reduced speed, and decreased reaction time. Notably, these changes in performance are more likely to be manifested by subjects’ performance on driver simulator tests as compared to assessment of subjects’ actual on-road driving performance. In general, cannabis-influenced variations in driving behavior are often the opposite of those effects exhibited by subjects under the influence of alcohol. Unlike subjects impaired by alcohol, individuals influenced by cannabis are typically aware of their impairment and “tend to compensate effectively” for it, either by driving more cautiously or by expressing an unwillingness to drive altogether. Further, numerous studies report that experienced cannabis users develop tolerance to many of the changes in cognitive or psychomotor performance associated with acute cannabis intoxication. The peak THC blood levels following inhalation do not consistently correspond with levels of peak behavioural impairment. Rather, subjects who inhale THC typically ascertain their highest THC blood levels within minutes, well before the substance’s adverse cognitive or psychomotor effects are at their most significant. Also, cannabis’ effect on psychomotor performance varies widely among individual subjects, particularly among those who are cannabis experienced versus those who are naïve. Experts have cautioned that an accumulation of THC in chronic consumers leads to cannabinoid concentrations in sober phases that resemble concentrations found in occasional users after acute cannabis use, therefore making the universal application of a specific blood/THC impairment standard in many cases inappropriate. However, because inhaled cannabis’ influence on psychomotor behaviour is often subtle, particularly in contrast to those associated with alcohol, and short-lived, consumers of the substance can greatly reduce their risk by refraining from driving for a period of several hours immediately following their cannabis use. Past use of cannabis, as defined by the detection solely of inactive cannabis metabolites in the urine of drivers, is not associated with an increased accident risk [12351].
Cannabis use in sports

Cannabinoids accounted for 13-14 percent of all AAFs reported by the IOC between 1998 and 2003. Since the enforcement of the 2004 WADA Code, when cannabinoids were prohibited in all sports, a significant decrease in cannabinoid-related AAFs from 16 percent to 8 percent was observed. These percentages place cannabinoids as the third most reported prohibited substance in 4 of the last 7 years of compiled WADA laboratory statistics (2003–9) and the second most reported in 2008–9 following anabolic agents. In a social context, cannabis is the most prevalent illicit drug abused in many countries. The use of cannabinoids peaks during the late teens to early twenties, decreasing thereafter to half peak prevalence by age 30. Based on these statistics, cannabis in sport would appear to reflect levels of recreational use reported in many countries, as elite athletes are in general young adults. Sanctions for a first positive result for cannabinoids range from a warning to a 2-year ban, with positive results on repeated occasions leading to a potential lifetime ban. In recent positive cannabinoid cases, there were serious consequences for the athlete’s image and sponsorship endorsement, even if the elite athlete stated the use was out of-competition. Based on WADA statistics, some sports report higher percentages of cannabis use than others, but there does not appear to be a pattern of abuse related to the degree of risk inherent in the sport, the abilities required for each type of sport or the psychological pressure related to exposure to the public. A specific culture of a sport and/or the athlete’s personal decision appears to be the primary motivation for using cannabis in sport. The significant decrease in AAFs for cannabinoids in-competition also stresses the importance of implementing such a tight anti-doping regulation in sport [11584].

Influence of nandrolone on cannabinoid dependence

The identification of the possible factors that might enhance the risk of developing drug addiction and related motivational disorders is crucial to reduce the prevalence of these problems. Here, it was examined in mice whether the exposure to the anabolic-androgenic steroid nandrolone would affect the pharmacological and motivational effects induced by Delta(9)-tetrahydrocannabinol (THC), the principal psychoactive component of Cannabis sativa. Mice received nandrolone using pre-exposure (during 14 days before THC treatment) or co-administration (1h before each THC injection) procedures. Both nandrolone treatments did not modify the acute antinociceptive, hypothermic and hypolocomotor effects of THC or the development of tolerance after chronic THC administration. Nandrolone pre-exposure blocked THC- and food-induced conditioned place preference and increased the somatic manifestations of THC withdrawal precipitated by the CB1 cannabinoid antagonist rimonabant (SR141617A). The aversive effects of THC were not changed by nandrolone. Furthermore, nandrolone pre-exposure attenuated the anxiolytic-like effects of a low dose of THC without altering the anxiogenic-like effects of a high dose in the lit/dark box, open field and elevated plus-maze. Biochemical experiments showed that chronic nandrolone treatment did not modify CB1 receptor binding and GTP-binding protein activation in the caudate-putamen and cerebellum. Taken together, the results suggest that chronic nandrolone treatment alters behavioural responses related to cannabinoid addictive properties [06227].

Cannabis in urine

The active substance in cannabis is tetrahydrocannabinol (THC). It is metabolised into its main metabolite carboxy-THC and conjugated, and then excreted chiefly in the urine. According to the World Anti-Doping Agency (WADA) standards, urine samples are considered positive for cannabis exposure if the sum of the concentrations of free and
conjugated carboxy-THC is greater than 15 microg/l, when determined by gas-chromatography/mass spectrometry. This threshold value distinguishes active users from passive smokers. It makes also less likely a positive result due to intake of commercial foods containing traces of cannabinoids. Since the mid-1990s, the ingestion of food products containing seeds or hemp seed oil has increased considerably in several Western countries. However, several US states have enacted regulatory limits for THC in foodstuffs and beverages, thus lowering THC intake from hemp containing foods and reducing the risk of a positive urine test. Regulations in Canada, the main supplier of hemp seeds to the USA, limit THC levels in hemp seed products to 10 parts per million (ppm). There are several other problems with carboxy-THC analysis in urine. The relevance of the 15 ng/ml cut-off value could be improved by correcting the THC concentration with the creatinine level. With this approach, the quantitative results would be corrected for urine dilution and possible adulteration. It will also be possible to predict repetitive or new marijuana use by analysis of the urinary cannabinoid to creatinine ratio in two consecutive urine samples collected at least 24 hours apart. Quantitation of carboxy-THC in urine alone cannot predict time of last marijuana use or suggest any relation between urine concentration and psychomotor effects. It has been found that occasional users of marijuana had positive urine specimens for three to four days after receiving a standard dose of marijuana. In heavy smokers, urine specimens have been shown to remain positive for seven to ten days after last drug use. It has also been reported an average time until the last positive result of 32 days (4–77 days) in urine for THC metabolites screened by immunoassay with a cut-off value of 20 ng/ml. According to some researchers determination of the time of last cannabis use is possible by measuring total THC in urine. Significant levels of THC could be measured by including a hydrolysis step in the extraction protocol. A concentration greater than 1.5 ng/mL would suggest marijuana use within a five hour time window, a time during which psychological and performance effects are known to occur [06226].

An abnormal result must be announced for cannabis if the main metabolite of THC, carboxy-THC, is discovered in a player's urine at a level in excess of 15 microg/l as a result of competition testing. This limit has been established to distinguish between active consumers and smokers of cannabis and players who may have been passively exposed to cannabis smoke. The limit also reduces the risk of a positive urine result after the consumption of some commercial products which contain traces of cannabis. The use of hemp seed and oil in food products has increased considerably in some Western countries since the mid-1990s. The authorities have acted to impose limits on THC levels such that the consumption of these commercial products will not bring about positive urine results. It should be noted that the elimination of THC metabolites from urine is a slow process and depends on individual physiology. In this way, a simple quantification of the urinary concentration of carboxy-THC reveals little information on the time elapsed since consumption. The quantity discovered in urine depends on various factors:

- dosage of the most recent consumption
- time elapsed between the most recent consumption and taking the urine sample
- manner of consumption (single dose or regular consumption)
- individual metabolism

When these factors are taken into account, it is extremely difficult to establish a relation between urinary concentration and the effects on an individual's psychomotor skills. Despite these considerations, some scientific studies have shown that on the occasional consumption of a normal dose (a "joint"), the user will have a positive result for carboxy-THC for three, four or even five days, depending on the smoker's body mass. In this way consuming marijuana with friends a few days before a match could be disastrous for a football player as there would be a considerable risk of failing a doping test. The pretext of
recreational use is no longer valid. Even if cannabis is taken without the intention of improving performance, the outcome will be a positive result if the urinary level exceeds the authorised threshold. For regular users – for example, those who smoke cannabis several times a week – urine samples would remain positive for a much longer time after the most recent consumption. Scientific research published on this subject has shown that the time until urine samples return negative outcomes can be as much as four weeks (two weeks on average) after the most recent consumption [06226].

Does cannabis have a doping effect?

The question often asked in the case of a positive result for cannabis is whether it was consciously used for doping purposes. The answer is that this substance can only indirectly improve performance – it can have a euphoric effect, reducing anxiety and increasing the sociability of a player who may be particularly nervous before an important match. It can also have a relaxing effect after the game. In this way, cannabis can be considered as a doping product that calms the mind. It has already been described that use of cannabis in sporting environments is basically motivated by the effects of relaxation and wellbeing, allowing the user to sleep more easily. However, if consumed regularly, it risks harming performance and motivation. It is when cannabis is consumed regularly that the signs become apparent in young athletes. There may be changes in behaviour during training as well as inconsistent performance, concentration, or motivation. Particular care should be taken when a player is vulnerable, for example if lacking support, during prolonged or repeated injury, when isolated from family or if subject to excessive pressure for results on the pitch. In these cases it is up to the club coaches and doctors to look out for any symptoms that may appear and to intervene tactfully, although firmly, when necessary. In some cases referral to a psychologist will be necessary [06226].

The ergogenic effects of marijuana are questionable, as its performance enhancing effect, if any, has yet to be established. Along these lines, very few studies have tested the effects of marijuana on performance. One of the first studies to evaluate the effects of marijuana tested the effects of marijuana smoking compared to placebo on several indices of exercise performance. Resting heart rate and both systolic and diastolic blood pressure were significantly elevated at rest after marijuana consumption compared to both control and placebo. Although there was no significant decrease in grip strength, physical work capacity at a heart rate of 170 decreased by 25 percent compared to placebo. It was tested subjects 10 min after smoking a marijuana cigarette (containing 1.7 % of delta⁹-THC) of 7 mg/kg of body weight, and noted a slight, but significant decrease in cycle ergometry time to exhaustion. It was demonstrated that double-blind administration of marijuana as 7.5 mg of delta⁹-THC or placebo did not affect blood pressure, ventilation or oxygen uptake during submaximal exercise (15 min at 50 % of VO₂max), however did increase heart rate and the rate-pressure product at rest and during both exercise and recovery. In another study it was hypothesized that the decrease in exercise performance may be due to its chronotropic effect leading to achievement of maximum heart rate at reduced workloads. Furthermore, detrimental effects on other aspects of performance have also been demonstrated. When subjects were acutely given THC orally (215 microg/kg) acutely, significant deficits in general performance, standing steadiness, reaction time and psychomotor performance were observed over a 5 hour period post-ingestion. Interestingly, in a case report, it was documented that in a patient with asthma, a condition characterized by bronchoconstriction, smoking marijuana prior to exercise testing led to bronchodilation and no defect in pulmonary function. Thus, if there is any positive effect of marijuana, it likely only indirectly improves performance. It is conceivable that cannabis may reduce an athlete’s feelings of pre-competition stress and anxiety as a result of the euphoric effect it may produce. Also, because cannabis diminishes alertness and has relaxing and sedative properties, use may
be driven by the effects of relaxation, well-being and improved sleep quality. For example, it has been reported that relaxing, pleasure, and improved sleeping were the main motives to use cannabis, with the rationale that adequate sleep and being relaxed before competition may lead to optimal performance. However, due to the trade-off of decreased exercise performance, possibly secondary to increases in heart rate and blood pressure, which may alter perceived exertion, marijuana may be considered an ergolytic agent [13008].

Overall, it appears that cannabis does not have ergogenic potential in sports activities and thus, its inclusion on the banned list is likely a function of its illicitness. As cannabis smoking impairs exercise and psychomotor performance (such as sedative effect, slower reaction times and other psychomotor effects), its ability to serve as an ergogenic aid has been questioned, and is generally considered to be an ergolytic drug. This is likely due to increase in heart rate and blood pressure, decline of cardiac output and reduced psychomotor activity that have been demonstrated in prior studies [13008].

<table>
<thead>
<tr>
<th>Acute effect</th>
<th>Effect on performance</th>
<th>Cannabis dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting heart rate and both systolic/diastolic blood pressure significantly elevated at rest</td>
<td>Physical work capacity at a heart rate of 170 decreased by 25 % compared to placebo</td>
<td>18.2 mg of delta$^9$-THC</td>
</tr>
<tr>
<td>Induced tachycardia at rest</td>
<td>VE, VO$_2$ and VCO$_2$ were increased above control at ≥50 % max effort; small, but significant reduction in maximal exercise duration; tachycardia up to 80 % of maximum effort and during recovery</td>
<td>7 mg/kg marijuana (containing 1.7 % delta$^9$-THC)</td>
</tr>
<tr>
<td>Increased heart rate and the rate-pressure product at rest</td>
<td>No effect on blood pressure, ventilation or oxygen uptake during submaximal exercise (15 min at 50 % of VO$_{2\text{max}}$); increased heart rate and the rate-pressure product during recovery</td>
<td>Smoking 7.5 mg of delta$^9$-THC</td>
</tr>
</tbody>
</table>

### Endogenous

Marijuana is the most commonly used illegal drug, particularly in Western societies. The discovery of an endogenous cannabinoid system (ECS) highlighted new molecules in various physiological processes. The ECS consists of G-protein-coupled cannabinoid receptors that can be activated by small lipid mediators, termed endocannabinoids (eCBs) and cannabis-derived drugs, plus the associated biochemical machinery (precursors, synthesis and degradative enzymes, and transporters). Several biochemical, pharmacological and physiological studies have shown that endocannabinoid system elements are widely distributed throughout the body, with regional variations and organ-specific actions. This review portrays the endocannabinoid "family" on new studies concerning eCB storage, release and functional roles and on the growing importance of its bioactive metabolites. Those findings reinforce and confirm the importance of ECS. Strategies for manipulating the system for the treatment of human disease will require a thorough understanding of the roles of the different eCBs and their sources [13491].
The endocannabinoid system has been implicated in the regulation of a variety of physiological processes, including a crucial involvement in brain reward systems and the regulation of motivational processes. Behavioral studies have shown that cannabinoid reward may involve the same brain circuits and similar brain mechanisms with other drugs of abuse, such as nicotine, cocaine, alcohol and heroin, as well as natural rewards, such as food, water and sucrose, although the conditions under which cannabinoids exert their rewarding effects may be more limited. The purpose of one review was to briefly describe and evaluate the behavioral and pharmacological research concerning the major components of the endocannabinoid system and reward processes. Special emphasis is placed on data received from four procedures used to test the effects of the endocannabinoid system on brain reward in animals; namely, the intracranial self-stimulation paradigm, the self-administration procedure, the conditioned place preference procedure and the drug-discrimination procedure. The effects of cannabinoid 1 (CB1) and cannabinoid 2 (CB2) receptor agonists, antagonists and endocannabinoid modulators in these procedures are examined. Further, the involvement of CB1 and CB2 receptors, as well the fatty acid amid hydrolase (FAAH) enzyme in reward processes is investigated through presentation of respective genetic ablation studies in mice. We suggest that the endocannabinoid system plays a major role in modulating motivation and reward processes. Further research will provide us with a better understanding of these processes and, thus, could lead to the development of potential therapeutic compounds for the treatment of reward-related disorders [13492]

“Spice”

Over 60 cannabinoids are present in cannabis, with delta9-tetrahydrocannabinol (THC) the main psychoactive constituent and responsible for the observed toxic effects after smoking, while other cannabinoids are responsible for minor effects, such as cannabiol (CBN), which is 10 percent as psychoactive as THC. THC is lipophilic and stores in several organs, especially in adipose tissue; this extensive body burden explains the prolonged cannabinoid detection rate in blood and urine for at least 4 weeks in chronic daily cannabis smokers. The WADA establishes a 15 ng/mL urinary 11-nor-9-carboxy-THC (THCCOOH) threshold; urine analyses involves THCCOOH-glucuronide conjugates cleavage, which significantly increases free THCCOOH concentrations and detection time. Urinary THCCOOH concentrations above the 15 ng/mL threshold are considered Adverse Analytical Findings and may be interpreted as a violation of anti-doping rules. Studies showed that even occasional and single cannabis smoking might yield a THCCOOH positive result (≥15 ng/mL) for up to 5 days. Thus, consuming cannabis even weeks before a match may imply a considerable risk of being detected in a doping test. In light of this considerable risk, some users started using a new preparation of herbal smoking blends named “Spice.” Such substances are highly potent cannabinoid analogs, with unknown and potentially harmful toxicological properties that may cause prolonged intoxication. These substances mimic or worsen cannabis’ toxic effects provoking cognitive and motor impairment [13489].

CP 47,497, a potent cannabinoid receptor type 1 agonist, is the main active ingredient in the herbal mixture "Spice" sold in European countries. The illegal use of "Spice" for its psychoactive effects has become a social issue. In this study, the in vitro metabolism of CP 47,497 was investigated in human liver microsomes to characterize the metabolic fate of CP 47,497. CP 47,497 was incubated with human liver microsomes, and the reaction mixture was analyzed using liquid chromatography-tandem mass spectrometry. A total of eight metabolites were detected in human liver microsomes and structurally characterized based on mass spectral data. The main metabolic pathways involved hydroxylations or oxygenations. The identified metabolites were mono-oxygenated metabolites (M1 and M4), mono-hydroxylated metabolites (M3, M5, M6, M7, and M8), and a di-oxygenated metabolite
The detection of these metabolites could confirm the presence of CP 47,497 in biological samples; therefore, collectively, they would be excellent indicators of "Spice" drug abuse [13493].

Synthetic cannabinoid receptor agonists are becoming increasingly popular with adolescents as an abused substance. Chronic use of these drugs can lead to addiction syndrome and withdrawal symptoms similar to cannabis abuse. Due to their potential health risk, several countries have banned these substances. It was reported the clinical presentation and legislation status of synthetic cannabinoids in “Spice” products and alert the health care community about the identification and risk assessment problems of these compounds. It was retrospectively reviewed cases presenting to our Emergency Department (ED) during a 3-month period with chief complaints of Spice drug use before arrival. Six cases presented to our ED after using Spice drugs. Two patients were admitted after reporting seizures. All but one presented with tachycardia. Two patients had hallucinations. The average length of ED observation was 2.8 h. No patient with seizures had recurrent episodes. Spice drugs can cause potentially serious health care conditions that necessitate ED evaluation. Most cases can be discharged from the ED after a period of observation. Legal issues surrounding these drugs are yet to be finalized in the United States [13494].

The non-psychoactive cannabidiol (CBD) is anxiolytic in humans following a single dose; decreased anxiety and fear memories extinction after oral CBD intake may enhance sports performance with no “violation” of the Code, as no THCCOOH is detected in urine. One way to protect athletes’ health and to promote health, fairness, and equality in sports is to include any illicit drugs, their constituents and analogs in the anti-doping program. The sports may assist to create educational program for youth and athletes as an alternative to keep them away from drugs and to preserve the intrinsic value about the “spirit of sport” [13489].

**Occasional or frequent smokers**

There is extended urinary excretion of delta9-tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), and 11-nor-9-carboxy-THC (THCCOOH) in abstinent frequent cannabis smokers. It was characterized THC, 11-OH-THC, THCCOOH, cannabidiol, cannabinol, THC-glucuronide, and THCCOOH-glucuronide disposition in urine of frequent and occasional cannabis smokers, and we propose a model to predict recent cannabis smoking. Frequent and occasional smokers resided on a closed research unit and smoked one 7 percent THC cannabis cigarette ad libitum. Urinary cannabinoids were quantified in each void by liquid chromatography-tandem mass spectrometry within 24 h of collection. No urine samples had measureable THC, 11-OH-THC, cannabidiol, or cannabinol. THCCOOH, THC-glucuronide, and THCCOOH-glucuronide were measurable in all frequent smokers’ urine and 60, 100, and 100 percent of occasional smokers’ urine samples, respectively. Pre- and postdose maximal concentrations (non- and creatinine normalized) and probability of being positive were significantly higher in frequent smokers' samples. THC-glucuronide concentrations peaked 0.6-7.4 h after smoking; THCCOOH and THCCOOH-glucuronide concentrations were highly variable. At the newly adopted THCCOOH 175 microg/L World Anti-Doping Agency decision limit, only 50 percent of frequent smokers were positive 0-6 h postdose; no occasional smokers' samples were positive. An absolute percentage difference of ≥50 percent between 2 consecutive THC-glucuronide-positive samples with a creatinine-normalized concentration of ≥2 microg/g in the first sample predicted cannabis smoking with efficiencies of 93 percent in frequent and 77 percent in occasional smokers within 6 h of first sample collection. These controlled urinary cannabinoid data provide a possible means of identifying recent cannabis intake in cannabis smokers' urine within a short collection time frame after smoking [13500].
Side effects

In March 2012, the Wyoming Department of Health was notified by Natrona County public health officials regarding three patients hospitalized for unexplained acute kidney injury (AKI), all of whom reported recent use of synthetic cannabinoids (SCs), sometimes referred to as "synthetic marijuana." SCs are designer drugs of abuse typically dissolved in a solvent, applied to dried plant material, and smoked as an alternative to marijuana. AKI has not been reported previously in users of SCs and might be associated with a previously unrecognized toxicity, a contaminant or a known nephrotoxin present in a single batch of drug, or a new SC compound entering the market. After the Wyoming Department of Health launched an investigation and issued an alert, a total of 16 cases of AKI after SC use were reported in six states. Review of medical records, follow-up interviews with several patients, and laboratory analysis of product samples and clinical specimens were performed. The results of the investigation determined that no single SC brand or compound explained all 16 cases. Toxicologic analysis of product samples and clinical specimens (available from seven cases) identified a fluorinated SC previously unreported in synthetic marijuana products: (1-(5-fluoropentyl)-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl) methanone, also known as XLR-11, in four of five product samples and four of six patients' clinical specimens. Public health practitioners, poison center staff members, and clinicians should be aware of the potential for renal or other unusual toxicities in users of SC products and should ask about SC use in cases of unexplained AKI [13495].

Marijuana is the most widely used illicit drug by pregnant women in the world. In utero exposure to 79-tetrahydrocannabinol (79-THC), a major psychoactive component of marijuana, is associated with an increased risk for anencephaly and neurobehavioural deficiencies in the offspring, including ADHD (attention deficit hyperactivity disorder), learning disabilities and memory impairment. The developing central nervous system (CNS) is susceptible to the effects of 79-THC and other cannabimimetics, including the psychoactive ingredients of “Spice”. These exocannabinoids interfere with the function of an endocannabinoid (eCB) system [12349].

Heart

As with cocaine, marijuana and hashish are used by athletes, not so much for their ergogenic action, as because of addiction. Cases of acute myocardial infarction, arrhythmias, and sudden death have been described among athletes who use them. The cardiovascular complications are mainly due to sympathomimetic action and to suppression of peripheral vasoreflexes. These pathophysiological mechanisms lead to increased demand and at the same time decreased supply of oxygen, as well as the presence of arrhythmias [12126].

Coronary artery disease (CAD) remains a major public health problem in the world. Acute coronary syndromes (ACS) mainly affect patients with cardiovascular risk factors. It was reported a case of an exercise-induced ACS in a 24-year-old soccer player without any classic cardiovascular risk factor, but with a history of massive cannabis addiction. Coronary angiography showed complete occlusion of the proximal right coronary artery and intravascular ultrasound revealed an atherosclerotic plaque disruption. Thromboaspiration and antithrombotic treatment were successful and coronary stenting was not performed. The respective roles of cannabis toxicity and physical activity as triggers for ACS in young people are discussed [13498].

Ischemic stroke. It was reported on a 16-year-old body builder who suffered from an acute ischemic stroke. In the urine, cannabis metabolites as well as metabolites of the oral androgenic-anabolic steroid methandrostenolone were detected, both known to be
associated with stroke events. One report highlights the role of cannabis and steroid abuse that induce strokes in the absence of arteriopathy, cardioembolism or thrombophilia. Owing to new upcoming socio-behavioral aspects of late childhood and early adolescent life, this formally rare abuse of cannabis and/or anabolic steroids as well as their associations with strokes becomes more current than ever [13496].

**Laboratory techniques**

Testing for cannabis in the form of marijuana, hashish or other cannabis containing products is performed by urine analysis. The target molecule detected in urine analysis is 11-nor-9-carboxy-delta²⁷-THC, with the limit for a positive test at >15 ng/mL. Detection is determined by gas-chromatography/mass spectrometry, and this threshold distinguishes active users from passive smokers and foods that contain traces of cannabinoids [13008].

The identification of 11-nor-delta9-tetrahydrocannabinol-9-carboxylic acid (THCCOOH) in hair represents an exceptional forensic analytical challenge due to low target concentrations in a complex matrix. Several dedicated techniques (gas chromatography – negative chemical ionization-tandem mass spectrometry (GC-NCI-MS/MS) or GC-GC-MS couplings) were specifically introduced into forensic toxicology aiming to a selective and sensitive identification of THCCOOH in hair. The combination of liquid-chromatography (LC) and MS/MS gained an outstanding relevance in forensic toxicology (including the detection of cannabinoids). However, its application to hair matrix is characterized by a lack of specificity which is due to the unspecific decarboxylation as most abundant fragmentation reaction. Therefore, various chemical modifications of the carboxyl and/or phenolic hydroxyl groups were examined to improve the selectivity. The selective methylation of the 9-carboxyl-group proved to be the most efficient derivatization procedure. Hair extracts were redissolved in acetonitrile and after addition of few milligrams of solid sodium carbonate derivatized with 25 microL methyl iodide. The resulting THC-9-carboxymethylester was separated by conventional reverse phase LC and selectively detected using negative electrospray ionization by recording the fragmentation reactions 357➔325 and 357➔297. Resulting limits of quantification were below 100 fg/mg. A further significant improvement was achieved by application of the multistage MS3 fragmentation 357➔325➔297. To verify the validity of this procedure, a systematic quantitative comparison of THCCOOH concentrations in hair with data from a well established GC-NCI-MS/MS technique was performed. Both techniques proved to be in good accordance and equally suitable for hair testing of THCCOOH [13499].

A rapid and sensitive determination of cannabinoids in urine is important in many fields, from workplace drug testing over toxicology to the fight against doping. The detection of cannabis abuse is normally based on the quantification of the most important metabolite 11-nor-Δ⁹-tetrahydrocannabinol-9-carboxylic acid (THCA) in urine. In most fields THCA needs to be present at a concentration of exceeding 15 ng/mL before a positive result can be reported. The method described in this paper, combines a 4min GC-MS/MS method with a fast sample preparation procedure using microwave assisted derivatisation in order to complete the quantification of THCA in urine in 30 min, using only 1 mL of urine. The method is selective, linear over the range 5-100 ng/mL and shows excellent precision and trueness and hence, the estimated measurement uncertainty at the threshold level is small. The method also complies with applicable criteria for mass spectrometry and chromatography. Therefore the method can be used for rapid screening and confirmatory purposes [12354].
Most substances on WADA’s Prohibited List are banned at all times; however, compounds belonging to the sections S6-S9 or P1 and P2 are not relevant for doping controls in out-of-competition periods. Within a two-year monitoring program (2006 and 2007), the prevalence of so-called non-specified stimulants in athletes’ urine samples was assessed yielding 0.36% adverse analytical findings in approximately 25 000 analyzed specimens, demonstrating that cocaine was by far the most frequently detected “prohibited” substance. These numbers led to the conclusion that no systematic abuse of stimulants during out-of-competition periods prevails [12017].

Tropane alkaloids like atropine are antidotes applied against organophosphorus intoxications. Atropine is toxic itself and should be closely monitored during treatment. Hence, simple, fast, and sensitive determination methods for tropane alkaloids in serum are desirable. Mostly adopted methods of analysis are gas chromatography (GC); high performance liquid chromatography (HPLC), and capillary electrophoresis (CE). Various liquid and solid capillary fillings used in micellar electrokinetic chromatography, microemulsion electrokinetic chromatography, capillary electrophoresis, and enantioseparation provide high versatility to CE applications. In HPLC, specialised columns enhance separation efficacy. Ultraviolet light detection is common practise, but recently sensitivity and analyte identification were enhanced by coupling GC, HPLC, and CE to mass spectrometry. Apart from medical treatment, tropane alkaloids, cocaine in particular, are abused with various intentions. Forensic analysis of tropane alkaloids and their metabolites comprises the additional difficulty of unequivocal drug identification. Because of severe legal consequences, sophisticated analytical methods were developed and may provide additional techniques for therapeutic drug monitoring. Examples from forensic cocaine analysis and from doping analysis were included in the review [10195].

An accurate and precise method for the quantification of 11-nor-delta9-tetrahydro-cannabinol-9-carboxylic acid (THCA) in urine by liquid chromatography/tandem mass spectrometry (LC/MS/MS) for doping analysis purposes has been developed. The method involves the use of only 200 microL of urine and the use of D9-THCA as internal standard. No extraction procedure is used. The urine samples are hydrolysed using sodium hydroxide and diluted with a mixture of methanol/glacial acetic acid (1:1). Chromatographic separation is achieved using a C8 column with gradient elution. All MS and MS/MS parameters were optimised in both positive and negative electrospray ionisation modes. For the identification and the quantification of THCA three product ions are monitored in both ionisation modes. The method is linear over the studied range (5-40 ng/mL), with satisfactory intra- and inter-assay precision, and the relative standard deviations (RSDs) are lower than 15 percent. Good accuracy is achieved with bias less than 10% at all levels tested. No significant matrix effects are observed. The selectivity and specificity are satisfactory, and no interferences are detected. The LC/MS method was applied for the analysis of 48 real urine samples previously analysed with a routine gas chromatography/mass spectrometry (GC/MS) method. A good correlation between the two methods was obtained) with a slope close to 1 [10196].

It is frequently questioned whether cannabinoids are detectable in urine from individuals having been passively exposed to hashish or marihuana smoke. Literature was reviewed to shed light on an issue that is often debated in the health services, judicial system and in sports. A Medline search with the index terms "cannabis", "hashish", "marihuana" was conducted in September 2007. Summaries, abstracts and reference lists of selected articles were screened for relevancy. Seven experimental studies with humans were identified. Cannabinoids were detected in urine in two studies where the subjects had been exposed to high smoke levels. In studies conducted under less extreme conditions no urine samples were positive for more than a few hours after exposure and the measured cannabinoid levels
were low. When cannabinoids are detected in urine with conventional methods and limits of quantification the results are commensurate with active smoking [09291].

One study was designed to determine the effect of delta-9-tetrahydrocannabinol (THC) on susceptibility to stress. It was reported that THC significantly prolonged the immobility time during the forced swim-stress. The selective cannabinoid CB1 receptor antagonist O-2050 significantly reduced the enhancement of immobility by THC. It was investigated the effect of THC on levels of stress hormone corticosterone under non-stress and forced swim-stress conditions. THC did not affect plasma corticosterone levels under non-stress conditions. However, THC, together with forced swim-stress, significantly increased plasma corticosterone levels. This effect was inhibited by O-2050. This evidence suggests that THC, under stressful conditions, enhances the susceptibility of the hypothalamus-pituitary-adrenal-axis to stress via the CB1 receptor, thereby increasing the risk of depression [09292].

Cocaine has been used as a medicine for many years. It was also one of the original ingredients of Coca-Cola until it was removed in 1903. Its therapeutic indication is as a local anaesthetic, although misuse is linked to its euphoric effects and feeling of decreased fatigue. Its potential for use as a recreational drug emphasises the lifestyle pressures faced by some athletes. In some disciplinary sports, such as sprinting, cocaine is likely to increase production of heat and lactic acid, which, coupled with vasoconstriction, could contribute to fatal cardiac arrest [06171].

Cocaine is the most potent stimulant of natural origin. As opposed to amphetamines, which are pure synthetic compounds, cocaine is primarily obtained from Coca species and its notoriety belies the fact that the drug has been used as a stimulant for thousands of years. The Incas used to chew Coca leaves to fight against tiredness; cocaine was used in a number of patent medicines and even in soft drinks. In its pure form, cocaine is a white crystalline powder extracted from the leaves of the South American Coca plant. Pure cocaine was first used medicinally in the 1880s as a local anaesthetic in eye, nose, and throat surgery because of its ability to provide anaesthesia as well as to constrict blood vessels and limit bleeding. Many of its therapeutic applications are obsolete due to the development of safer drugs. Cocaine can be snorted, smoked, or injected. When snorted, cocaine powder is inhaled through the nose and absorbed into the bloodstream through the nasal tissues. When injected, a needle is used to release the drug directly into the bloodstream. Smoking involves inhaling cocaine vapour or smoke into the lungs, from where absorption into the bloodstream is as rapid as by injection. Each of these methods of administration pose great risks to the user. Crack is cocaine that has been processed from cocaine hydrochloride to a free base for smoking. The most popular route of administration is snorting, which produces peak effect in 5-15 minutes, lasting for up to one hour. Inhalation of free-base cocaine produces peak effects in less than one minute and a short lived physiological effect measured in minutes [06171].

Most substances on WADA's Prohibited List are banned at all times; however, compounds belonging to the sections S6-S9 or P1 and P2 are not relevant for doping controls in out-of-competition periods. Within a two-year monitoring program (2006 and 2007), the prevalence of so-called non-specified stimulants in athletes' urine samples was assessed yielding 0.36 percent adverse analytical findings in approximately 25 000 analyzed specimens, demonstrating that cocaine was by far the most frequently detected "prohibited" substance. These numbers led to the conclusion that no systematic abuse of stimulants during out-of-competition periods prevail and that the current structures of the Prohibited List (considering two scenarios with in- and out-of-competition testing) are justified [13012].

**Environmental factors important for abuse**
Decades of experimentation with a variety of pharmacological treatments have identified some effective therapy for heroin addiction but not for cocaine addiction. This may be due, at least in part, to our incomplete understanding of the factors involved in the differential vulnerability to these addictions. Cocaine addiction and heroin addiction are often considered mere variations of the same disorder, and the preference for one drug or another has been variously attributed to factors such as drug availability or price, to the addict's lifestyle, or even to chance. Yet there is evidence of substance-specific influences on drug taking. In particular, data from twin registries suggest that a sizeable portion of the variability in the susceptibility to drug abuse is due to environmental factors that are unique to opiates or to psychostimulants. Very little is known about the underpinnings of these epidemiological data. We report here original data indicating that the setting of drug taking exert a differential influence on heroin versus cocaine use in human addicts. It was also reviewed additional clinical and pre-clinical data pointing to fundamental differences in the way in which the environment interacts with cocaine relative to heroin and other addictive drugs. These findings – as well as other evidence, including the lack of pharmacological treatments effective for both cocaine and heroin addiction – support the notion that much is to be gained by taking into account the substance-specific aspects of drug addiction. At a therapeutic level, for example, it appears reasonable to propose that cognitive-behavioral approaches should be tailored in a substance-specific manner in order to allow the addict to anticipate, and cope with, the risks associated to the various environmental settings of drug use [13520].

**Effects**

Cocaine is a strong CNS stimulant and is probably the most addictive agent known. Its recreational use is widespread, and it is highly addictive with its effect mediated through dopamine release. For ethical and practical reasons, most of the knowledge of the pharmacology of cocaine comes from animal studies or from addict reports. Classic physical effects of cocaine use include constricted blood vessels, dilated pupils and increased temperature, heart rate and blood pressure. It also increases motor activity and talkativeness and is a strong inducer of euphoria. The duration of cocaine's immediate euphoric effects (hyperstimulation, reduced fatigue, and mental clarity) depends on the route of administration. The faster the absorption, the more intense are the effects and the shorter the duration of action. The effects from snorting may last 15-30 minutes whereas the effects from smoking may last 5-10 minutes. Increased use can reduce the period of time a user feels high and increases the risk of addiction. Cocaine users usually feel an initial “rush” or sense of well-being, of having more energy and being more alert. This effect quickly wears off, often leaving the user feeling more “down” or depressed than before. This down feeling leads the addict to use more cocaine, sometimes just to feel “normal”. Over a period of time, the amount of cocaine needed and the frequency of use to achieve a “high” have to be increased. Cocaine is more addictive than amphetamine and the increasingly higher doses used by addicts may lead to a state of irritability, restlessness, anxiety, and paranoia. Other complications associated with cocaine use include disturbances in heart rhythm and heart attacks, chest pain and respiratory failure, strokes, seizures and headaches, and gastrointestinal complications such as abdominal pain and nausea. Cocaine misuse is strongly associated with cerebrovascular accidents arising either from rupture or spasm of cerebral blood vessels. Different means of taking cocaine can produce different adverse effects. Regular snorting, for example, can lead to loss of sense of smell, nosebleeds, problems with swallowing, hoarseness, and a chronically runny nose. Ingesting cocaine can cause severe bowel gangrene due to reduced blood flow. People who inject cocaine can experience severe allergic reactions and, as with any injecting drug user, are at increased
risk for contracting human immunodeficiency virus (HIV) infection and other bloodborne diseases [06171].

**Cocaine in sport**

Despite the popular myth, cocaine does not really enhance performance, whether in the job, in sports, at school, or during sex. On the contrary, long term use can lead to loss of concentration, irritability, loss of memory, paranoia, loss of energy, anxiety, and a loss of interest in sex. In particular, several studies have shown that cocaine has no beneficial effect on running times and reduces endurance performance. Furthermore, at all doses, cocaine significantly increases glycogen degradation while increasing plasma lactate concentration without producing consistent changes in plasma catecholamine levels. The controlling effect of cocaine on an addict's life can lead to exclusion of all other facets of life. Nevertheless, despite these apparently detrimental effects, cocaine continues to be misused in sport. It may be that cocaine only affects activities of short duration requiring a burst of high intensity energy output. It is possible that the central nervous stimulatory effect may be more important than its action on peripheral metabolism. It has been suggested that athletes are drawn to cocaine because of the effects of heightened arousal and increased alertness, achieved principally at low doses. Federal regulations for cocaine were introduced in December 1914. This act banned non-medical use of cocaine, prohibited its importation and selling. Cocaine can currently be administered by a doctor for legitimate medical use, such as for local anaesthetic use for some eye, ear, and throat surgeries. Cocaine is banned by both WADA and IOC, including its use as a local anaesthetic. Like amphetamines, it comes under category S6 of the prohibited substances in competition. The presence of cocaine and/or its metabolites (benzoylecgonine and methylecgonine) in urine can be described as severe doping offence [06171].

**Side effects**

A number of dramatic fatalities associated with coronary occlusion have occurred in athletes misusing cocaine, usually those who have been exercising intensely following drug administration. Many sportspeople who misuse cocaine complain of negative central effects such as perceptual misjudgments and time disorientation that sometime reduce their athletic performance. Furthermore, cocaine addicts frequently turn to other drugs to relieve the down feeling when more cocaine is not available. When used together, these drugs and cocaine can prove even more deadly than when used alone. Some fatalities have also occurred when cocaine misuse has been mixed with alcohol or anabolic steroids. The joint misuse of alcohol and cocaine is extremely cardiotoxic. These practices increase the risk of sudden death by cardiac arrest or seizures followed by respiratory arrest [06171].

**Heart**

Cocaine is a particularly toxic substance for the cardiovascular and respiratory system, so its use can lead to serious complications. Among these, the most tragic is SCD, which may be due to coronary artery vasospasm, lethal arrhythmia, direct toxic action on the myocardium, or severe suppression of the respiratory centre. Ischaemia and/or acute myocardial infarction are common complications, both in individuals with normal coronary vessels and, to a greater extent, on a substrate of atherosclerosis, and are not always related to the cocaine dosage. The main pathophysiological mechanism that leads to the appearance of ischaemia is the strong sympathomimetic and parasympatholytic effects of the drug, which are enhanced by the increased adenylate cyclase activity. The consequence of this is an increase in oxygen demand, because of the tachycardia, and in blood pressure, and at the same time a reduction in oxygen supply because of the vasospasm, while also promoting the
thrombogenic process. Also common is the manifestation of rhythm disorders, because of sudden vasospasm, or its local anaesthetic effect from obstruction of the Na⁺/K⁺ pump, or its toxic effect on the myocardium. Because of this there is an increase in endothelial permeability in the pulmonary capillaries, which can result in pulmonary oedema. Finally, other complications that have been described are infective endocarditis, myocarditis and dilated cardiomyopathy (chemical cardiomyopathy), rupture of aortic aneurysms, arterial and pulmonary hypertension, and thromboembolic vascular episodes [12126].

The use of cocaine has been associated with acute and chronic cardiovascular disease. Cocaine inhibits norepinephrine reuptake in the sympathetic system leading to overstimulation and may cause release of catecholamines from central and peripheral stores. Acute coronary syndromes (including myocardial ischemia and infarction) are the commonest cardiac events secondary to cocaine abuse. This may be due to coronary artery spasm, increase in myocardial oxygen consumption from increases in heart rate and blood pressure, and a prothrombotic state. Most myocardial infarctions occur in the absence of atherosclerotic coronary disease and are unrelated to the dose and frequency of cocaine use. Cocaine abuse may however also lead to premature coronary disease sometimes with quite rapid onset. Cocaine abuse may also lead to coronary artery aneurysms, aortic dissection, rupture, vasculitis and stroke. Arrhythmias are not common with cocaine use, but sinus tachycardia, sinus bradycardia, supraventricular, ventricular arrhythmias and bundle branch blocks have been reported. A dilated cardiomyopathy can be caused by cocaine use and a cocaine-induced myocarditis has been reported at postmortem in 20-30 percent of cases. Myocarditic changes, however, may be fully reversible if identified early and abstinence from further cocaine abuse occurs. In the case study detailed the ECG changes with a significant cardiac event are shown in a young professional athlete who has a cocaine abuse history [12114].

Brain projections of cardiovascular disease induced by cocaine

Neural plasticity has been observed in the bed nucleus of the stria terminalis (BNST) following exposure to both cocaine and androgenic-anabolic steroids. Here we investigated the involvement of the BNST on changes in cardiovascular function and baroreflex activity following either single or combined administration of cocaine and testosterone for 10 consecutive days in rats. Single administration of testosterone increased values of arterial pressure, evoked rest bradycardia and reduced baroreflex-mediated bradycardia. These effects of testosterone were not affected by BNST inactivation caused by local bilateral microinjections of the nonselective synaptic blocker CoCl2. The single administration of cocaine as well as the combined treatment with testosterone and cocaine increased both bradycardiac and tachycardiac responses of the baroreflex. Cocaine-evoked baroreflex changes were totally reversed after BNST inactivation. However, BNST inhibition in animals subjected to combined treatment with cocaine and testosterone reversed only the increase in reflex tachycardia, whereas facilitation of reflex bradycardia was not affected by local BNST treatment with CoCl2. In conclusion, the present study provides the first direct evidence that the BNST play a role in cardiovascular changes associated with drug abuse. Our findings suggest that alterations in cardiovascular function following subchronic exposure to cocaine are mediated by neural plasticity in the BNST. The single treatment with cocaine and the combined administration of testosterone and cocaine had similar effects on baroreflex activity, however the association with testosterone inhibited cocaine-induced changes in the BNST control of reflex bradycardia. Testosterone-induced cardiovascular changes seem to be independent of the BNST [13522].

Laboratory techniques

1449
The use of thin-film solid-phase microextraction (SPME) as the sampling preparation step before direct analysis in real time (DART) was evaluated for the determination of two prohibited doping substances, cocaine and methadone, in urine samples. Results showed that thin-film SPME improves the detectability of these compounds: signal-to-blank ratios of 5 (cocaine) and 13 (methadone) were obtained in the analysis of 0.5 ng/ml in human urine. Thin-film SPME also provides efficient sample cleanup, avoiding contamination of the ion source by salt residues from the urine samples. Extraction time was established in 10 min, thus providing relatively short analysis time and high throughput when combined with a 96-well shaker and coupled with DART technique [13523].

In doping controls, the detection of cannabinoid misuse is based on the analysis of the non-psychoactive metabolite 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (carboxy-THC). The determination of values greater than 15 ng/mL in urine represents an adverse analytical finding; however, no accurate prediction of the time of application is possible as the half-life of carboxy-THC ranges between three and four days. Consequently the detection of carboxy-THC in doping control urine samples collected in competition might also result from cannabis use in out-of-competition periods. The analysis of the glucuronide of the pharmacologically active delta 9-tetrahydrocannabinol (THC-gluc) may represent a complementary indicator for the detection of cannabis misuse in competition. An assay for the determination of THC-gluc in human urine was established. The sample preparation consisted of liquid-liquid extraction of urine specimens, and extracts were analysed by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Authentic doping-control urine samples as well as specimens obtained from a controlled smoking study were analysed and assay characteristics such as specificity, detection limit (0.1 ng/mL), precision (>90 %), recovery (approximately 80 %), and extraction efficiency (90 %) were determined [09293].

**MALDI-TOF/TOF mass spectrometry**

The analysis of doping agents in biological fluids is of top significance in clinical and forensic toxicology. Herein it was described the study of a screening method for the detection of a mixture of drugs of potential abuse including cocaine and its metabolites. By using matrix-assisted laser desorption/ionization MALDI-TOF/TOF mass spectrometry. This screening procedure to detect the presence of different drugs, avoiding time consuming procedures could be useful in different fields of forensic analytical toxicology, including antidoping analysis [12348].

**Analysis of hair**

In forensic toxicology, apart from urine and blood samples, different complex matrices are also of interest such as various tissues or especially hair samples. Mass spectrometry coupled with gas or liquid chromatography (GC-MS or LC-MS) has been utilized to analyze drugs of abuse in such matrices. A large number of biomolecules ranging from large proteins to small pharmaceuticals have been visualized by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) or MALDI MS Imaging (MALDI-MSI) in cells and tissues. MALDI-MS combined with spatial information in which the total ion current (TIC) or extracted ion current (XIC) is sorted with respect to the individual x/y-positions on the sample allow one to create MS images of various compounds in the sample, for example, tissue or hair. This technique has demonstrated to be a widely applicable tool for the description and elucidation of the spatial distribution of various compounds (peptides/proteins, but also small molecules) within biological tissues. MALDI-MSI has turned out to be a powerful tool to investigate the distribution of proteins and small molecules within biological systems by in situ analysis of tissue sections. In a preliminary test, four single hairs of a drug abuser were analyzed for the presence of drugs by MALDI Fourier transform mass spectrometry (MALDI-FTMS). Washed hair strains were directly fixed on a sample plate using pressure stable, double-sided adhesive tape; α-cyano-4-hydroxycinnamic acid matrix was manually spotted onto the hair.
strains. FTMS full scans were obtained moving from the hair root region towards the hair tip. Cocaine (accurate m/z ratio 304.15433) was identified mostly from the root of the hair and then later again towards the hair tip. This was confirmed by analysis of a second hair. Additionally cocaine metabolites with m/z ratio 290.13868 (benzoylecgonine), and m/z 318.16998 (cocaethylene) were detected for plausibility control. Using the MALDI technique, time-related information was obtained concerning the behavioural pattern of the consumer with high resolution compared to conventional procedures. However, in two hairs of the same individual which were analyzed under the same conditions, negative results were achieved. These preliminary results confirm the applicability of MALDI-MS for the determination of drugs and pharmaceuticals in hair samples being useful in forensic toxicology. The high chronological resolution allows an enhanced interpretation concerning the periods of drug administration. However, the negative results with two negative hairs have also demonstrated that hair analysis of single hairs can lead to misinterpretation. Different growth rates have to be considered, and particularly the phenomenon of different growth phases (anagen, catagen, telogen) require attention [13521].

**Methylenedioxymethamphetamine (MDMA; Ecstasy)**

The recreational and illicit use of amphetamine designer compounds, specially 3,4-methylenedioxymethamphetamine (MDMA; Ecstasy), is of concern worldwide. Such psychostimulating drugs are frequently present as complex mixtures in “rave' pills” making concomitant polysubstance use a common trend. However, the understanding of possible combination effects with these substances is still scarce. The present study was aimed at predicting the cytotoxic effects of mixtures of four amphetamine derivatives: MDMA, methamphetamine, 4-methylthioamphetamine and d-amphetamine in a human hepatoma cell line. Concentration-response curves for all single-mixture components were recorded by the MTT assay. Data obtained for individual agents were then used to compute the additivity expectations for mixtures of definite composition, using the pharmacological models of concentration addition (CA) and independent action. By comparing the predicted calculations with the experimentally observed effects, we concluded that CA accurately predicts the combination of amphetamines, which act together to generate additive effects over a large range of concentrations. Notably, we observed substantial mixture effects even when each drug was present at low concentrations, which individually produced unnoticeable effects. Nonetheless, for all tested mixtures, a small deviation from additivity was observed towards higher concentrations, particularly at high effect levels. A possible metabolic interaction, which could explain such deviation, was investigated, and it was observed that at higher mixture concentrations increased MDMA metabolism could be contributing to divergences from additivity. In conclusion, the present work clearly demonstrates that potentially harmful interactions among amphetamine drugs are expected when these drugs are taken concomitantly [13487].

3,4-Methylenedioxymethamphetamine (MDMA) is a racemic drug of abuse and its R- and S-enantiomers are known to differ in their dose-response curve. The S-enantiomer was shown to be eliminated at a higher rate than the R-enantiomer most likely explained by stereoselective metabolism that was observed in various in vitro experiments. The aim of this work was the development and validation of methods for evaluating the stereoselective elimination of phase I and particularly phase II metabolites of MDMA in human urine. Urine samples were divided into three different methods. Method A allowed stereoselective determination of the 4-hydroxy-3-methoxymethamphetamine (HMMA) glucuronides and only achiral determination of the intact sulfate conjugates of HMMA and 3,4-dihydroxymethamphetamine (DHMA) after C18 solid-phase extraction by liquid
chromatography-high-resolution mass spectrometry with electrospray ionization. Method B allowed the determination of the enantiomer ratios of DHMA and HMMA sulfate conjugates after selective enzymatic cleavage and chiral analysis of the corresponding deconjugated metabolites after chiral derivatization with S-heptafluorobutyrylpromyl chloride using gas chromatography-mass spectrometry with negative-ion chemical ionization. Method C allowed the chiral determination of MDMA and its unconjugated metabolites using method B without sulfate cleavage. The validation process including specificity, recovery, matrix effects, process efficiency, accuracy and precision, stabilities and limits of quantification and detection showed that all methods were selective, sensitive, accurate and precise for all tested analytes [13351].

The serotonin transporter (SERT) has been associated to diverse functions and diseases, though seldom to memory. Therefore, we made an attempt to summarize and discuss the available publications implicating the involvement of the SERT in memory, amnesia and anti-amnesic effects. Evidence indicates that Alzheimer's disease and drugs of abuse like d-methamphetamine (METH) and (+/-)3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”) have been associated to decrements in the SERT expression and memory deficits. Several reports have indicated that memory formation and amnesia affected the SERT expression. The SERT expression seems to be a reliable neural marker related to memory mechanisms, its alterations and potential treatment. The pharmacological, neural and molecular mechanisms associated to these changes are of great importance for investigation [13352].

To improve our understanding of the pharmacology of “ecstasy” in recreational environments; in particular, to describe the composition of ecstasy pills, patterns of ecstasy use and the relationship between dose of 3,4-methylenedioxymethamphetamine (MDMA) and resulting plasma concentrations. A naturalistic observational study of 56 experienced “ecstasy” users in recreational settings in Australia was performed. Drug use patterns (number of pills consumed, other drugs consumed). drug content of pills and resultant plasma concentrations of MDMA and related drugs were assessed by gas chromatography/mass spectrometry (GC/MS). Ecstasy pills generally contained MDMA, but this was often combined with other drugs such as 3,4-ethylendioxyethylamphetamine (MDEA) and methamphetamine. The dose of MDMA per pill ranged from 0 to 245 mg and users consumed from one-half to five pills, with the total dose consumed ranging up to 280 mg. Plasma concentrations of MDMA increased with number of pills consumed and cumulative MDMA dose. Use of larger numbers of pills was associated with extended exposure to the drug. It was concluded that MDMA is the major active drug in ecstasy pills, but there is a high degree of variation in doses. Use of multiple pills over the course of one session is common and results in a sustained increase in MDMA plasma concentrations over a number of hours. This is likely to lead to a much greater exposure of the brain to MDMA than would be predicted from controlled single-dose pharmacokinetic studies [13353].

Metabolites

3,4-Methylenedioxymethamphetamine (MDMA) is excreted in human urine as unchanged drug and phase I and II metabolites. Previous urinary excretion studies after controlled oral MDMA administration have been performed only after conjugate cleavage. Therefore, it was investigated intact MDMA glucuronide and sulfate metabolite excretion. It was used LC-high-resolution MS and GC-MS to reanalyze blind urine samples from 10 participants receiving 1.0 or 1.6 mg/kg MDMA orally. It was determined median C(max),t(max), first and last detection times, and total urinary recovery; calculated ratios of sulfates and glucuronides; and performed in vitro-in vivo correlations. Phase II metabolites of 3,4-
dihydroxymethamphetamine (DHMA), 4-hydroxy-3-methoxymethamphetamine (HMMA), 3,4-dihydroxyamphetamine (DHA), and 4-hydroxy-3-methoxymethamphetamine were identified, although only DHMA sulfates, HMMA sulfate, and HMMA glucuronide had substantial abundance. Good correlation was observed for HMMA measured after acid hydrolysis and the sum of unconjugated HMMA, HMMA glucuronide, and HMMA sulfate. More than 90 percent of total DHMA and HMMA were excreted as conjugates. The analytic with the longest detection time was HMMA sulfate. Median HMMA sulfate/glucuronide and DHMA 3-sulfate/4-sulfate ratios for the first 24 h were 2.0 and 5.3, respectively, in accordance with previous in vitro calculations from human liver microsomes and cytosol experiments. It was concluded that human MDMA urinary metabolites are primarily sulfates and glucuronides, with sulfates present in higher concentrations than glucuronides. This new knowledge may lead to improvements in urine MDMA and metabolite analysis in clinical and forensic toxicology, particularly for the performance of direct urine analysis [11509].

Laboratory testing

Qualitative urinalysis can verify abstinence of drug misuse but cannot detect changes in drug intake. For drugs with slow elimination, such as methamphetamine (MA), a single episode of abuse can result in up to 5 days of positive urine drug screens. Thus, interventions that produce substantial decreases in drug use but do not achieve almost complete abstinence are classified as ineffective. Using nonpharmacologic doses of deuterium-labeled l-methamphetamine (l-MA-d3) we have developed a simple, robust method that reliably estimates changes in MA intake. Twelve subjects were dosed with 5 mg of l-MA-d3 daily and challenged with 15, 30, and 45 mg of nonlabeled d-MA (d-MA-d0) after reaching plasma steady status of l-MA-d3. Urinary concentration ratios of d-MA-d(0) to l-MA-d(3) provided clear separation of the administered doses with as little as 15-mg dose increments. Administered doses could not be resolved using d-MA-d0 concentrations alone. In conclusion, the urinary concentration of d-MA-d0 l-MA-d3 provides a quantitative, continuous measure of illicit MA exposure. The method reliably detects small, clinically relevant changes in illicit MA intake from random urine specimens, is amenable to deployment in clinical trials, and can be used to quantify patterns of MA abuse [13354].

There is evidence that the use of MDMA (methylenedioxymethamphetamine), colloquially known as “ecstasy” particularly among late adolescents and young adults is increasing. Despite recent government-sponsored public education programs, there is a perception that recreational use of MDMA is much less harmful than other illicit substances like heroin. Recent seizures by police in Australia underline the extent of the demand for MDMA and how lucrative trafficking in MDMA has become. In two recent Australian cases, appellate courts considered the legislative intent of both State and Commonwealth legislation and held that a quantity-based penalty regime applied which distinguished between “traffickable” and “commercial” quantities of illicit drugs and that no distinction turned on the relative “harmfulness” of MDMA. In examining the question of harmfulness, this column summarises the pharmacology and morbidity of MDMA and considers the links between MDMA and other substances of abuse and the implications for further prevention programs [09294].

The abuse of ecstasy-type drugs such as 3,4-methylenedioxyamphetamine (MDMA) and 3,4-methylenedioxymethamphetamine (MDA) is generally associated with young adults attending "Rave" parties. Little toxicological information has been reported regarding ecstasy usage by individuals undergoing monitoring in other settings in the United States. The goal of this study was to determine the prevalence and patterns of licit and illicit drugs in urine specimens of ecstasy users. A survey of laboratory data over the years 2005-2007 revealed that 198 urine specimens were confirmed positive (cutoff concentration 100 ng/mL) for
MDMA and/or MDA from the following types of donors (positive specimens): correctional (159); sports (19); workplace (9); pain patients (8); and special test requests (3). Of these, 122 (62 %) were positive for MDMA and MDA, 70 (35 %) were positive for MDMA, and 6 (3 %) were positive for MDA. A majority (84 %) of the specimens contained multiple drugs and/or metabolites in addition to MDMA and MDA. The median number of drugs/metabolites reported for these ecstasy users was 5 (range, 1-9). In addition to MDMA/MDA, the most commonly identified drug groups (%) were cannabis (THCCOOH) (62 %); amphetamine/methamphetamine (38 %); benzylecgonine (31 %); diazepam-related (10 %); opiates (7 %); alprazolam (6 %); and others (6%). Although multidrug ingestion appears to be common amongst ecstasy users, caution is recommended in interpretation. Illicit ecstasy in the United States and Canada frequently contains methamphetamine and other active substances, and multidrug use may not have been intentional [09295].

Bruxism is a recognized side effect of several licit and illicit drugs. In one report, this phenomenon was illustrated in three patients suffering from 3,4-methylenedioxymethamphetamine (ecstasy) abuse [10491].

The aim of this study was to investigate the influence of ecstasy (MDMA) administration on body temperature and soleus muscle histology in exercised and non-exercised mice. Charles-River mice were distributed into four groups: Control (C), exercise (EX), MDMA treated (M), and M + EX. The treated animals received an i.p. injection (10 mg/kg) of MDMA (saline for C and EX), and the exercise consisted of a 90 min level run at a velocity of 900 m/h, immediately after the MDMA or saline administration. Body temperature was recorded every 30 min via subcutaneous implanted transponder. Animals were sacrificed 1.5, 25.5, and 49.5 h after i.p. injection and the soleus muscles were removed and processed for light and electron microscopy. The MDMA-treated animals showed a significant increase in body temperature (similar in M and M + EX groups), reaching the peak 90 min after i.p. administration; their temperature remained higher than control for more than 5 h. The EX group evidenced a similar and parallel, yet lower temperature increase during exercise and recovery. Morphological signs of damage were rarely encountered in the EX group; they were more pronounced in M group and even aggravated in M + EX group. In conclusion, MDMA and exercise per se increased body temperature but in conjunction did not have a cumulated effect. However, ecstasy and concomitant physical activity might severely accumulate with regard to skeletal muscle toxicity and may lead to rhabdomyolysis [10492].

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is one of the most widely abused illegal drugs. Some users self-report euphoria and an increased perception and feeling of closeness to others. When taken in warm environments, MDMA users may develop acute complications with potential fatal consequences. In rodents, MDMA increases locomotor activity and, depending on ambient temperature, may produce a dose-dependent, potentially lethal hyperthermia. Like most other recreational drugs, MDMA is frequently taken in combination with other substances including tobacco, EtOH, marijuana, amphetamines, cocaine and, caffeine. Although polydrug use is very common, the understanding of the effects of this multiple substance use, as well as the analysis of consequences of different drug-drug associations, received rather little attention. The purpose of one review was to summarize our current knowledge about the changes on MDMA-related behavior, pharmacology, and neurotoxicity associated with co-consumption of other drugs of abuse and psychoactive agents [11508].

**gamma-Aminobutyric acid (GABA)**
gamma-Aminobutyric acid, GABA, is synthesized through the decarboxylation of glutamate by the enzyme glutamic acid decarboxylase. Glutamate is the main excitatory neurotransmitter, and GABA is the major inhibitory neurotransmitter in the mature brain. GABA became a popular supplement with bodybuilders in the early 1980s after a study showed it increased plasma growth hormone (GH) levels in man. GABA acts primarily by activating Cl⁻ channels called GABAₐ receptors, and by eliciting metabotropic G-protein mediated responses by GABAₐ receptors. GABA is considered to act as a natural tranquilizer and antiepileptic agent in the brain. GABAₐ receptors are the site of action of benzodiazepines, barbiturates and anaesthetics and known to mediate sedation. GABAₐ agonists may be useful for the treatment of pain and drug dependence. Baclofen, the first synthetic GABAₐ receptor agonist, is used clinically for the treatment of spasticity and skeletal muscle rigidity. GABAₐ antagonists on the other hand have shown antidepressant and cognition-enhancing effects. Baclofen, in rats, has been shown to prolong time to fatigue, possibly because of a boost in glycogen owing to the effect of interleukin 6 release in the muscle. Recently it was shown that GABA ingestion at rest increases immunoreactive GH (irGH) and immunofunctional GH (ifGH) secretion, which may enhance the skeletal muscle response to resistance training. Moreover, when GABA ingestion was combined with exercise, concentrations of irGH and ifGH rose even higher. Although some effects have been found, specifically for the response to resistance training, much more research regarding the effects of GABAergic manipulations on exercise performance is needed to elucidate the role of GABA [10527].

Influence of anabolic steroids

Anabolics androgenic steroids (AAS) comprise a large and growing class of synthetic androgens used clinically to promote tissue-building in individuals suffering from genetic disorders, injuries, and diseases. Despite these beneficial therapeutic applications, the predominant use of AAS is illicit: these steroids are self-administered to promote athletic performance and body image. Hand in hand with the desired anabolic actions of the AAS are untoward effects on the brain and behavior. While the signaling routes by which the AAS
impose both beneficial and harmful actions may be quite diverse, key endpoints are likely to include ligand-gated and voltage-dependent ion channels that govern the activity of electrically excitable tissues. It was reviewed the known effects of AAS on molecular targets that play critical roles in controlling electrical activity, with a specific focus on the effects of AAS on neurotransmission mediated by GABA<sub>A</sub> receptors in the central nervous system [12336].

Anabolic androgenic steroids (AAS), synthetic testosterone derivatives that are used for ergogenic purposes, alter neurotransmission and behaviors mediated by GABA<sub>A</sub> receptors. Some of these effects may reflect direct and rapid action of these synthetic steroids at the receptor. The ability of other natural allosteric steroid modulators to alter GABA<sub>A</sub> receptor-mediated currents is dependent upon the phosphorylation state of the receptor complex. Here we show that phosphorylation of the GABA<sub>A</sub> receptor complex immunoprecipitated by beta<sub>2</sub>/beta<sub>3</sub> subunit-specific antibodies from the medial preoptic area (mPOA) of the mouse varies across the estrous cycle; with levels being significantly lower in estrus. Acute exposure to the AAS, 17alpha-methyltestosterone (17alpha-MeT), had no effect on the amplitude or kinetics of inhibitory postsynaptic currents in the mPOA of estrous mice when phosphorylation was low, but increased the amplitude of these currents from mice in diestrus, when it was high. Inclusion of the protein kinase C (PKC) inhibitor, calphostin, in the recording pipette eliminated the ability of 17alpha-MeT to enhance currents from diestrous animals, suggesting that PKC-receptor phosphorylation is critical for the allosteric modulation elicited by AAS during this phase. In addition, a single injection of 17alpha-MeT was found to impair an mPOA-mediated behavior (nest building) in diestrus, but not in estrus. PKC is known to target specific serine residues in the beta<sub>3</sub> subunit of the GABA<sub>A</sub> receptor. Although phosphorylation of these beta<sub>3</sub> serine residues showed a similar profile across the cycle, as did phosphoserine in mPOA lysates immunoprecipitated with beta<sub>2</sub>/beta<sub>3</sub> antibody (lower in estrus than in diestrus or proestrus), the differences were not significant. These data suggest that the phosphorylation state of the receptor complex regulates both the ability of AAS to modulate receptor function in the mPOA and the expression of a simple mPOA-dependent behavior through a PKC-dependent mechanism that involves the beta<sub>3</sub> subunit and other sites within the GABA<sub>A</sub> receptor complex [12337].

gamma-Butyrolactone (GBL)
The aim of one report was to describe morbidity associated with gamma-butyrolactone (GBL) dependence, and outcomes of withdrawal. The setting was a specialist out-patient clinic and affiliated in-patient detoxification unit. Patients with home support were offered the option of out-patient withdrawal management, based on high-dose diazepam and baclofen, titrated against withdrawal severity in an initial session lasting approximately 4 hours. Patients were then reviewed daily during the first 3 days of treatment, offered weekly follow-up for 4 weeks, and telephoned 2-4 months later. Drug history and social functioning were obtained by self-report in clinical interviews with a single clinician. Treatment completion, outcomes and adverse events associated with withdrawal are reported. Patients reported impaired social functioning associated with GBL dependence and difficulty in accessing treatment. Nineteen patients commenced detoxification; 17 initially declined admission and were treated as outpatients. Mean diazepam dose in the first 24 hours was 75 mg (range 40-110 mg). Sixteen patients completed withdrawal, although several had lapses to GBL use during treatment. One patient developed delirium and required transfer to the in-patient detoxification unit. Most patients had persisting insomnia, anxiety and depression for weeks after withdrawal. GBL withdrawal can be managed in ambulatory settings, but needs to be backed up with seamless access to in-patient treatment if required [11235].

Popularity of gamma-hydroxybutyric acid (GHB) is fairly stable among drug users, while the consumption of its chemical precursor, gamma-butyrolactone (GBL), is a growing phenomenon. Although conventional analytical methods allow to detect this substance in various matrices, linking a trace and a source is still a difficult challenge. However, as several synthesis pathways and chemical precursors exist for the production of GBL, its carbon isotopic signature may vary extensively. For that purpose, a method has been developed to determine the carbon isotopes content of GBL by means of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). The delta$^{13}$C-values of 19 bulk samples purchased worldwide were in the range from -23.1 to -45.8 per thousand (SD<0.3 per thousand). Furthermore, testing on the purification of GBL by distillation has not been found to be consistent with such a large range of delta$^{13}$C-values, which are likely to result from the isotopic composition of the organic precursors used to produce GBL together with the kinetic isotope effect associated with the synthesis routes. Finally, inter- and intra-variability measurements of the delta$^{13}$C-values demonstrated the high potential of IRMS for discriminating between seizures of GBL and for source determination [10197].

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**gamma-Butyrolactone and gamma-butyrolactone**
Gamma-hydroxybutyrate (GHB) and its precursor gamma-butyrolactone (GBL) are commonly abused drugs with a narrow therapeutic index. Therefore, overdoses occur readily with recreational use, and severe poisoning can occur after deliberate self-poisoning. It was reported the sequelae in a patient who ingested a massive dose of GBL, with suicidal intent. Severe metabolic acidosis and an asystolic cardiac arrest were successfully treated with standard resuscitation, supportive care, and continuous venovenous hemodiafiltration. Plasma GHB concentrations were the highest reported to date. The acidosis was attributed to rapid systemic absorption of GBL, followed by rapid metabolism to GHB [11360].

Gamma-hydroxybutyrate (GHB) is naturally present in the human body, but may also be used as an intoxicating drug. Information from several sources has suggested its increased availability and use in Norway. There have also been reports of an increasing use of the chemical precursor gamma-butyrolactone (GBL). There is currently a need for knowledge on symptoms, addictiveness and overdoses, as well as targeted preventive measures. One article was based on a discretionary selection of articles resulting from a literature search in PubMed, as well as reports from Norwegian and European authorities and research institutions. An intake of small amounts of GHB produces an intoxicating effect, whereas higher doses can result in poisoning. Deaths have been reported. The effect may be variable, due to a steep dose-response curve and interaction with alcohol and other intoxicants. Treatment of poisoning is symptomatic and supportive. Treatment of abstinence is also supportive, while delirium may be treated as delirium tremens [11510].

Gamma-hydroxybutyric acid (GHB) was first synthesized in 1960 as a derivative of the endogenous neurotransmitter gamma-aminobutyric acid (GABA) to pass the blood-brain barrier. In some European countries, GHB is used as an anaesthetic drug, but it has insufficient analgesic effects that necessitate its combination with an analgesic; adverse effects, such as seizures or vomiting, frequently occur. Moreover, controlling dosage and duration of effects is difficult. Later, GHB was also used in the treatment of alcohol and heroin withdrawal. Since studies have shown that GHB has a positive effect on patients with narcolepsy and cataplexy, the substance was licensed for their use in the United States in 2002 and in Europe in 2005, despite the fact that these therapies have many side-effects. As early as the 1980s, GHB was offered in the USA as a dietary supplement. Bodybuilders, in particular, used GHB because of its suggested anabolic muscle-building effects through the increased release of growth hormones. It was also consumed for its supposed aphrodisiac and weight-loss effects, and as a sleeping aid. After numerous cases of intoxication occurred over the following years, the US Food and Drug Administration (FDA) banned its sale to consumers in 1990. However, this did not put a stop to misuse of the drug. GHB gained in popularity; its users consumed “liquid ecstasy” or “soap” on the club scene as a party drug or at private events, as an alternative to, or in combination with, alcohol [11585].

Abuse of gamma-hydroxybutyric acid (GHB) has been known since the early 1990’s, but is not as widespread as the consumption of other illegal drugs. However, the number of severe intoxications with fatal outcomes is comparatively high; not the least of which is brought about by the consumption of the currently legal precursor substances gamma-butyrolactone (GBL) and 1,4-butanediol (1,4-BD). In regards to previous assumptions, addiction to GHB or its analogues can occur with severe symptoms of withdrawal. Moreover, GHB can be used for drug-facilitated sexual assaults. Its pharmacological effects are generated mainly by interaction with both GABAB and GHB receptors, as well as its influence on other transmitter systems in the human brain. Numerous analytical methods for determining GHB using chromatographic techniques were published in recent years, and an enzymatic screening method was established. However, the short window of GHB detection in blood or urine due to its rapid metabolism is a challenge. Furthermore, despite several studies addressing this problem, evaluation of analytical results can be difficult: GHB is a metabolite of GABA.
(gamma-aminobutyric acid); a differentiation between endogenous and exogenous concentrations has to be made. Apart from this, in samples with a longer storage interval and especially in postmortem specimens, higher levels can be measured due to GHB generation during this postmortem interval or storage time [11585].

Illicit forms of GHB have come under narcotics law in the USA since 2000 and are subject to the strictest categorization (Schedule I). In some parts of Europe (Germany, for example), GHB is still approved for treatment as an anaesthetic and for the treatment of narcolepsy. In March 2001, GHB was added to Schedule IV of the 1971 UN Convention on Psychotropic Substances, thereby binding all EU member states to control it under their legislation addressing psychotropic substances. However, GHB is not yet fully under national control in all countries [11585].

Quantitative data on the prevalence of GHB abuse is incomplete, but various qualitative measures indicate that a mini-epidemic of abuse began in the late 1980s and continues up to the present. Undoubtedly, the easy availability and low costs of GHB and its precursors have contributed to its popularity. According to a study by the European Monitoring Centre for Drugs and Drug Addiction, consumption of GHB or GBL is not as widespread as consumption of other illegal drugs. Studies have shown that the prevalence of young people’s leisure use of GHB in the past month rarely exceeds 3 percent. However, there are also signs that consumption is becoming more common in private spheres, especially in certain population subgroups and certain social environments or geographical areas. Depending on the particular study and target group interviewed, the lifetime prevalence is 3 percent to 19 percent (six studies were included from the UK, the Netherlands, and Austria with data from years 1999 to 2007). According to various national reporting systems in the USA, there has been an emergence of a problem with GHB abuse beginning no later than 1994 or 1995. Quantitative data on the prevalence in the population is not available due to the fact that the use of GHB is not queried specifically, but is included under “other drugs” in most surveys since 2001. Since the final active agent in the body is GHB, even in cases of GBL or 1,4-BD consumption, an exact discrimination between these drugs is not possible in most cases. In spite of the comparatively low popularity of GHB, cases of intoxication, somewith a lethal result, have been increasing. Since GHB is not yet detected in routine toxicological screening, the prevalence of GHB abuse is thought to be underestimated [11585].

GHB is a naturally occurring hydroxy carboxylic acid in the human brain, blood, urine, and peripheral tissue. It appears both as a metabolic product and as a precursor of GABA. In spite of numerous studies, its function has not yet been clarified in detail, but there is evidence that GHB has different effects on the balance of neurotransmitters. One possible mechanism of action in discussion is that GHB acts as a neuromodulator in the GABA system. Moreover, GHB causes a dose-response increase or decrease of dopamine levels in the brain and affects the cholinergic system (via GABAB receptors), as well as neurosteroids and, indirectly, opioids. Furthermore, GHB potentiates brain serotonin turnover and increases the secretion of growth hormones. In millimolar concentrations (above endogenous concentrations), GHB already activates GABAB receptors itself or by conversion to GABA and thereby inhibits the influx of Ca\(^{2+}\) and opens K\(^+\)-channels, as GABA does. In addition, the existence of a separate G-protein coupled GHB receptor in the brain has been proven. This receptor is thought to be stimulated already by endogenous concentrations of GHB (micromolar range). It is suggested that after exogenous admission, GHB will act both as a GABAB- and GHB-receptor agonist. GHB is administered intravenously for the purpose of sedation, and orally for most other indications. Misuse occurs mostly by oral administration in liquid form, neat or diluted in drinks. Occasionally, capsules are ingested, which often contain GHB in the form of its sodium salt (sodium oxybate). GHB induces abiphasic time profile with
an initial stimulant-like effect related to the simultaneous rise of plasma concentrations and a latter sedative effect not related to GHB kinetics. GHB has a narrow therapeutic index: the symptoms of poisoning are dose-dependent and partly similar to those of alcohol poisoning. Headache, nausea, vomiting, vertigo, and speech impairment may be present. Higher concentrations may lead to bradycardia, seizures, respiratory depression to the point of respiratory arrest, and impaired consciousness to the point of coma. In severe intoxication, myoclonus or seizures followed by loss of consciousness have often been described. A typical characteristic is a rapid onset and sudden clinical picture. Owing to the extremely fast metabolism of GHB, usually the effects last only 1-4 h, depending on the dose. This phenomenon is known as the “fast-in, fast-out” effect and causes a sudden awakening from (near) coma in most cases, with patients usually recovering within 6-8 h after receiving symptom-oriented treatment. Patients who developed coma (Glasgow Coma Scale. GCS, > 3) had serum levels that ranged from 72 to 300 mg/L with a median of 193 mg/L and regained consciousness as levels decreased to the range of 75 to 150 mg/L. In 15 patients with GCS <8, blood concentrations of 112 to 430 mg/L were determined (most of them co-ingested other drugs or ethanol). One patient was in a state of coma (GCS 7) after ingestion of 1,4-BD, corresponding blood levels were 82 mg/L for 1,4- BD and 103 mg/L for GHB. A woman who was admitted to an emergency department in a comatose state had 161 mg/L GHB in serum after ingestion of GBL from nail polish remover pads. After oral administration, GHB is absorbed very rapidly; maximum plasma concentrations are reached after 25-45 min. The distribution throughout the body is fast and follows a two-compartment model. The apparent distribution volume is reported with 0.19 to 0.4 L/kg and was not significantly affected by gender, food, or liver cirrhosis. GBL has potentially a higher intestinal flux after oral administration and a shorter onset, therefore causing higher C_max of GHB. 1,4-butanediol is also quickly absorbed and metabolized. It is extensively converted to GHB after oral administration, but significant inter-individual variability in the rate of metabolism, possibly related to genetic variants in the alcohol dehydrogenase, was observed. Moreover, conversion of 1,4-BD is blocked competitively by ethanol [11585].

Endogenous GHB is formed from GABA by means of succinic semialdehyde reductase via the intermediate product succinic semialdehyde (SSA). Via the enzyme GHB dehydrogenase, GHB can be oxidated back into SSA. After a further oxidation step, SSA reaches the citric acid cycle, mainly as succinic acid. Only a small proportion of GHB is excreted in an unchanged form through the kidneys (<2 %). GHB is eliminated rapidly; the terminal elimination half-life in plasma averages 20-60 min after single oral doses, but there are indications that the elimination kinetics of GHB is not linear after administration of therapeutic doses. Moreover, in some cases after ingestion of large doses, zero order kinetics seems to be more appropriate than first-order kinetics. Drug facilitated sexual assaults/chemical submission GHB and its precursors are colourless liquids, transparent, and almost entirely without taste. They can thus be mixed with drinks to administer to unsuspecting victims without their noticing, and are used with criminal intent to weaken the resistance of individuals, for example to exploit their property or body without their consent and without them having the slightest recollection afterwards of what happened (anterograde amnesia). Criminals tend to use substances such as GHB to facilitate the commitment of their offences. They also tend to use substances currently not under international control, such as ketamine, 1,4-BD and GBL, since they are easily available through legitimate channels. GHB can result in impaired consciousness, including coma with anterograde amnesia. Studies have shown that GHB is one of the substances most often used for the purpose of drug facilitated sexual assault (DFSA) in the USA.[5,36,37] Similar cases have also been reported in Germany and other European countries. Because of its depressant effect on the central nervous system, the use of GHB and its analogues is also important in cases of driving under the influence of drugs (DUID). For example, Jones et al. identified in their database 548 DUID cases with GHB (alone or together with other recreational drugs)
for the years 1997 to 2007 in Sweden. Fatalities have been reported with accidental overdoses of GHB, suicidal intent, or trauma as a result of impaired driving. Fatal overdoses occur also with the precursors GBL or 1,4-BD. Especially post-mortem analytical GHB results should be interpreted with caution. Furthermore, it should be recognized that abrupt GHB withdrawal can cause life-threatening clinical courses which can even lead to death. Initial studies after clinical administration of GHB in patients with narcolepsy have not shown any cases of misuse or of a developing tolerance. More recent studies, however, indicate the potential for the drug as mentally and physically addictive and suggest a high abuse liability. GHB abuse can lead to addiction with severe withdrawal complications; the same is valid for its precursors. GHB addiction is characterized by round-the-clock dosing, typically every 1-3 h. According to our experience, typical doses are 20 to 70 mL per day; reported doses of patients with GBL/1,4-BD withdrawal in the literature ranged from “two sips every night” up to 900 mL/day during five weeks to four years. Addiction can develop within a few weeks. Withdrawal symptoms after abrupt GHB discontinuation manifested very quickly within 2-6 h. Withdrawal complications of dependence can be associated with a range of neurological phenomena including tremor, anxiety attacks, confusion, epileptic fits, and memory loss. These initial symptoms may progress to severe delirium with auditory and visual hallucinations and autonomic instability. Cardiovascular effects included severe tachycardia and hypertension. Life-threatening clinical courses with admission to the intensive care unit have been documented. Most patients typically requested medical assistance for continued withdrawal symptoms from three days to up to 14 days or more after the last dose. Benzodiazepines, barbiturates, and antipsychotic medication are the most used drugs in the treatment of GHB withdrawal symptoms. However, in many cases, high doses of benzodiazepines and barbiturates proved ineffective. A sufficient medical treatment of the GHB withdrawal syndrome comparable to the treatment of alcohol or benzodiazepine addiction is not yet clearly defined [11585].

**gamma-Butyrolactone and 1,4-butanediol**

Since GHB is subject to the Narcotics Act, legal substitutes such as gamma-butyrolactone (GBL) or 1,4-butanediol (BD), the most commonly used GHB analogues, are currently being consumed. These substances are metabolized into GHB after oral administration or they are transformed into GHB before ingestion by means of a simple chemical reaction, either by the dealers or the users themselves. Instructions on how to synthesize these substances, as well as complete “chemistry kits”, are available on the Internet. Since the actual effective substance is GHB, consumption of these precursors results in the same effects, side effects, and symptoms of intoxication. In most countries, the GHB precursors (GBL and 1,4-BD) are not controlled under current drug or medicine legislation owing to their widespread use as solvents and the associated consequences for the chemical industry. Although three EU member states (Sweden, Italy, and Latvia) have chosen to control them under the same or similar legislation as that affecting GHB, this is not done all over EU. Recently in Germany, several dealers of GBL were punished based on the pharmaceutical law. They were accused of selling so-called “precarious pharmaceuticals” with a reasonable suspicion of harmful effects (on the basis of § 5 and § 95 of the German pharmaceutical act) [11585].

**gamma Hydroxybutyrate (GHB)**

GHB is a short-chain 4-carbon fatty acid found endogenously in the brain, mainly in the hypothalamus and basal ganglia, in the form of γ-hydroxybutyric acid. GHB has several precursors, including gamma-butyrolactone, which are metabolised into GHB upon ingestion through various pathways. Concurrently, GHB is transformed into the inhibitory
neurotransmitter GABA, with preference for the GABA\textsubscript{B} receptor. The precise mechanism of action of GHB remains unclear. Its properties indicate a role in the brain as a neurotransmitter or neuromodulator. Additional research has shown it could influence serotonergic and dopaminergic activity, both directly and indirectly through interaction with other systems (e.g. GABA\textsubscript{B} receptors). Primary effects include lowered inhibition, induced feelings of euphoria and increased libido. GHB was identified and synthesised more than 40 years ago as a central nervous system depressant: it has been used as an anaesthetic adjuvant and to improve sleep patterns. Links to athletic performance came from a study proposing that GHB administration increased GH release. The drug was then marketed as a nutritional supplement alleging enhanced muscle growth, better sleep quality and improved sexual performance. Simultaneously, GHB became popular during the 1980s with body builders due to the potential anabolic and performance-enhancing properties. These benefits have never been proven in athletic populations but, interestingly, a recent study that examined the effects of GHB on sleep and sleep-related GH release found that low-dose supplementation (2.5–3.5 g/day) caused a twofold increase in GH secretion during sleep. An exact mechanism was not identified, but it was suggested that GHB augments GH release by inducing deeper phases of sleep. GHB and its precursors are not typically present in the diet, but can be found in flavouring agents. It is typically taken as a liquid or powder, and dosages range hugely from 12.5–100 mg/kg/day. It is rapidly absorbed and metabolised, and effects occur almost instantaneously. After a single dose (12.5–50 mg/kg), maximal plasma concentrations are reached after approximately 30–40 min, and its elimination half-life ranges from 30–50 min. Side effects include drowsiness, alcohol-like inebriation, dizziness and induction of sleep. Overdose can cause coma, respiratory depression and death. Frequent and prolonged use has resulted in increased tolerance, and dependence. Currently, no available evidence supports the use of GHB in the athletic population [10527].

GHB, a short-chain 4-carbon fatty acid found in the brain, has also been promoted as a nutritional supplement alleging enhanced muscle growth, better sleep quality and improved sexual performance. GHB is found endogenously in the brain, mainly in the hypothalamus and basal ganglia, in the form of gamma-hydroxybutyric acid. GHB has several precursors, including γ-butyrolactone, which are metabolised into GHB upon ingestion through various pathways. Concurrently, GHB is transformed into the inhibitory neurotransmitter GABA, with preference for the GABA\textsubscript{B} receptor. The precise mechanism of action of GHB remains unclear. Its properties indicate a role in the brain as a neurotransmitter or neuromodulator. Additional research has shown it could influence serotonergic and dopaminergic activity, both directly and indirectly through interaction with other systems (e.g. GABA\textsubscript{B} receptors). Primary effects include lowered inhibition, induced feelings of euphoria and increased libido. GHB was identified and synthesised more than 40 years ago as a central nervous system depressant: it has been used as an anaesthetic adjuvant and to improve sleep patterns. Links to athletic performance came from a study proposing that GHB administration increased GH release. The drug was then marketed as a nutritional supplement alleging enhanced muscle growth, better sleep quality and improved sexual performance. Simultaneously, GHB became popular during the 1980s with body builders due to the potential anabolic and performance-enhancing properties. These benefits have never been proven in athletic populations but, interestingly, a recent study that examined the effects of GHB on sleep and sleep-related GH release found that low-dose supplementation (2.5–3.5 g/day) caused a twofold increase in GH secretion during sleep. An exact mechanism was not identified, but it was suggested that GHB augments GH release by inducing deeper phases of sleep. GHB and its precursors are not typically present in the diet, but can be found in flavouring agents. It is typically taken as a liquid or powder, and dosages range hugely from 12.5–100 mg/kg/day. It is rapidly absorbed and metabolised, and effects occur almost instantaneously. After a single dose (12.5–50 mg/kg), maximal plasma concentrations are reached after approximately 30–40 min, and its elimination half-life ranges from 30–50 min.
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Gamma hydroxybutyrate (GHB), also known as fantasy, liquid ecstasy, and was initially introduced as a general anesthetic. Due to dosing difficulty and side effects, regular use was discontinued. It was also used as a nutritional supplement for sleep inducement and to increase muscle mass, however, its use as a “club drug” and “date rape drug” led to US federal regulation of GHB. GHB analogs such as GBL (gamma-butyrolactone), BD (1,4-butanediol), GHV (gamma-hydroxyvalerate), and GVL (gamma-valerolactone), which are found legitimately in industrial solvents and paint strippers, are used for illicit consumption in an effort to produce effects similar to GHB. GHB is often used to increase feelings of euphoria, relaxation, sociability, and sexuality and to explore altered states of consciousness. However, consequences of use include loss of consciousness, overdose, amnesia, emotionality, loss of motor control, withdrawal, hospital admittance, sexual assault victimization, and engaging in risky behaviors, such as driving while under the influence and polysubstance use. Despite possible adverse effects, GHB has been used to treat sleep and addictive disorders including alcohol withdrawal. In the 1990s, it was promoted as a supplement and taken to improve mood and sex. GHB and its analogs (gamma butyrolactone and butanediol) were widely available until federal regulations were put into effect with mounting evidence of adverse events. One survey (n= 61) study was conducted to assess patterns, experiences, and functions of use. Much of what is understood regarding GHB treatment is based on hospital case studies for overdose and withdrawal. This study found that most respondents who began this web-based survey completed it without incident. It is possible to obtain a relatively diverse sample of GHB/analog users via the Internet, although most were men. Results indicate that over 76 percent of respondents had used these substances one or more times per day, and that it is not uncommon for GHB/analogs to be obtained through persons not known well to the consumer. Furthermore, results indicate that 42 to 58 percent of persons consuming these substances did not know there were government warnings regarding adverse events associated with these drugs. The sample generally endorsed use of GHB/analogs for recreational purposes, to improve health, and to assist with sleep difficulty. Respondents also generally appeared to have significant withdrawal symptoms including mood disturbance and insomnia. Although the sample reported relatively few consequences due to use, the dollar amounts associated with arrests, jail time, hospital care, and annual purchase cost of the drug are exorbitant and range from USD 900 to USD 36,000. Of note, 50 respondents had engaged in driving under the influence. Had these 50 respondents been arrested even once, at USD 10,000 per episode, the costs of for the entire sample would have been USD 500,000 (without injury or accident costs). A substantial proportion of participants are unmotivated to alter use. Relaxation and having fun were seen as important in making decisions to use, and similarly, not wanting to disappoint others was seen as important to making decisions to not use. A substantial proportion of participants associate with friends who encourage use, as compared to their family members who discourage use. Although many respondents were thinking about changing use or working on changing use, they are more vulnerable to using GHB/analogs when socializing, when feeling pressured by others, when they feel an urge to use, or when they are physically uncomfortable. What’s more, respondents seemed overly reliant on cognitive coping skills to avoid use, rather than on more active behavioral coping strategies. These findings suggest that the public needs to be better informed of the potential adverse events associated with GHB/analogs. The data also suggest that efforts to reduce use might focus on increasing contacts with family members who are nonusers, and increasing efficacy and behavioral skills to cope with social pressure, urges, and physical discomfort. Motivation to reduce use is associated with acknowledging more use-related problems, more coping
skills, and fewer dependence symptoms. More withdrawal symptoms are associated with fewer coping skills, and more lifetime dependence symptoms. Most frequent use in the past 12 months is associated with more use-related problems and lower confidence to resist use; whereas having more confidence to resist is associated with fewer problems. Confidence to resist is associated with 12-month dependence symptoms, which may suggest that persons are over-confident in their ability to resist use or alternatively, that previous symptoms abated with recently improved confidence to resist. As expected, most frequent use in the last 12 months is associated with most frequent lifetime use, and surprisingly, it is also related to low dependence symptom count for both 12-month and lifetime. Similarly, most frequent lifetime use was related to low 12-month and lifetime dependence symptom count. In addition, 12-month and lifetime dependence are associated with acknowledging fewer use-related problems. It is difficult to reconcile these findings, and more research is needed to see if results are spurious or if there are subsets of users with varying vulnerabilities to development of dependence symptoms. Persons using in relation to socializing have fewer dependence symptoms, and see more benefits to use. Tentatively this suggests that there may be a portion of users who do not experience difficulties with GHB/analogs when used in the context of social settings; however more detailed studies are needed to understand if this is true and potential mediating factors. Persons using in relation to perceived heath benefits employ fewer coping skills and have more 12-month dependence symptoms. The view that GHB/analogs should be legal and freely accessible is associated with perceiving fewer use-related problems, use of fewer coping skills, and more 12-month dependence symptoms. This suggests that persons more vulnerable to developing dependence symptoms begin to use for health benefits and do not believe in limiting access; however, again, more detailed studies are needed to determine if this is true as well as potential mediating factors [11233].

Gamma-hydroxybutyric acid (GHB) is an endogenous short-chain fatty acid popular as a recreational drug due to sedative and euphoric effects, but also often implicated in drug-facilitated sexual assaults owing to disinhibition and amnesic properties. Whilst discrimination between endogenous and exogenous GHB as required in intoxication cases may be achieved by the determination of the carbon isotope content, such information has not yet been exploited to answer source inference questions of forensic investigation and intelligence interests. However, potential isotopic fractionation effects occurring through the whole metabolism of GHB may be a major concern in this regard. Thus, urine specimens from six healthy male volunteers who ingested prescription GHB sodium salt, marketed as Xyrem®, were analysed by means of gas chromatography/combustion/isotope ratio mass spectrometry to assess this particular topic. A very narrow range of delta^{13}C values was observed, whilst mean delta^{13}C value of Xyrem® corresponded to -2.5 percent. Since urine samples and prescription drug could not be distinguished by means of statistical analysis, carbon isotopic effects and subsequent influence on delta^{13}C values through GHB metabolism as a whole could be ruled out. Thus, a link between GHB as a raw matrix and found in a biological fluid may be established, bringing relevant information regarding source inference evaluation. Therefore, the study supports a diversified scope of exploitation for stable isotopes characterized in biological matrices from investigations on intoxication cases to drug intelligence programmes [11234].

Gamma-hydroxybutyrate (GHB) is naturally present in the human body, but may also be used as an intoxicating drug. Information from several sources has suggested its increased availability and use in Norway. There have also been reports of an increasing use of the chemical precursor gamma-butyrolactone (GBL). There is currently a need for knowledge on symptoms, addictiveness and overdoses, as well as targeted preventive measures. One article was based on a discretionary selection of articles resulting from a literature search in PubMed, as well as reports from Norwegian and European authorities and research institutions. An intake of small amounts of GHB produces an intoxicating effect, whereas
higher doses can result in poisoning. Deaths have been reported. The effect may be variable, due to a steep dose-response curve and interaction with alcohol and other intoxicants. Treatment of poisoning is symptomatic and supportive. Treatment of abstinence is also supportive, while delirium may be treated as delirium tremens [11510]

Gamma-hydroxybutyric acid (GHB) was first synthesized in 1960 as a derivative of the endogenous neurotransmitter gamma-aminobutyric acid (GABA) to pass the blood-brain barrier. In some European countries, GHB is used as an anaesthetic drug, but it has insufficient analgesic effects that necessitate its combination with an analgesic; adverse effects, such as seizures or vomiting, frequently occur. Moreover, controlling dosage and duration of effects is difficult. Later, GHB was also used in the treatment of alcohol and heroin withdrawal. Since studies have shown that GHB has a positive effect on patients with narcolepsy and cataplexy, the substance was licensed for their use in the United States in 2002 and in Europe in 2005, despite the fact that these therapies have many side-effects. As early as the 1980s, GHB was offered in the USA as a dietary supplement. Bodybuilders, in particular, used GHB because of its suggested anabolic muscle-building effects through the increased release of growth hormones. It was also consumed for its supposed aphrodisiac and weight-loss effects, and as a sleeping aid. After numerous cases of intoxication occurred over the following years, the US Food and Drug Administration (FDA) banned its sale to consumers in 1990. However, this did not put a stop to misuse of the drug. GHB gained in popularity; its users consumed “liquid ecstasy” or “soap” on the club scene as a party drug or at private events, as an alternative to, or in combination with, alcohol [11585].

Abuse of gamma-hydroxybutyric acid (GHB) has been known since the early 1990’s, but is not as widespread as the consumption of other illegal drugs. However, the number of severe intoxications with fatal outcomes is comparatively high; not the least of which is brought about by the consumption of the currently legal precursor substances gamma-butyrolactone (GBL) and 1,4-butanediol (1,4-BD). In regards to previous assumptions, addiction to GHB or its analogues can occur with severe symptoms of withdrawal. Moreover, GHB can be used for drug-facilitated sexual assaults. Its pharmacological effects are generated mainly by interaction with both GABAB and GHB receptors, as well as its influence on other transmitter systems in the human brain. Numerous analytical methods for determining GHB using chromatographic techniques were published in recent years, and an enzymatic screening method was established. However, the short window of GHB detection in blood or urine due to its rapid metabolism is a challenge. Furthermore, despite several studies addressing this problem, evaluation of analytical results can be difficult: GHB is a metabolite of GABA (gamma-aminobutyric acid); a differentiation between endogenous and exogenous concentrations has to be made. Apart from this, in samples with a longer storage interval and especially in postmortem specimens, higher levels can be measured due to GHB generation during this postmortem interval or storage time [11585].

Illicit forms of GHB have come under narcotics law in the USA since 2000 and are subject to the strictest categorization (Schedule I). In some parts of Europe (Germany, for example), GHB is still approved for treatment as an anaesthetic and for the treatment of narcolepsy. In March 2001, GHB was added to Schedule IV of the 1971 UN Convention on Psychotropic Substances, thereby binding all EU member states to control it under their legislation addressing psychotropic substances. However, GHB is not yet fully under national control in all countries [11585].

Quantitative data on the prevalence of GHB abuse is incomplete, but various qualitative measures indicate that a mini-epidemic of abuse began in the late 1980s and continues up to the present. Undoubtedly, the easy availability and low costs of GHB and its precursors have contributed to its popularity. According to a study by the European Monitoring Centre for
Drugs and Drug Addiction, consumption of GHB or GBL is not as widespread as consumption of other illegal drugs. Studies have shown that the prevalence of young people’s leisure use of GHB in the past month rarely exceeds 3 percent. However, there are also signs that consumption is becoming more common in private spheres, especially in certain population subgroups and certain social environments or geographical areas. Depending on the particular study and target group interviewed, the lifetime prevalence is 3 percent to 19 percent (six studies were included from the UK, the Netherlands, and Austria with data from years 1999 to 2007). According to various national reporting systems in the USA, there has been an emergence of a problem with GHB abuse beginning no later than 1994 or 1995. Quantitative data on the prevalence in the population is not available due to the fact that the use of GHB is not queried specifically, but is included under “other drugs” in most surveys since 2001. Since the final active agent in the body is GHB, even in cases of GBL or 1,4-BD consumption, an exact discrimination between these drugs is not possible in most cases. In spite of the comparatively low popularity of GHB, cases of intoxication, somewhith a lethal result, have been increasing. Since GHB is not yet detected in routine toxicological screening, the prevalence of GHB abuse is thought to be underestimated.

GHB is a naturally occurring hydroxy carboxylic acid in the human brain, blood, urine, and peripheral tissue. It appears both as a metabolic product and as a precursor of GABA. In spite of numerous studies, its function has not yet been clarified in detail, but there is evidence that GHB has different effects on the balance of neurotransmitters. One possible mechanism of action in discussion is that GHB acts as a neuromodulator in the GABA system. Moreover, GHB causes a dose-response increase or decrease of dopamine levels in the brain and affects the cholinergic system (via GABAB receptors), as well as neurosteroids and, indirectly, opioids. Furthermore, GHB potentiates brain serotonin turnover and increases the secretion of growth hormones. In millimolar concentrations (above endogenous concentrations), GHB already activates GABAB receptors itself or by conversion to GABA and thereby inhibits the influx of Ca\(^{2+}\) and opens K\(^{-}\)-channels, as GABA does. In addition, the existence of a separate G-protein coupled GHB receptor in the brain has been proven. This receptor is thought to be stimulated already by endogenous concentrations of GHB (micromolar range). It is suggested that after exogenous admission, GHB will act both as a GABAB- and GHB-receptor agonist. GHB is administered intravenously for the purpose of sedation, and orally for most other indications. Misuse occurs mostly by oral administration in liquid form, neat or diluted in drinks. Occasionally, capsules are ingested, which often contain GHB in the form of its sodium salt (sodium oxybate). GHB induces biphasic time profile with an initial stimulant-like effect related to the simultaneous rise of plasma concentrations and a latter sedative effect not related to GHB kinetics. GHB has a narrow therapeutic index; the symptoms of poisoning are dose-dependent and partly similar to those of alcohol poisoning. Headache, nausea, vomiting, vertigo, and speech impairment may be present. Higher concentrations may lead to bradycardia, seizures, respiratory depression to the point of respiratory arrest, and impaired consciousness to the point of coma. In severe intoxication, myoclonus or seizures followed by loss of consciousness have often been described. A typical characteristic is a rapid onset and sudden clinical picture. Owing to the extremely fast metabolism of GHB, usually the effects last only 1-4 h, depending on the dose. This phenomenon is known as the “fast-in, fast-out” effect and causes a sudden awakening from (near) coma in most cases, with patients usually recovering within 6-8 h after receiving symptom-oriented treatment. Patients who developed coma (Glasgow Coma Scale. GCS, > 3) had serum levels that ranged from 72 to 300 mg/L with a median of 193 mg/L and regained consciousness as levels decreased to the range of 75 to 150 mg/L. In 15 patients with GCS <8, blood concentrations of 112 to 430 mg/L were determined (most of them co-ingested other drugs or ethanol). One patient was in a state of coma (GCS 7) after ingestion of 1,4-BD, corresponding blood levels were 82 mg/L for 1,4- BD and 103 mg/L for GHB. A
woman who was admitted to an emergency department in a comatose state had 161 mg/L GHB in serum after ingestion of GBL from nail polish remover pads. After oral administration, GHB is absorbed very rapidly; maximum plasma concentrations are reached after 25-45 min. The distribution throughout the body is fast and follows a two-compartment model. The apparent distribution volume is reported with 0.19 to 0.4 L/kg and was not significantly affected by gender, food, or liver cirrhosis. GBL has potentially a higher intestinal flux after oral administration and a shorter onset, therefore causing higher C\text{max} of GHB. 1,4-butanediol is also quickly absorbed and metabolized. It is extensively converted to GHB after oral administration, but significant inter-individual variability in the rate of metabolism, possibly related to genetic variants in the alcohol dehydrogenase, was observed. Moreover, conversion of 1,4-BD is blocked competitively by ethanol [11585].

Endogenous GHB is formed from GABA by means of succinic semialdehyde reductase via the intermediate product succinic semialdehyde (SSA). Via the enzyme GHB dehydrogenase, GHB can be oxidated back into SSA. After a further oxidation step, SSA reaches the citric acid cycle, mainly as succinic acid. Only a small proportion of GHB is excreted in an unchanged form through the kidneys (<2 %). GHB is eliminated rapidly; the terminal elimination half-life in plasma averages 20-60 min after single oral doses, but there are indications that the elimination kinetics of GHB is not linear after administration of therapeutic doses. Moreover, in some cases after ingestion of large doses, zero order kinetics seems to bemore appropriate than first-order kinetics. Drug facilitated sexual assaults/chemical submission GHB and its precursors are colourless liquids, transparent, and almost entirely without taste. They can thus be mixed with drinks to administer to unsuspecting victims without their noticing, and are used with criminal intent to weaken the resistance of individuals, for example to exploit their property or body without their consent and without them having the slightest recollection afterwards of what happened (anterograde amnesia). Criminals tend to use substances such as GHB to facilitate the commitment of their offences. They also tend to use substances currently not under international control, such as ketamine, 1,4-BD and GBL, since they are easily available through legitimate channels. GHB can result in impaired consciousness, including coma with anterograde amnesia. Studies have shown that GHB is one of the substances most often used for the purpose of drug facilitated sexual assault (DFSA) in the USA.[5,36,37] Similar cases have also been reported in Germany and other European countries. Because of its depressant effect on the central nervous system, the use of GHB and its analogues is also important in cases of driving under the influence of drugs (DUID). For example, Jones et al. identified in their database 548 DUID cases with GHB (alone or together with other recreational drugs) for the years 1997 to 2007 in Sweden. Fatalities have been reported with accidental overdoses of GHB, suicidal intent, or trauma as a result of impaired driving. Fatal overdoses occur also with the precursors GBL or 1,4-BD. Especially post-mortem analytical GHB results should be interpreted with caution. Furthermore, it should be recognized that abrupt GHB withdrawal can cause life-threatening clinical courses which can even lead to death. Initial studies after clinical administration of GHB in patients with narcolepsy have not shown any cases of misuse or of a developing tolerance. More recent studies, however, indicate the potential for the drug as mentally and physically addictive and suggest a high abuse liability. GHB abuse can lead to addiction with severe withdrawal complications; the same is valid for its precursors. GHB addiction is characterized by round-the-clock dosing, typically every 1-3 h. According to our experience, typical doses are 20 to 70 mL per day; reported doses of patients with GBL/1,4-BD withdrawal in the literature ranged from “two sips every night” up to 900 mL/day during five weeks to four years. Addiction can develop within a few weeks. Withdrawal symptoms after abrupt GHB discontinuation manifested very quickly within 2-6 h. Withdrawal complications of dependence can be associated with a range of neurological phenomena including tremor, anxiety attacks, confusion, epileptic fits, and memory loss. These initial symptoms may progress to severe delirium with auditory and visual
hallucinations and autonomic instability. Cardiovascular effects included severe tachycardia and hypertension. Life-threatening clinical courses with admission to the intensive care unit have been documented. Most patients typically requested medical assistance for continued withdrawal symptoms from three days to up to 14 days or more after the last dose. Benzodiazepines, barbiturates, and antipsychotic medication are the most used drugs in the treatment of GHB withdrawal symptoms. However, in many cases, high doses of benzodiazepines and barbiturates proved ineffective. A sufficient medical treatment of the GHB withdrawal syndrome comparable to the treatment of alcohol or benzodiazepine addiction is not yet clearly defined [11585].

The discovery of gamma-hydroxybutyrate (GHB) over 40 years ago led to its immediate use as a general anesthetic agent. Subsequent research demonstrated that GHB is an endogenous compound in the mammalian brain and current research suggests that GHB is a probable neurotransmitter. In the United States, reports of anabolic effects lead to its misuse among body builders during the 1980's while the intoxicating properties of the drug lead to its popularization as a substance of abuse during the 1990's. GHB became associated with reports of drug-facilitated sexual assault and cases of physical dependence and withdrawal. Efforts to ban GHB caused increased use of GHB analogues and pro-drugs. Against this backdrop, GHB was being developed for the treatment of narcolepsy, leading to the approval of Xyrem (sodium oxybate) oral solution in 2002 for the treatment of cataplexy in patients with narcolepsy. A risk management program permits the safe handling and distribution of the approved product, minimizes the risk for diversion, provides professional and patient education about the risks and benefits of sodium oxybate, and includes physician and patient registries. Post-marketing surveillance indicates sodium oxybate has an acceptable safety profile and presents minimal risk for the development of physical dependence [06228].

Gamma-hydroxybutyrate (GHB) is a naturally occurring compound present in the brain and peripheral tissues of mammals. It is a minor metabolite and precursor of gamma-aminobutyric acid (GABA). Just as GABA, GHB is believed to play a role in neurotransmission. GHB was first synthesized in vitro in 1960, when it revealed depressive and hypnotic effects on the central nervous system. In 1960s it was used as an anaesthetic and later as an alternative to anabolic steroids, in order to enhance muscle growth. However, after it was shown that it caused strong physical dependence and severe side effects, GHB was banned. For the last fifteen years, GHB has been abused for its intoxicating effects such as euphoria, reduced inhibitions and sedation. Illicitly it is available as white powder or as clear liquid. Paradoxically GHB can easily be manufactured from its precursor gamma-butyrolactone (GBL), which has not yet been banned. Because of many car accidents and criminal acts in which it is involved, GHB has become an important object of forensic laboratory analysis. One paper described gas and liquid chromatography, infrared spectroscopy, microscopy, colourimetry and nuclear magnetic resonance as methods for detection and quantification of GHB in urine and illicit products [06229].

Gamma-hydroxybutyrate (GHB) is a drug of abuse that causes euphoria, anxiolysis, and hypnosis. The recent rise in the recreational intake of GHB, as well as its association with 'drug rape', has turned the attention to GHB in acute hospital settings. Acutely admitted GHB intoxicated patients may display various levels of sedation or coma, but may also show paradoxical agitation, combativeness, or self-injurious behaviors. The symptoms can be nonspecific and the definite diagnosis therefore normally relies on the detection of GHB in blood or body fluids, which is an analysis that may not be promptly available. As a basis for understanding the clinical features of GHB intoxication and abuse, it was reviewed the pharmacological and neurophysiological knowledge about GHB, which stems from decades of clinical and basic GHB research. In addition, it was discussed the latest discoveries in the quest for distinct GHB receptors in the brain, and their possible implications for future
therapies of GHB abuse [06230].

Gamma-hydroxybutyric acid (GHB; sodium oxybate) is approved for narcolepsy symptom treatment, and it is also abused. One study compared the participant-rated, observer-rated effects, motor/cognitive, physiological, and reinforcing effects of GHB and ethanol in participants with histories of sedative (including alcohol) abuse. Fourteen participants lived on a residential unit for 1 month. Sessions were conducted Monday through Friday. Measures were taken before and repeatedly up to 24 hours after drug administration. Participants were administered GHB (1, 2, 4, 6, 8, and 10 g/70 kg), ethanol (12, 24, 48, 72, 96, and 120 g/70 kg), or placebo in a double-blind, within-subjects design. For safety, GHB and ethanol were administered in an ascending dose sequence, with placebos and both drugs intermixed across sessions. The sequence for each drug was stopped if significant impairment or intolerable effects occurred. Only 9 and 10 participants received the full dose range for GHB and ethanol, respectively. The highest doses of GHB and ethanol showed onset within 30 minutes, with peak effects at 60 minutes. GHB effects dissipated between 4 and 6 hours, whereas ethanol effects dissipated between 6 and 8 hours. Dose-related effects were observed for both drugs on a variety of measures assessing sedative drug effects, abuse liability, performance impairment, and physiological effects. Within-session measures of abuse liability were similar between the two drugs. However, postsession measures of abuse liability, including a direct preference test between the highest tolerated doses of each drug, suggested somewhat greater abuse liability for GHB, most likely as a result of the delayed aversive ethanol effects (e.g. headache) [13534].

To avoid the detection of small fragmentation products of gamma-hydroxybutyrate (GHB), a liquid chromatography-tandem mass spectrometry GHB quantification method in human serum supported by adduct formation was developed and validated. The continuous infusion of GHB/GHB-D6 made the identification of two adducts possible and GHB/GHB-D6 sodium acetate adduct fragmentation was used as target mass transition. A Luna 5 μm C18 (2) 100 A, 150 mm × 2 mm analytical column and elution with a programmed flow of the mobile phase consisting of 10 percent A (H₂O/methanol = 95/5, v/v) and 90 percent B (H₂O/methanol = 3/97, v/v), both with 10 mM ammonium acetate and 0.1percent acetic acid (pH = 3.2), were used. Protein precipitation with 1 mL of the mobile phase B was used as the sample preparation. The calculated limit of detection/quantification was 1 microg/mL. The presented study shows that the fragmentation of GHB sodium acetate adducts is an effective way of quantification of this small molecule and is an interesting alternative to other methods based on the detection of ions smaller than 85 Da. This fact together with the short analysis time of 3 min and the fast sample preparation make this method very attractive for forensic/clinical application [13535].

Gamma-hydroxybutyric acid and gamma-butyrolactone are illegal drugs with potentially fatal outcomes that are entering wider use in Scandinavia. Gamma-hydroxybutyric acid-dependent persons with withdrawal symptoms often require forceful withdrawal treatment provided in psychiatric units. Data were collected from interviews with 15 registered nurses working in specialised dependency units in psychiatric wards. The data collected were analysed through a descriptive, qualitative analysis. The registered nurses' narratives revealed four main areas of convergence: feelings of anxiety and despair, preparation for unpredictable and precarious situations, striving for good relationship and striving to optimise and develop nursing care. The interviews revealed that registered nurses reflect on and discuss their feelings about their patients' situations with colleagues; prepare themselves for potential aggressiveness and unpredictable situations; improve their care through conscious attitude adjustment and relationship-forming behaviours; and strive to increase their personal knowledge, maintain a hopeful outlook and exhibit a positive approach. These themes were found in all nine categories and sixteen subcategories. The findings based on the registered
nurses' narratives indicated that the registered nurses experienced their work situation when caring for these patients to be very complex and demanding. It was concluded that the study revealed that registered nurses worked extensively to craft their approach and attitude towards their patients. It is clear that registered nurses use themselves as tools or instruments for the creation of good relationships, thus providing the best care possible. Registered nurses should be given more education, clearer guidelines and better guidance to assist them in facing such challenging and often problematic situations. One-on-one shadowing provides the possibility to create and develop relationship [135].

GHB occurs naturally in the human body primarily in the central nervous system. It is a metabolite of brain neurotransmitter GABA. Chemically, GHB is called gamma-hydroxybutyric acid, 4-hydroxybutyric acid, or gamma-hydroxybutyrate. GHB has a structure that consists of a chain of four carbon atoms, with an alcohol group (-C-OH) at one end and a carboxylic acid group (-C-OOH) at the other end. GHB is usually exists either as a free acid, or as a sodium salt. The sodium salt is called Sodium Oxybate and is soluble in water and methanol. It is available as tablet, light-colored powder; colorless liquid, odorless, tasteless in small vials. It was first developed for its calming actions and was found out to have the ability to induce sleep. This made it to be used as an anesthetic in 1960s but was withdrawn due to its side effects like seizures and coma. Due to its euphoric effects, it is nowadays used as “recreational drugs”. The vials contain as much as 10 hits and are often slipped into an unknowing victim's drink when they aren’t noticing GHB is most commonly available in liquid form and is taken orally. It is also available in powder, putty, capsule and gel forms. Street names are Liquid ecstasy, Fantasy, GBH, Georgia Homeboy, Great hormones at bedtime, Liq. E, Liq. X, salty water, Soap, Everclear, Sodium Oxybate, Scoop, Poor man heroine. Trade names include Alcover (Italy), GammaOH (France), Somsanit (Germany) and Xyrem (US, Canada). GHB was used clinically as an anesthetic in the 1960s but was withdrawn due to side effects that included seizures and coma. GHB has been implicated in a number of crime types; most notably in drug-facilitated sexual assault. GHB is abused by three main groups of users: Body builders who use the substance believing that it stimulated the release of growth hormone; sexual predators who covertly administer the drug for its sedative and amnesic effects and club-goers (rave parties) who take the drug for its euphoric effects. The short-lived hypnotic effects, relative safety and widespread availability of the drug have made it particularly well suited to this role. The drug has an addictive potential if used for long term. The primary effects of GHB use are those of a CNS depressant and therefore range from relaxation, to euphoria, confusion, amnesia, hallucinations, and coma. Despite the increased regulation, GHB remains widely available through the Internet where one can easily purchase the necessary reagents as well as recipes for home production. There are reports of patients being unresponsive to painful stimuli and cases of oral self-mutilations linked to the abuse of GHB, though quiet rare. Such cases should remind odontologists that intra-oral lesions may be the result of self-mutilation either due to mental illness or altered states caused by the use of prescription or non-prescription drugs. It is well documented in the dental literature that certain cultures practice deliberate mutilation and non-therapeutic extractions of the dentition to satisfy a variety of social, religious and cultural imperatives. Reasons for such activities include esthetic adornment, class identification and a means of facilitating oral sex. In Western cultures, deliberate mutilation however is considered pathologic. Oral self-mutilation may be classified as either organic or functional. In organic self-mutilation, the person injures himself unknowingly and compulsively. The occurrence of oral self-mutilation resulting from a functional cause may be motivated by secondary gains, facultial or neurotic self-excoriations and self-mutilation during a psychotic episode. The purpose of this article is to highlight the fact that though, oral self-mutilation, particularly auto-extraction, is rare, such cases should remind odontologists that intra-oral lesions, such as extraction sockets, may be the result of self-mutilation either due to mental illness or altered states caused by the use of prescription or non-prescription drugs [130].
**Metabolism and neuromodulatory properties**

The most important synthetic pathway for GHB involves conversion of GABA to succinic semialdehyde by the molecule GABA aminotransferase, followed by reduction of succinic semialdehyde to GHB by cytosolic succinic semialdehyde reductase. Mitochondrial succinic semialdehyde dehydrogenase converts succinic semialdehyde to succinate. A minor pathway for GHB production involves partial oxidation of 1, 4 butanediol. Systemically administered gamma-butyrolactone (GBL) is converted by a circulating lactonase to GHB. The lactonase is not present in brain tissue. The most significant catabolic pathway for GHB degradation is the oxidation of GHB to succinic semialdehyde by NADP⁺-linked succinic semialdehyde reductase. The resultant succinic semialdehyde undergoes further metabolism to either GABA or succinate. There is disagreement as to whether there is significant metabolism of GHB through a beta oxidation scheme. GHB exerts ubiquitous pharmacologic and physiological effects when it is administered systemically to animals. However, GHB also has many of the vital properties of a neurotransmitter or neuromodulator and neurodepressant. This neurodepressant effect may be mediated by a specific GHB receptor, binding to GABA receptors, modulation of GABA levels, or interactions with other neurotransmitters. There is compelling evidence that a novel GHB receptor exists in the CNS. These receptors are saturated at the levels of GHB achieved after exogenous administration. In addition to its own receptor; GHB is known to bind to the GABA₆ receptor, although with a much lesser affinity. Physiologic levels of GHB would not bind this receptor sufficiently to cause pharmacologic effect. However, supraphysiologic levels achieved after exogenous administration could cause considerable binding of the GABA₆ receptor leading to membrane hyperpolarization and depression of the CNS. Thus, experimental evidence to date suggests that the high concentrations of GHB in brain tissue that would be predicted to mount up from exogenous administration of this compound as occurs in the clinical scenarios of GHB intoxication, addiction and abuse may wield their protean pharmacologic, toxicologic and behavioral effects chiefly through mechanisms mediated by the GABA₆ receptor [13010].

**Positive aspect of GHB**

GHB can be easily manufactured at home with very little knowledge of chemistry, as it only involves the mixing of its two precursors, GBL and an alkali hydroxide (such as sodium hydroxide) to form the resulting GHB salt. Due to the ease of manufacture, it can even be produced in private homes by low level producers. It can be purchased overseas via Internet and then shipped to the purchaser. GHB is generally taken orally and is rapidly absorbed from the gastrointestinal tract. The onset of GHB’s effects is delayed and systemic effects occur generally within 15 min [13010].

**Cardiovascular and respiratory effects**

It was observed a constant but short drop in blood pressure in rabbits after administration of GHB, but in dogs there was either no effect or a slight progressive increase in blood pressure. In all animals, a constant bradycardia was observed. It was also observed a strong hepatic and renal vasodilating action, particularly during hemorrhagic shock in animals, indicating that GHB has “antishock activity”. In man, there appeared to be no effect on blood pressure. Laborit also observed that at low hypnotic doses of GHB, a decrease in ventilatory rate was reported with an increase in amplitude. At high (sleep-inducing) doses of GHB, a Cheyne-Stokes rhythm appeared [13010].

1471
Central nervous system effects

Based on behavioral and electroencephalographic criteria, GHB-induced sleep has been described as being indistinguishable from natural sleep, i.e. unlike coma, the natural stages of sleep 1-2-3-4-REM (rapid eye movement) all occur in their normal sequence. The effect of GHB-enhanced sleep appears to wear off after 3-4 hours at “normal” doses [13010].

GHB in obstetrics

GHB had a spectacular action on the dilation of the cervix and furthermore was beneficial in obstetric surgery due to the absence of respiratory depression in the infant and its antishock property against possible cardiac anoxia [13010].

Sexual enhancing effects of GHB

GHB is “aphrodisiac” in man. It has four sexual-enhancing effects; disinhibition (e.g. relaxation), heightened sense of touch, enhancement of male erectile capacity and increased power of orgasm. These drugs also appear to promote high-risk sexual behaviors that have been associated with increased HIV infection [13010].

GHB use in alcohol and opiate withdrawal

The use of GHB in alcohol withdrawal has been investigated by various researchers and was found to reduce tremors after consumption of alcohol. GHB has also been shown to inhibit voluntary ethanol consumption. The exact mechanism of GHB-enhanced alcohol and opiate withdrawal is unknown. However, a profound inhibition of dopamine output in the nucleus accumbens and ventral caudate nucleus has been associated with alcohol and opiate withdrawal syndromes, and increased dopamine output is known to be involved in the rewarding effects of morphine and alcohol. Therefore, it is possible that GHB suppresses these symptoms as it increases the dopamine levels in these regions of the brain and maintains the dopamine reward pathway [13010].

Interaction with alcohol

Gamma-hydroxybutyrate (GHB) is a common drug of abuse that can produce serious toxicity, particularly when used with other sedatives. It was examined the individual and combined effects of GHB and ethanol in human volunteers. Sixteen healthy adults (7 men) were given 50 mg/kg GHB (Xyrem), 0.6 g/kg ethanol in 2 doses, alone and combined in a double-blind, placebo-controlled, crossover study. Plasma concentrations, heart rate (HR), blood pressure (BP), and oxygen saturation (O_{2sat}) were serially monitored for 24 hours. Adverse events included 2 instances of hypotension and 6 episodes of vomiting with GHB-plus-ethanol ingestion. Oxygen saturation was decreased by GHB and ethanol individually, and maximally decreased by the drugs combined. Compared with baseline, systolic and diastolic BP were significantly decreased, and HR was increased by ethanol but not affected by GHB alone. Ethanol coingestion resulted in 16 percent higher GHB maximal plasma concentration and 29 percent longer elimination half-life, indicating possible enhanced bioavailability or reduced clearance of GHB caused by ethanol, however, these effects were not statistically significant. It was concluded that modest doses of GHB do not affect hemodynamic function, but O_{2sat} was decreased. Gamma-hydroxybutyrate-plus-ethanol resulted in more adverse effects, including gastrointestinal disturbances, hypotension, and decreased O_{2sat}, but only minimal pharmacokinetic interactions were observed [06231].
Pharmacokinetics and pharmacodynamics

gamma-Hydroxybutyrate (GHB) is a potent sedative/hypnotic and drug of abuse. Tolerance develops to GHB's sedative/hypnotic effects. It is hypothesized that GHB tolerance may be mediated by alterations in central nervous system pharmacokinetics or neurotransmitter response. Rats were dosed daily with GHB (548 mg/kg s.c. q.d. for 5 days), and sleep time was measured as an index of behavioral tolerance. Plasma and brain GHB pharmacokinetics on days 1 and 5 were monitored using blood and microdialysis sampling. Extracellular (ECF) striatal dopamine levels were measured by microdialysis as a pharmacodynamic endpoint of tolerance. Pharmacokinetic (PK)/pharmacodynamic (PD) modeling was performed to describe the plasma and brain disposition using an indirect response model with inhibition of dopamine synthesis rate to describe the pharmacodynamic response. GHB plasma and brain ECF concentration versus time profiles following acute or chronic exposure were not significantly different. GHB sedative/hypnotic tolerance was observed by day 5. Acute GHB administration resulted in a decrease in striatal ECF dopamine (DA) levels compared with baseline levels. GHB tolerance was reflected by a 60% decrease in dopamine area under the curve (effect and baseline): acute, 10.1 ± 15.3 percent basal DA/min/10^−3 versus chronic, 4.73 ± 1.49 percent basal DA/min/10^−3. The PK/PD model revealed an increase in the IC50 following chronic exposure indicating decreased dopaminergic sensitivity toward the inhibitory effects of GHB. The findings indicate that GHB pharmacokinetics do not contribute to behavioral tolerance; however, changes in neurotransmitter responsiveness may suggest specific neurochemical pathways involved in the development and expression of tolerance [06232].

Despite gamma-hydroxybutyrate (GHB) therapeutic uses and the increasing concern about its toxicity, few studies have addressed GHB dose-related effects under controlled administration and their relationship with its pharmacokinetics. The study design was double-blind, randomized, crossover, and controlled. As a pilot pharmacology phase I study, increasing doses of GHB were given. Single oral sodium GHB doses (40, 50, 60, and 72 mg/kg) were administered to eight volunteers. Plasma and urine were analyzed for GHB by gas chromatography-mass spectrometry. Physiological effects, psychomotor performance, and subjective effects were examined simultaneously. GHB produced dose-related changes in subjective effects as measured by questionnaires and VAS. GHB showed a mixed stimulant-sedative pattern, with initially increased scores in subjective feeling of euphoria, high, and liking followed by mild-moderate symptoms of sedation with impairment of performance and balance. Mean peak GHB plasma concentrations were 79.1, 83.1, 113.5, and 130.1 mug/L for 40, 50, 60, and 72 mg/kg, respectively. GHB-mediated physiological and subjective effects were dose dependent and related to GHB plasma concentrations. GHB urinary excretion was mainly related to administered doses. GHB-mediated subjective and physiological effects seem dose dependent and related to GHB plasma concentrations. Results suggest a high abuse liability of GHB in the range of dose usually consumed [06233].

1,4-Butanediol (BD) is converted to gamma-hydroxybutyrate (GHB) after ingestion, and is associated with cases of dependence, coma, and death. The pharmacology of BD after oral ingestion has not been described in humans. Eight healthy volunteers (five men) were administered 25 mg/kg BD in a single oral dose after an overnight fast in a double-blinded, placebo-controlled, crossover study. Vital signs were monitored, and serial blood samples collected over 24 h for gas chromatography-mass spectrometry analysis of BD and GHB levels. Subjective mood and symptoms responses were assessed by visual analog scale. All subjects completed the study without significant adverse effects. BD was quickly absorbed and cleared, with time to maximal plasma concentration of 24+/−12 min, and elimination half-
life ($T_{1/2}$) of 39 ± 11 min. BD was extensively converted to GHB, with a mean maximum GHB concentration of 46 ± 20 mg/l reached 39 ± 11 min after BD ingestion. GHB $T_{1/2}$ averaged 32 ± 7 min. Some subjects exhibited slow oral clearance of BD, which tended to correlate with a variant haplotype of the alcohol dehydrogenase gene ADH-IB G143A. Mean CL/F was 152 ± 177 mL/min kg for four subjects with variant haplotype versus 599 ± 447 mL/min kg for four wild-type subjects. Subjects reported feeling less awake and alert, less able to concentrate, and more lightheaded in the first 90 min after BD ingestion. Pulse oximetry readings were lower 45 min after BD dosing with a mean oxygen saturation of 98.5 percent with BD versus 99.6 percent with placebo. Transient increases in mean systolic and diastolic blood pressure were observed, but other vital signs remained unchanged. BD was extensively converted to GHB after oral administration, but significant inter-individual variability in the rate of metabolism, possibly related to variants in ADH-IB, was observed. At the modest dose studied, significant clinical effects were not seen [06234].

In the urine
Gamma-hydroxybutyric acid (GHB) is used as an illicit drug and is implicated in drug-facilitated sexual assault, but it also has some therapeutic uses. Detection of GHB in urine is important for forensic testing and could be of clinical benefit in overdose management. Urine GHB concentration-time profiles have not been well-characterized or correlated with doses used therapeutically. GHB levels were measured by gas chromatography-mass spectrometry in urine collected over 24 h from 16 adults administered single doses of 50 mg/kg GHB (Xyrem) alone and combined with 0.6 g/kg ethanol. Peak GHB urine concentrations averaged 150-200 mg/L and occurred in the 0-3 h urine collection. Significant variability in GHB urine levels between individuals was observed. Caucasians had lower urine concentrations than other races/ethnicities. Men had lower GHB levels than women in the first 3 h after dosing. Coingestion of ethanol did not significantly affect renal clearance of GHB, but urine GHB concentrations were lower in the first 3 h when ethanol and GHB were coingested. At a proposed cut-off of 10 mg/L to distinguish endogenous versus exogenous GHB levels, 13 percent of the samples collected from 3 to 6 h, 81 percent of samples collected from 6 to 12 h, and 100 percent of urine specimens collected from 12 to 24 h were below this level. It was concluded that the detection time for GHB in urine may be shorter than the previously reported 12-h window in some people taking therapeutic doses of GHB [06235].

One study was designed to supplement previous studies that documented in vitro production of gamma-hydroxybutyrate (GHB) in urine samples. Urine samples were provided by subjects who reported that they had never used GHB (n=31). The specimens were stored under standard conditions of refrigeration (5 degrees C) without any preservatives added. All specimens were repeatedly analyzed for the presence of endogenous GHB over a 6-month period using a previously reported headspace GC-MS method. Significant elevations in GHB were observed in many of the urine samples as storage time increased. As a result, the in vitro production of GHB may increase the apparent GHB concentrations in urine during storage. This potential for an artificial increase in GHB concentration must be appreciated when establishing the threshold between endogenous and exogenous concentrations of GHB [06236].

Other negative aspect of GHB

GHB is an extremely dangerous drug as the dose range between safe and toxic is very narrow. After being swallowed the Ecstasy pill begins to disintegrate. It starts to enter the bloodstream through the lining of the small intestine. Once in the bloodstream the active ingredient of GHB will be pumped into the heart. After about 30 minutes, the GHB will be small enough to cross the capillaries into the brain and the “high” will start to be felt. GHB
makes nerves in the brain release serotonin. This serotonin then causes a gland in the brain to release oxytocin also known as ‘the cuddle chemical’. Oxytocin creates the feeling of euphoria and the illusion of strong connection with others. The brain also releases dopamine which allows the user to focus hard on one thing, like beat of a song in a club. It also affects the hypothalamus which controls the body temperature. GHB users are a high risk for overheating. GHB makes you high but it also drags you down. The way the drug affects a person can be compared to the swinging pendulum. The “feel good” emotions for the abuser go far beyond their usual boundaries. But the person feels far worse than usual as the pendulum of emotions swings back in the other direction. The main adverse effects include respiratory depression, amnesia, apnea, hypotonia, aggressiveness, hallucinations, dizziness, euphoria, confusion and seizures. Resolution of CNS depression occurs abruptly with patients going from unresponsive to agitated and combative over very short period of time [13010].

GHB abuse

Due to the various effects of GHB and the various groups of people using the compound, it has a wide-ranging abuse potential. Various myths have come up from time to time which has made the usage of GHB even more adverse. Many people use it for weight lifting. A few credit GHB for keeping them young. According to some it is the safest of all euphoric drugs, in terms of having zero negative side effects. However some are of opinion that it is good!!! Better than alcohol as you need not overload the liver with processing excess amounts of alcohol. A few even use it as a vitamin supplement. Physical dependence has been observed at prolonged high dosage. Reports indicate that GHB is abused for various reasons and by various sections of society [13010].

Body builders who believe that by taking this drug could improve muscle mass or improve exercise performance but there is no evidence of such effects. Therefore it is illicitly sold and distributed in gymnasiums. Some people erroneously refer to GHB as an anabolic steroid, which is not the case, as its chemical structure does not resemble a steroid. Conversely, other people sometimes use GHB as an apparent appetite suppressant or weight loss product, although there is very little definite scientific data to support these claims. Second group includes the sexual predators who covertly administer drug for its sedative and amnesic effects and lastly club goers who take the drug for its euphoric effects. GHB use is very often in night clubs and is an alternative to ecstasy. Most patients are transported from night clubs with 84 [13010].

GHB withdrawal is similar to withdrawal from other sedative/hypnotic agents such as ethanol. GHB’s significant “likelihood of abuse” is evident with its sixth place ranking out of 19 hypnotic drugs compared. It is ranked second only to pentobarbital with respect to toxicity taking into account withdrawal severity. Onset of withdrawal symptoms typically occurs within a few hours after cessation of GHB or its prodrugs. The withdrawal symptoms include: insomnia, anxiety, tremor, delirium, seizures and in extreme cases even, death. Wernicke-Korsakoff syndrome has also been associated with GHB withdrawal. In the majority of GHB-related deaths, the concentration in post mortem blood has been found to be high. Furthermore, in living persons, similar concentrations have been detected in unconscious patients who awake a few hours later with no obvious side effects. Due to the rapid absorption and metabolism of GHB, however, it is difficult to predict how much of the original dose such post mortem concentrations represent. Hence more research and thorough analysis of GHB in fatalities and poisonings are still required before the true involvement of GHB can be established and accurate mortality and morbidity figures produced [13010].
**GHB detection**

GHB can be detected in hair even months after GHB ingestion. To detect GHB in urine, the sample must be taken within 8-12 hours of GHB ingestion. In some cases, however, extensive drug screening is performed and presence of GHB has been confirmed by gas chromatography and mass spectrometry [13010].

**Laboratory technique**

A gas chromatographic-tandem mass spectrometric (GC-MS-MS) method for determining trace concentrations of gamma-hydroxybutyrate (GHB) in blood and urine has been developed. Multiple reaction monitoring was used to detect parent and daughter ions of GHB, 233 and 147, respectively, following liquid-liquid extraction with acetonitrile and derivatisation with N,O-bis[trimethylsilyl]trifluoroacetamide (BSTFA). Deuterated GHB was used as an internal standard. The assay produced excellent linearity and sensitivity without conversion to gamma butyrolactone. The lower limit of quantitation (LLOQ) in 50 microL of sample was 2.5 microg/mL. The expanded uncertainty values for intra- and interassay results were ± 0.097 and ± 0.123 ng/mL at a confidence level of 95 percent for blood and urine, respectively. Endogenous concentrations of GHB were found to be in the range of 0.3 to 6 microg/mL in urine and 0.5 to 2.3 microg/mL in blood, confirming previously suggested cut-off values for forensic analysis [06237].

One study was designed to supplement previous attempts to establish an accurate range of normal endogenous gamma-hydroxybutyrate (GHB) concentrations in random antemortem urine samples. Furthermore, its purpose was to ascertain the effect of gender, race, age, medications, and select medical conditions on endogenous concentrations of GHB in urine and the proposed endogenous urinary GHB cutoff of 10 microg/mL. Urine samples (n=207) were provided by subjects who reported that they had never used GHB. As part of the collection process, subjects also completed a short survey to collect information about gender, race, age, orally ingested medications, and select medical conditions. All specimens were analyzed in duplicate for the presence of endogenous GHB using a previously reported headspace gas chromatography-mass spectrometry method. The data were analyzed for tendencies among different population groups. GHB concentrations ranged from 0.00 to 2.70 microg/mL in all specimens, with a median concentration of 0.24 microg/mL. Males (n=130) had an average endogenous GHB concentration of 0.27 microg/mL (0.00-2.70 microg/mL), whereas females (n=77) averaged 0.29 microg/mL (0.00-0.98 microg/mL). Select medical conditions and participants’ race, age ranges, and medications that were used within 48 h prior to collection were also evaluated. The results of this study will aid the interpretation of low GHB concentrations measured in urine samples, particularly in investigations of drug-facilitated crimes [06238].

**beta-Hydroxy-beta-methylbutyrate (HMB)**

HMB is a byproduct of leucine metabolism in the human body. HMB is currently marketed as calcium-HMB-monohydrate. Although the ergogenic mechanism is unknown, investigators speculate that HMB may be incorporated into cell components or may influence cellular enzyme activity, in some way inhibiting the breakdown of muscle tissue during strenuous exercise. Several animal studies involving poultry, cattle, and pigs have indicated HMB supplementation may increase lean muscle mass and decrease body fat. Studies on humans are limited, but the first published report indicated that HMB supplementation (1.5-3.0 g/day)
significantly increased lean tissue and strength in untrained males who initiated a resistance-training program for three weeks. Research was limited in the following years. In a year 2000 review of research evaluating claims that HMB supplementation can enhance exercise performance, it was indicated that at that time there were only two reports published in peer-reviewed journals, and the remaining eight papers were published as abstracts only and had not undergone the peer-review process. Based on these data, they indicated that although there is some support for the claims, at least in young, untrained individuals, the response of resistance-trained individuals is less clear and that there is a need for more tightly controlled, longer duration studies to evaluate the ergogenic effect of HMB supplementation. Peer-reviewed studies published subsequent to year 2000 are equivocal regarding the ergogenic effect of HMB supplementation on exercise performance in untrained individuals. For example, it was reported that short-term HMB supplementation (3 grams daily for 4 weeks) during resistance training significantly increased upper body strength in both untrained males and females. In another report older subjects engaged in 8 weeks of resistance training and consuming 3 grams HMB daily experienced a significant decrease in body fat, but no significant change in fat-free mass. In a placebo-controlled study, compared the effects of HMB (3 grams daily) and creatine (20 grams for 10 days; 10 grams for 14 days), both separately and in combination, on body composition and strength of subjects undergoing resistance training for 3 weeks. All subjects gained body mass and strength with training, but the supplement groups gained more than the placebo group. However, the only significant effect on body mass was that of the creatine group compared to the placebo group. Overall these findings with untrained individuals are equivocal, meriting additional research. In general, studies using resistance-trained individuals as subjects report no significant effects of HMB supplementation. In one study it was provided forty experienced strength-trained athletes with 0, 3, or 6 grams of HMB daily during four weeks of resistance training, and reported no significant effects of HMB on muscle tissue breakdown, fat-free body mass, or strength gains in the bench press and leg press. It was also studied the effect of daily HMB supplementation on body composition, muscular strength, and markers of muscle damage and muscle protein turnover following 3 and 6 weeks of training. While both the placebo and HMB groups increased lean body mass and strength with the training, the HMB supplementation provided no additional benefits. In a review, it was indicated that studies of HMB supplementation have shown minimal gains in strength and lean body mass in specific populations, mainly untrained athletes. However, HMB use in athletes involved in regular high-intensity exercise has not been proven to be beneficial when multiple variables are evaluated. It was concluded that HMB cannot be recommended as an ergogenic until further studies in larger groups reproduce early data [06239].

One study investigated the effects of 6 weeks of dietary supplementation of beta-hydroxy-beta-methylbutyrate (HMB) and HMB combined with creatine monohydrate (HMBCr) on the muscular strength and endurance, leg power, and anthropometry of elite male rugby league players. The subjects were divided into a control group (n=8), a HMB group (n=11; 3 g/day)) or a HMBCr group (n=11; 12 g/day with 3 g HMB, 3 g Cr, 6 g carbohydrates). Three repetition maximum lifts on bench press, deadlifts, prone row, and shoulder press, maximum chin-up repetitions, 10-second maximal cycle test, body mass, girths, and sum of skinfolds were assessed pre- and postsupplementation. Statistical analysis revealed no effect of HMB or HMBCr on any parameter compared with presupplementation measures or the control group. HMB and HMBCr were concluded to have no ergogenic effect on muscular strength and endurance, leg power, or anthropometry when taken orally by highly trained male athletes over 6 weeks [07214].

The aim of one study was to determine the effects of oral beta-hydroxy-beta-methylbutyrate (HMB) supplementation (3 g/d) on selected components of aerobic performance and body composition of active college students. Subjects were randomly assigned to either an HMB
(n=8) or a placebo (PLA) group (n=8) for a 5-weeks supplementation period during which they underwent interval training 3 times a week on a treadmill. Aerobic-performance components were measured using a respiratory-gas analyzer. Body composition was determined using dual-energy X-ray absorptiometry. After the intervention, there were significant differences between the 2 groups in gains in maximal oxygen consumption (+8% for PLA and +13% for HMB). Regarding body composition, there were no significant differences. The authors concluded that HMB supplementation positively affects selected components of aerobic performance in active college students [07215].

The purpose of one study was to determine the effects of short-term supplementation with the free acid form of beta-hydroxybeta-methylbutyrate (HMB-FA) on indices of muscle damage, protein breakdown, recovery and hormone status following a high-volume resistance training session in trained athletes. A total of twenty resistance-trained males were recruited to participate in a high-volume resistance training session centred on full squats, bench presses and dead lifts. Subjects were randomly assigned to receive either 3 g/d of HMB-FA or a placebo. Immediately before the exercise session and 48 h post-exercise, serum creatine kinase (CK), urinary 3-methylhistadine (3-MH), testosterone, cortisol, and perceived recovery status (PRS) scale measurements were taken. The results showed that CK increased to a greater extent in the placebo (329%) than in the HMB-FA group (104%). There was also a significant change for PRS, which decreased to a greater extent in the placebo than in the HMB-FA group. Muscle protein breakdown, measured by 3-MH analysis, numerically decreased with HMB-FA supplementation and approached significance. There were no acute changes in plasma total or free testosterone, cortisol or C-reactive protein. In conclusion, these results suggest that an HMB-FA supplement given to trained athletes before exercise can blunt increases in muscle damage and prevent declines in perceived readiness to train following a high-volume, muscle-damaging resistance-training session [13524].

Beta-hydroxy-beta-methylbutyrate (HMB), a metabolite of the branched-chain amino acid leucine, is extensively used by athletes and bodybuilders in order to increase strength, muscle mass and exercise performance. It was performed a systematic review of the clinical literature on the effectiveness of HMB supplementation in healthy and pathological conditions (i.e. training programs, aging, acute and chronic diseases, and after bariatric surgery). It was reviewed all clinical trials indexed in Medline that tested HMB supplementation as well as all the experimental data regarding HMB intracellular mechanisms of action. Search terms included: randomized controlled trials, controlled clinical trials, single- and double-blind method, HMB, proteolytic pathways, muscle atrophy, cachexia, and training. It was found out 13 studies testing HMB in healthy young trained subjects, 11 in healthy young untrained subjects, 9 in patients affected by chronic diseases (i.e. cancer, HIV, chronic obstructive pulmonary disease), and 6 in elderly subjects. The indexed studies support that HMB is effective in preventing exercise-related muscle damage in healthy trained and untrained individuals as well as muscle loss during chronic diseases. Most of the selected studies showed the effectiveness of HMB in preventing exercise-related muscle damage in healthy trained and untrained individuals as well as muscle loss during chronic diseases. The usual dose of 3 g/day may be routinely recommended to maintain or improve muscle mass and function in health and disease. The safety profile of HMB is unequivocal. Further, well-designed clinical studies are needed to confirm effectiveness and mode of action of HMB, particularly in pathological conditions [13525].

Beta-hydroxy-beta-methylbutyrate (HMB) is a five-carbon organic acid and a derivative, in vivo, of the essential amino acid leucine (LEU) via its metabolite alpha-ketoisocaproate (alpha-KIC). LEU is a potent anti-catabolic compound and a regulator of protein metabolism. In fact, muscle loss in atrophic conditions can be reversed by LEU supplementation. High
LEU doses counteract muscle proteolysis, while low LEU doses enhance muscle protein synthesis. Almost 80 percent of LEU is normally employed for protein synthesis while the remainder is converted to α-KIC and only a small proportion of LEU (5%) is converted into HMB. LEU may be transaminated to alpha-KIC by two different pathways. The first one consists of transformation of alpha-KIC into HMB by the liver cytosolic enzyme KIC dioxygenase with cytosolic HMB subsequently converted into beta-hydroxy-beta-methylglutaryl-CoA (HMG-CoA) which can be directed for cholesterol synthesis in liver and in muscle. The second pathway consists of liver α-KIC oxidation into isovaleryl-CoA by the mitochondrial branched-chain ketoacid dehydrogenase (BCKD); finally, HMG-CoA is produced by the HMG-CoA synthase. HMB supplementation is claimed to exert positive effects both in healthy (i.e., increasing sport performance as well as reducing exercise-related muscle damage) and pathological conditions (i.e., preserving and increasing muscle mass) perhaps by reducing protein degradation and enhancing protein synthesis. In cachectic conditions (i.e., cancer, sepsis, acquired immunodeficiency syndrome, malnutrition, myopathies, congestive heart failure, renal failure, and chronic obstructive pulmonary disease) skeletal muscle loss is mainly consequent to a shift towards protein breakdown. The proteolytic systems, i.e. the ubiquitin–proteasome and the caspase systems, may initiate myofibrillar proteolysis thus playing a pivotal role in muscle wasting diseases. HMB may influence protein metabolism as shown by changes in proteasome dependent proteolysis and protein synthesis in experimental models. HMB administration to murine cell culture attenuated the tumor factor proteolysis-inducing factor (PIF)-induced activation of protein kinase C (PKC), the subsequent degradation of nuclear factor-kappaB inhibitor-alpha (IκBα) and nuclear accumulation of nuclear factor kappaB (NFκB). HMB also attenuated PIF-induced phosphorylation of p24/44 mitogen-activated protein kinase (MAPK) thereby interfering with proteasome expression. Moreover, expression of the proteasome 20S alpha or beta subunits was reduced by 50 percent as well as the ATPase subunits of the 19S proteasome regulatory subunit and the E2 ubiquitin-conjugating enzyme [13525].

Muscle protein degradation also occurs following activation of caspase-3 and caspase-8 by tumor necrosis factor-alpha (TNF-alpha), interferon-gamma (IFN-gamma), angiotensin II (ANG II) and lipopolysaccharide (LPS). The subsequent autophosphorylation and activation of protein kinase R (PKR) increases reactive oxygen species (ROS) formation via activation of p38 MAPK. ROS formation stimulates NF-κB-mediated induction of the ubiquitin–proteasome pathway. HMB completely attenuated the increase in ROS formation, caspase-3 and caspase-8 activity and PKR autophosphorylation in murine myogenic cell culture. In addition, HMB stimulated protein synthesis in murine myotubes treated with cachectic stimuli (PIF, TNF-alpha/IFN-gamma, ANG II and LPS) through enhancement of the phosphorylation and activation of the mammalian target of rapamycin (mTOR) that, in turn, phosphorlates and activates the 70-kDa ribosomal S6 kinase (p70S6k). Cachectic stimuli also depress protein synthesis by activating PKR with subsequent phosphorylation of eukaryotic initiation factor 2 (eIF2) on the α-subunit and eukaryotic elongation factor 2 (eEF2). HMB attenuated phosphorylation of eEF2 by increasing phosphorylation of mTOR and attenuated phosphorylation of eIF2alpha by preventing autophosphorylation and activation of PKR. In presence of HMB, phosphorylation of the initiation factor 4E-binding protein 1 (4EBP1) was increased; so, the increased association of eukaryotic initiation factor 4E (eIF4E) with eIF4G resulted in the increase of the active eIF4G-eIF4E complex consequently to the reduction of the inactive 4E-BP1-eIF4E complex. Together, these effects active the translation machinery and attenuate PIF-induced depression of protein synthesis in murine myotubes. Thus, HMB attenuation of muscle and body weight loss in experimental cancer cachexia may rely on HMB-mediated decrease in phosphorylated eIF2α and increase in phosphorylated p70S6k and phosphorylated mTOR. The HMB anabolic properties are consistent with muscle hypertrophy and increase in serum insulin levels, expression of mTOR and phosphorylation...
of p70S6k in healthy and sedentary rats. In addition to the anabolic properties above, the increase of muscle glycogen, ATP content and citrate synthase (CS) activity after HMB supplementation to Wistar rats confirm HMB-related changes in oxidative metabolism improving muscle strength generation and performance during intense contractions [13525].

Tumor weight and tumor cell proliferation, ex vivo, were reduced in Walker 256 tumor-bearing rats treated with HMB. These animals also expressed significant increase in IκBα, Bax/Bcl-2 protein expression ratio, phagocytic capacity and H2O2 production rates in blood polymorphonuclear cells, decrease in NFκB p65 subunit content, and an intense infiltration of leukocytes and activated granulocytes in tumor necrotic regions. HMB also decreased the extent of human peripheral blood mononuclear cell proliferation and cytokine production, in vitro. However, when added to human serum-starved myoblasts HMB induced cell proliferation, MyoD (a marker for activated satellite cells) expression, the phosphorylation of mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK), muscle differentiation factors (myogenin and MEF2) expression, an increase in insulin-like growth factor-1 (IGF-1) mRNA levels and accelerated cell fusion. HMB also reduced serum-starvation- or staurosporine-induced cell apoptosis and myonuclear apoptosis during recovery from hind limb suspension-induced muscle fiber atrophy in aged rats, perhaps via a reduction in pro-apoptotic protein (Bax and cleaved caspase-3) levels and increase in anti-apoptotic protein levels (Bcl-2 and Bcl-X). As the myoblast proliferation would be related to the mediation of MAPK/ERK pathway, the promotion of cell differentiation and fusion as well as the prevention of apoptosis would be related to the activation of phosphoinositide 3′-kinase (PI3 K)/Akt pathway. In fact, HMB enhances the association of the p85 subunit of PI3 K with tyrosine-phosphorylated proteins and PI3 K-dependent Akt phosphorylation thereby conditioning cell survival via inhibition of pro-apoptotic proteins. Because HMB administration increased growth hormone (GH) and IGF-I mRNA levels in vitro and in vivo models, GH/IGF-I axis may mediate some of HMB’s effects on myoblasts proliferation, differentiation and survival [13525].

Some of HMB’s reparative effects on damaged tissues may be due to its metabolite HMG-CoA which represents a carbon source for cholesterol synthesis allowing for cell growth, function and regenerative capability of the cell membrane. Therefore, HMB may prevent leakage of muscle enzymes by repairing the cell membranes damaged after physical exercise. In addition, HMB supplementation was recently shown to be effective in attenuating dexamethasone-induced muscle atrophy and to reduce total and low-density lipoprotein cholesterol in humans. Noteworthy, despite the increase in cholesterol synthesis HMB supplementation does not influence serum testosterone levels in healthy males [13525].

Supplementing the diet with the amino acid leucine in combination with resistance training may increase lean body mass (LBM), strength and decrease body fat. Moreover, leucine appears to decrease skeletal muscle soreness following eccentric exercise, and prevent declines in both circulating testosterone and skeletal muscle power following an overreaching cycle. Leucine has been thought to augment adaptations to strength training by acting as the primary signal to activate protein synthesis (e.g. regulation of translation initiation). Additionally, for over three decades this amino acid has been known to exert antiproteolytic effects. However, the effects of leucine on muscle proteolysis are maximized at 10–20 times (5-10 mM/L) the concentration required to maximally stimulate muscle protein synthesis. Thus, it is probable that these effects are partly mediated by the conversion of leucine to a specific metabolite. One strong candidate is the leucine-derived metabolite, beta-hydroxy-beta-methylbutyrate (HMB). In 1996, it was first demonstrated that supplementation with HMB lowered muscle proteolysis following resistance training, and augmented gains in LBM and strength in a dose-dependent manner. Since that time HMB has been studied in a variety of anaerobic and aerobic training conditions. While numerous studies have supported
the efficacy of HMB supplementation for enhancing recovery, LBM, strength, power, and aerobic performance, there have also been conflicting results. The International Society of Sports Nutrition (ISSN) therefore bases the following position stand on a critical analysis of the literature on the use of beta-hydroxy-beta-methylbutyrate (HMB) as a nutritional supplement. The ISSN has concluded the following [13526]:

- HMB can be used to enhance recovery by attenuating exercise induced skeletal muscle damage in trained and untrained populations
- If consuming HMB, an athlete will benefit from consuming the supplement in close proximity to their workout
- HMB appears to be most effective when consumed for 2 weeks prior to an exercise bout
- 38 mg/kg bodymass daily of HMB has been demonstrated to enhance skeletal muscle hypertrophy, strength, and power in untrained and trained populations when the appropriate exercise prescription is utilized
- Currently, two forms of HMB have been used: Calcium HMB (HMB-Ca) and a free acid form of HMB (HMB-FA). HMB-FA may increase plasma absorption and retention of HMB to a greater extent than HMB-Ca. However, research with HMB-FA is in its infancy, and there is not enough research to support whether one form is superior
- HMB has been demonstrated to increase LBM and functionality in elderly, sedentary populations
- HMB ingestion in conjunction with a structured exercise program may result in greater declines in fat mass
- HMB’s mechanisms of action include an inhibition and increase of proteolysis and protein synthesis, respectively
- Chronic consumption of HMB is safe in both young and old populations

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Metabolism

HMB is naturally produced in animals and humans from the amino acid leucine. The first step in production of HMB is the reversible transamination of leucine to alpha-keto-isocaproate (KIC) by the enzyme branched chain amino acid transferase. After leucine is metabolized to
KIC, KIC is either metabolized into isovaleryl-CoA by the enzyme α-ketoacid dehydrogenase in the mitochondria, or into HMB in the cytosol, by the enzyme α-ketoisocaproate dioxygenase. KIC is primarily metabolized into isovaleryl-CoA, with only approximately 5 percent of leucine being converted into HMB. To put this into perspective, an individual would need to consume over 600 g of high quality protein to obtain the amount of leucine (60 grams) necessary to produce the typical 3 g daily dosage of HMB used in human studies. Since consumption of this amount of protein is impractical, HMB is typically increased via dietary supplementation. As a dietary supplement, HMB has been commercially available as a mono-hydrated calcium salt, with the empirical formula Ca(HMB)$_2$$\cdot$H$_2$O (HMB-Ca). The magnitude and rate of appearance of HMB following ingestion is dependent on the dose, and whether or not it is consumed with additional nutrients. It is likely that the addition of glucose slowed gastric emptying, or improved HMB clearance. Recently a new delivery method of HMB, administered as a free acid, has been investigated. The free acid form is called beta-hydroxy-beta-methylbutyric acid and can be designated as HMB-free acid (HMB-FA). The initial research studies have utilized HMB-FA associated with a gel, containing a buffering mechanism (K$_2$CO$_3$) that raises the pH to 4.5. Commercially, HMB has only been available in the calcium salt form (HMB-Ca) as a powder, which has generally been supplemented in capsule form. Moreover, it was previously thought that because calcium dissociated relatively easily from HMB-Ca (10-5 minutes in the gut), there would be no difference in digestion kinetics between HMB-Ca and HMB-FA. The half-life of HMB in plasma when given as HMB-FA and HMB-Ca were found to be approximately three- and two and a half hours, respectively [13526]

**HMB safety**

The safety of HMB has been widely studied. In a study conducted in compliance with Food and Drug Administration Good Laboratory Practice, rats consuming a diet of up to 5 percent HMB-CA for 91 days did not exhibit any adverse effects vis a vis clinical observations, hematology, clinical chemistry or organ weights. In addition, two meta-analyses, one with HMB supplementation alone and another with HMB supplementation combined with glutamine and arginine, have concluded that HMB is safe and does not result in any adverse effects. Evidence to date indicates that that consumption of HMB is safe in both young and old populations; however, future studies examining the effects of HMB on insulin sensitivity in humans are warranted [13526].

**Effects on skeletal muscle damage, protein breakdown, and recovery**

HMB is presently thought to work by speeding regenerative capacity of skeletal muscle following high intensity or prolonged exercise. Researchers have used a number of dependent measures to examine this attribute including serum indices of skeletal muscle damage (creatine kinase [CK], and lactate dehydrogenase [LDH]), and urinary indicators of protein breakdown (3-methyl-histidine [3-MH] and urea nitrogen). Perceived recovery and skeletal muscle soreness have also been investigated following training with, and without HMB supplementation. However, results across studies have not been consistent [13526].

**Effects of training status**

Training status has been a variable that has received a great deal of interest in the literature. When training and/or diet are controlled, a number of studies have demonstrated that HMB can lower indices of skeletal muscle damage and protein breakdown in a dose dependent fashion in untrained populations. However, research indicates that in trained populations it is critical that the exercise stimulus is of adequate intensity and volume to cause skeletal muscle damage. If these conditions are lacking, HMB is not likely to be It has been found
effects of pre exercise administration of HMB-FA to resistance trained athletes on muscle damage, and perceived recovery following a high volume resistance training bout centered around squats, deadlifts, and bench press. HMB-FA supplementation blunted the rise in CK levels and protein breakdown following a workout compared to the placebo group. Moreover, HMB-FA improved the perceived recovery score, suggesting that HMB-FA enhanced recovery [13526].

Duration of supplementation, dose, and timing

The duration, dosage, and timing of HMB supplementation have notably varied in the literature. The effects appeared to be greater when ingesting 3 g of HMB-CA compared to lower doses of the supplement (1.5 g of HMB-CA). Other investigations who have supplemented with HMB-Ca for two or more weeks have generally reported that the supplement lowers indices of skeletal muscle damage and soreness, while those supplementing for shorter periods of time have not. These findings suggest that HMB-Ca supplementation may be optimized when ingestion begins two weeks prior to the onset of a new training period or change in training workload. In the majority of studies, however, researchers have had subjects consume HMB-Ca with breakfast, lunch, and dinner, without any regard to how the supplement is timed relative to exercise. Currently only two studies have reported HMB’s acute effects on skeletal muscle damage and recovery. Collectively these findings lead us to suggest the following: HMB supplementation appears to speed recovery in untrained and trained individuals if the exercise stimulus is high intensity, and/or high volume in nature. For untrained individuals this would likely occur with the introduction of most exercise regimens; however, in a trained population the exercise stimulus will likely need to center on free weights and compound movements. In regards to optimizing HMB supplementation, it appears that HMB has both acute and chronic effects. HMB’s acute effects likely depend upon supplementation pre-exercise. If taking HMB-Ca, the recommendation would be to consume 3 g, at least 60 minutes prior to intense exercise. If consumed with glucose it may need to be taken as long as two hours prior to training. HMB in the HMB-FA form may have an overall faster and greater effect based upon the rise in plasma levels. Thus, athletes could consume the supplement in HMB-FA form 30-60 minutes prior to exercise. Finally, in order to optimize HMB’s chronic effects, the recommendation would be to consume 3 g daily, divided into three equal servings for a minimum of two weeks prior to a potentially damaging skeletal muscle event [13526].

The effects on skeletal muscle hypertrophy in healthy untrained and trained adults

Similar to its reported effects on skeletal muscle damage, a wide range of subject populations (untrained vs resistance trained; male vs female) and training protocols have been examined. Training protocols have varied in duration (10 days to 12 weeks), periodization scheme, and training modalities (machines and free weights vs free weights only). To confound the situation further, some researchers have designed and monitored the resistance-training protocol, while others have left it up to subjects to train on their own. In other cases, subjects have participated in unspecified training protocols reportedly provided by various team coaches or training camps. In addition, studies have provided a variety HMB doses ranging from 1.5 to 6 g daily. Moreover, some studies have supplemented HMB along with creatine monohydrate or arginine and glutamine. Further, some researchers have controlled for diet, while the majority have not. Lastly, the outcome measures for indices of skeletal muscle mass have varied from less accurate indirect indices (skin fold and bioelectrical impedance measures), to dual x-ray absorptiometry (DXA) to determine fat free mass (FFM) and LBM, respectively. Thus, in order to make any overall conclusions on HMB’s effectiveness, the validity and reliability of each of these measures needs to be considered. In both trained and untrained individuals the majority of studies using HMB have
lasted four weeks or less. In untrained individuals supplementation with HMB has been demonstrated to increase FFM, as well as strength in as little as three weeks. These findings are not surprising if HMB operates through speeding recovery of damaged skeletal muscle tissue. In particular, research indicates that the initial weeks of training result in the highest magnitude of damage in an untrained population. Research supports that rate of improvement in novice lifters decline as their training experience increases, however, the majority of studies using HMB were not periodized. For these reasons HMB’s magnitude of effect over a placebo in novices only slightly increases when analyzing results over eight weeks versus three to four weeks utilizing a linear resistance training model. Finally, in untrained individuals it appears that 3 g of HMB per day produces greater gains than 1.5 g of HMB per day; though, 6 g of HMB per day was not shown to further increase HMB’s effectiveness over 3 g of HMB per day. However, only one study has examined a daily dose of 6 g HMB, therefore no definitive recommendation on (upper limit) dosing can be provided until additional research is conducted. According to the available science, the effectiveness of HMB appears to be optimized under conditions of continually changing loading patterns. The rate of adaptation in strength, power, and hypertrophy in trained and untrained individuals markedly differs. For example it was found that 21 weeks of resistance training resulted in 21 and 4 percent increases in strength in untrained and highly strength trained athletes, respectively. In these subjects, HMB appears to augment adaptations following unaccustomed high intensity training protocols. Because the rate of adaptation is markedly slowed in trained populations it is likely that HMB’s effects in this population will be optimized over longer duration protocols (>6 weeks). To date, few studies have examined monitored resistance training in trained athletes. Collectively the findings lead us to the following conclusions [13526]:

- In untrained individuals, HMB can enhance muscle hypertrophy and dynamic strength in as little as three weeks
- For trained individuals it is important to realize that adaptations occur at a slower rate than in untrained individuals. For this reason, HMB will likely be most beneficial over longer training durations (> 6 weeks) in trained individuals.
- HMB supplementation has been demonstrated to result in modest increases in strength during unsupervised, resistance training programs greater than six weeks in duration.
- Presently, available literature suggests 38 mg/kg BM daily, divided into two to three servings provides an adequate amount of HMB to enhance adaptive processes in muscle.

**HMB in athletes training in an energy restricted state**

The effects of HMB supplementation on regenerative capacity and fat metabolism make it a unique candidate for a number of special situations in which skeletal muscle wasting is indicated. One situation in particular concerns caloric (energy) restriction. Restricting calories prior to competition is commonly used by bodybuilders and those in weight-classified sports. However, research demonstrates that calorie restriction can cause decreases in lean mass and exercise performance. Findings suggest that individuals who are moderately calorically restricted may augment fat loss and prevent declines in LBM by supplementing with HMB [13526]

**HMB supplementation in youth and adolescent populations**

Research in infants using HMB has yet to be done using human models. However, there is recent epigenetic data in animal models to suggest that HMB given during pregnancy can result in prenatal programming of skeletal muscle tissue. Specifically, maternal
supplementation of HMB during pregnancy resulted in greater weight and lean mass gain in piglets than those not under maternal treatment. Moreover, research in growing, pre-adolescent rats suggests that HMB supplementation was able to stimulate skeletal muscle hypertrophy in the extensor digitorum longus and soleus muscles. There is little research examining the effects of HMB in human adolescent populations. However, this population may be an ideal model for HMB supplementation as resources required to augment their training adaptations compete with resources needed for normal growth of organs, bones, and muscle tissue. No changes in hormone status (testosterone, cortisol, IGF-1, growth hormone) or inflammatory mediators (IL-6 and IL-1 receptor antagonist) occurred with HMB-Ca supplementation [13526].

**HMB supplementation in aging and masters athletes**

Skeletal muscle loss is a part of the aging process and approximately 30 percent of skeletal muscle mass is lost between the 5th and 8th decades of life. This reduction in skeletal muscle mass occurs for several reasons, including maintaining a sedentary lifestyle, malnutrition, insulin resistance, oxidative stress, and alterations in skeletal muscle metabolism and repair. In addition, the elderly exhibit impaired anabolic and anti-catabolic responsiveness to resistance exercise and amino acid feeding, termed anabolic resistance. Anabolic resistance can be overcome by supplementation of leucine, and it has been hypothesized that this may be due to the conversion of leucine to HMB. These data suggest a potential benefit of HMB supplementation in aging individuals. HMB supplementation was able to prevent the loss of skeletal muscle fiber size in very old as compared to young rats. These studies suggest that HMB alone can decrease body fat and increase skeletal muscle mass and strength in aging populations. The efficacy of HMB supplementation in conjunction with a strength-training program has also been investigated in aging populations. Considering the paucity of available research on HMB ingestion and resistance exercise in older adults, additional investigations are warranted [13526].

**HMB improves indices of aerobic performance, fat loss, and energy metabolism**

While HMB has long been touted as an anti-catabolic agent that may aid recovery and improve performance, recent evidence has identified additional metabolic benefits of HMB supplementation related to energy metabolism. The mechanisms for these benefits of HMB on aerobic performance and fat loss are poorly understood. However, recent evidence demonstrated that HMB supplementation improves fatty acid oxidation, adenosine monophosphate kinase (AMPK), Sirt1 (Silent information regulator transcripts) and Sirt3 activity in 3T3-L1 adipocytes and in skeletal muscle cells. Consequently, this recent evidence has shown that HMB supplementation increases mitochondrial biogenesis and fat oxidation. Exactly how HMB induces changes in Sirt proteins, AMPK, and mitochondria remains unclear. However, these results could have implications for obesity, insulin resistance, and diabetes, as well as for athletes seeking to improve body composition and aerobic performance [13526].

**Proposed mechanisms of action**

Skeletal muscle protein turnover is the product of skeletal muscle protein synthesis and skeletal muscle protein degradation. When protein synthesis exceeds protein degradation, there is a net synthesis of skeletal muscle protein. However, when protein degradation exceeds protein synthesis, there is a net breakdown of skeletal muscle protein. HMB has been shown to affect both protein synthesis and degradation pathways in skeletal muscle. HMB has been shown to stimulate protein synthesis in skeletal muscle. This has been hypothesized to occur through stimulation of mTOR, a protein kinase responsive to
mechanical, hormonal, and nutritional stimuli. Mammalian target of rapamycin has a central role in the control of cell growth, primarily by controlling mRNA translation efficiency. Indeed, previous studies have observed that HMB supplementation increases phosphorylation of mTOR and its downstream targets ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor-4 binding protein-1. The growth hormone (GH) and insulin-like growth factor 1 (IGF-1) axis may also play a key role in the stimulation of protein synthesis, and it is possible HMB may stimulate protein synthesis through changes in the activity of GH/IGF-1 axis. It is possible that the GH/IGF-1 axis signaling may require a large change in plasma HMB levels. At this point, it is not clear whether a threshold response to a specific concentration of plasma HMB exists [13526].

Skeletal muscle regeneration

In addition to the direct effects on protein synthesis, HMB has been shown to affect satellite cells in skeletal muscle. When myoblasts were cultured with HMB, the mRNA expression of myogenic regulatory factor D (MyoD), a marker of cell proliferation, was increased in a dose responsive manner. Moreover, the addition of various concentrations of HMB (25-100 microg/mL) to the culture medium for 24 hours resulted in a marked increase of myogenin and myocyte enhancer factor-2 (MEF2) expression, markers of cell differentiation. As a result, there was a significant increase in the number of cells, suggesting a direct action of HMB upon the proliferation and differentiation of myoblasts [13526].

Skeletal muscle proteolysis

Skeletal muscle proteolysis is increased in catabolic states such as fasting, immobilization, aging, and disease. HMB has been shown to decrease skeletal muscle protein degradation both in vitro and in vivo. The ubiquitin-proteasome system is an energy-dependent proteolytic system that degrades intracellular proteins. The activity of this pathway is significantly increased in conditions of exacerbated skeletal muscle catabolism, such as fasting, immobilization, bed rest and disease. Therefore, inhibition of this proteolytic system could explain the attenuation of skeletal muscle protein losses observed during treatment with HMB [13526].

HMB in young trained subjects

Several studies tested the metabolic effects of HMB supplementation to young trained subjects. Augmentation of muscle mass, strength and anaerobic properties with no effects on aerobic capacity and hormonal and inflammatory mediators during the initial phases of the training season by HMB administration to elite adolescent volleyball players. Pre-exercise HMB supplementation significantly lowered lactate dehydrogenase (LDH) and creatine kinase (CK) activities thus mitigating exercise-induced muscle damage. Maximal oxygen consumption (\(\text{VO}_{2\text{peak}}\)) and lactate accumulation peak were unaffected by HMB administration to endurance-trained cyclists, but HMB resulted in a greater time to reach \(\text{VO}_{2\text{peak}}\) and an increase in the onset of blood lactate accumulation (OBLA). Also HMB free acid supplementation was shown to improve markers of exercise-induced muscle damage and ameliorated recovery in resistance-trained men. On the contrary, a trivial effect on combined averaged strength measures, FM, BM and FFM was observed with HMB supplementation during resistance training. Moreover, no short duration HMB-related ergogenic benefit during high-intensity training, nor significant differences between supplemental and placebo groups as for anaerobic power, CK, cortisol, testosterone and myoglobin levels were shown. Additionally, three different studies failed to demonstrate HMB-related influences on muscle function and damage parameters during a strenuous
exercise program [13525].

Three g/day of HMB supplementation to untrained subjects promoted a greater increase in FFM and peak isometric torque while a larger dose (6 g/day) produced a greater increase in peak isokinetic torque without compromising liver function, renal function, immune system or lipid profile during resistance training. HMB supplementation increased maximal oxygen consumption (VO\textsubscript{2max}) and respiratory compensation point (RCP), i.e. components of aerobic performance, suggesting increased capacity to tolerate intense physical activity over a long period of time. Subjects receiving HMB as a free acid gel presented quicker and greater plasma concentrations and improved clearance of plasma HMB versus those receiving calcium HMB (CaHMB) gelatine capsules. This new gel formulation could improve HMB availability and efficacy to tissue. Short-term HMB supplementation had no effect on the severity of swelling, muscle soreness or the subsequent recovery of muscle torque measures following an eccentric exercise bout [13525].

**HMB in trained versus untrained subjects**

A significant HMB-related increase in FFM and weight lifted with resistance training was associated with reduced 3-methylhistidine (3-MH), i.e. a marker of exercise-induced muscle proteolysis, and CK, a marker of muscle damage has been noticed demonstrated a greater increase in upper body strength when combined with an exercise program; plasma CK levels tended to be suppressed in the HMB group. Moreover, the HMB group tended to increase FFM and decrease percent fat. These studies found no significant influence of prior training status (trained versus untrained) or gender on the effects of HMB on body composition, strength and muscle damage [13525].

**HMB mixed with other molecules in young trained subjects**

In two different studies, no ergogenic effect of HMB or HMB/CR supplementation on muscular strength, endurance, leg power, aerobic and anaerobic ability or anthropometry were measured in rugby players over a 6-weeks resistance training program. There were no adverse effects on indices of health although blood bicarbonate, blood monocytes and lymphocytes that were significantly different from the control but still within normal ranges [13525].

**HMB mixed with other molecules in young untrained subjects**

An amino acid-based formula (containing HMB, ARG, GLN, taurine and dextrose) significantly augmented the positive benefits (improved FFM, muscle strength and muscle power) by 12 weeks of resistance exercise when compared with the placebo group. This nutritional formula promoted increased resting and exercise-induced testosterone and resting GH concentrations and reduced pre-exercise cortisol concentrations thereby improving the anabolic-to-catabolic hormone ratio. Moreover, it was associated with a decrease in plasma CK, malondialdehyde (MDA, i.e. a marker of free radical formation and lipid peroxidation which are responsible for exercise-induced membrane disruption) and percent body fat. Another study revealed a significant effect in FFM gains with CR supplementation and a trend with HBM supplementation during a weight-training program; CR- and HBM-related effects on FFM were additive. Creatine, HMB, CR/HMB supplementation caused accumulative strength increases above the placebo group. HMB alone significantly suppressed the exercise-induced rise in serum CK but CR antagonized this effect. HMB also decreased urine urea nitrogen (UUN) and blood urea nitrogen (BUN) which were not affected by CR supplementation. HMB/KIC supplementation for 14 days before a single bout of
eccentrically biased resistance exercise significantly attenuated the CK response, the percentage decrement in concentric one repetition maximum (1RM), the percentage increase in limb girth and delayed onset muscle soreness (DOMS) that are signs and symptoms of exercise-induced muscle damage [13525].

Dose and safety of treatment

Normally, an individual metabolizes 60 g of L-LEU to obtain 3 g of HMB but a 70 kg person produces 0.2-0.4 g of HMB per day, depending on the dose of LEU in the diet. The dose of HMB provided to the treatment groups varied between trials but in the majority 3 g/day of HMB were provided. Up to 3 g of HMB could improve strength and FFM and reduce muscle damage in a dose dependent manner, while higher doses, such as 6 g, had no additional benefits. The 3 g (or 38 mg/kg of body weight per day) dose may be an optimal dosage but too few studies have investigated the efficacy of higher dosages of HMB. Currently, all studies reported no adverse effects from the daily use of HMB. Renal, hematological, hepatic, endocrine functions were not negatively affected as a result of the intake of the nutrient supplement as well as any marker of tissue damage. BUN increase was reported in only two studies. This effect was possibly caused by the additional nitrogen consumed or perhaps ureagenesis induced by arginine and glutamine supplementation. It was determined the influence of oral glucose ingestion upon the time course kinetics of HMB in humans. That study reported no major differences between concomitant glucose/HMB and HMB supplementation alone, except for the longer interval required for the HMB concentration to peak and the longer plasma half-life when HMB was consumed with glucose. All studies employed capsule, drink or powder form of CaHMB salt. Two studies used HMB free acid form, which produced a different plasma kinetic profile. CaHMB has a potential role as a phosphate binder in uremia as demonstrated in vitro. It might contribute to the management of hyperphosphatemia in uremic patients. In one recent experimental study it was observed that HMB supplementation induced hyper-insulinemia [13525].

HMB versus glucocorticoids

Glucocorticoid (GC) excess alters glucose homeostasis and promotes modifications in murinometric and anthropometric parameters in rodents and humans, respectively. beta-Hydroxy-beta-methylbutyrate (HMB), a leucine metabolite, has been proposed as a nutritional strategy for preventing muscle wasting, but few data regarding its effects on glucose homeostasis are available. Here, it was analyzed whether the effects of GC excess on glucose homeostasis may be attenuated or exacerbated by the concomitant ingestion of HMB. Adult Wistar rats (90-days-old) were assigned to four groups: (1) vehicle treated (Ctl), (2) dexamethasone (DEX) treated (Dex), (3) HMB treated (Hmb), and (4) DEX plus HMB treated (DexHmb). Dex groups received DEX (1 mg/kg body weight (BW), intraperitoneal) for 5 consecutive days. HMB groups ingested HMB (320 mg/kg BW, oral gavage) for the same 5 days. HMB ingestion did not attenuate the effects of DEX on food intake and body weight loss, changes in masses of several organs, insulin resistance, and glucose intolerance. In fact, in DexHmb rats, there was increased fasting glycemia and exacerbated glucose intolerance with the main effect attributed to DEX treatment. HMB exerted no attenuating effect on plasma triacylglycerol levels from DexHmb rats, but it seems to attenuate the lipolysis induced by bwta-adrenergic stimulation (20 micromol/L isoproterenol) in fragments of retroperitoneal adipose tissue from DexHmb rats. Therefore, HMB does not attenuate the diabetogenic characteristics of GC excess. In fact, the data suggest that HMB may exacerbate GC-induced glucose intolerance [13527].
**Attenuation of muscle loss during sustained energy deficit**

To investigate the efficacy and underlying mechanisms of beta-hydroxy-beta-methylbutyrate (HMB) on body composition, muscle mass and physical performance under catabolic versus normal training conditions mice were divided into four groups (n=10/group): (1) ALT=ad libitum+trained (1 h/d for 3 d/wk); (2) ALTH=ALT+HMB (0.5 g/kg BW/d); (3) C=calorie restricted (-30%)+trained (6 h/d, 6 d/wk); and (4) CH=C+HMB. Repeated in vivo assessments included body composition, grip strength and sensorimotor coordination before and after the experimental protocol, while in vitro analyses included muscle wet weights, expression of selected genes and proteins regulating muscle mass, and myofiber cross-sectional area. ALTH had greater lean mass than ALT and sensorimotor function increased in ALTH, but decreased in ALT under normal training conditions. Grip strength decreased only in C, but was maintained in CH. Gastrocnemius mass and myofiber CSA were greater in CH than C following catabolic conditions. Gastrocnemius atrogin-1 mRNA expression was elevated in C but not in CH compared to all other groups whereas atrogin-1 protein levels showed no significant changes. It was concluded that HMB improves body composition and sensorimotor function during normal training and attenuates muscle mass and strength loss during catabolic conditions [13528].

**Influence of HMB on protein synthesis**

Many low-birth-weight infants experience failure to thrive. The amino acid leucine stimulates protein synthesis in skeletal muscle of the neonate, but less is known about the effects of the leucine metabolite beta-hydroxy-beta-methylbutyrate (HMB). To determine the effects of HMB on protein synthesis and the regulation of translation initiation and degradation pathways, overnight-fasted neonatal pigs were infused with HMB at 0, 20, 100, or 400 micromol/kg body wt/h for 1 h (HMB 0, HMB 20, HMB 100, or HMB 400). Plasma HMB concentrations increased with infusion and were 10, 98, 316, and 1,400 nmol/ml in the HMB 0, HMB 20, HMB 100, and HMB 400 pigs. Protein synthesis rates in the longissimus dorsi (LD), gastrocnemius, soleus, and diaphragm muscles, lung, and spleen were greater in HMB 20 than in HMB 0, and in the LD were greater in HMB 100 than in HMB 0. HMB 400 had no effect on protein synthesis. Eukaryotic initiation factor (eIF)4E-eIF4G complex formation and ribosomal protein S6 kinase-1 and 4E-binding protein-1 phosphorylation increased in LD, gastrocnemius, and soleus muscles with HMB 20 and HMB 100 and in diaphragm with HMB 20. Phosphorylation of eIF2α and elongation factor 2 and expression of system A transporter (SNAT2), system L transporter (LAT1), muscle RING finger 1 protein (MuRF1), muscle atrophy F-box (atrogin-1), and microtubule-associated protein light chain 3 (LC3-II) were unchanged. Results suggest that supplemental HMB enhances protein synthesis in skeletal muscle of neonates by stimulating translation initiation [13529].

**Influence on free acids**

The purpose of this study was to examine the effect of beta-hydroxy-beta-methylbutyrate-free acid (HMB-FA) and cold-water immersion (CWI) on circulating concentrations of TNF-alpha and monocyte TNF-alpha receptor 1 (TNFR1) expression. Forty resistance-trained men (22 ± 2 years) were randomized into four groups [placebo (PL), HMB-FA, CWI, and HMB-FA-CWI] and performed an acute, intense exercise protocol (four sets of up to 10 repetitions of the squat, dead lift, and split squat). Participants also performed four sets of up to 10 repetitions of the squat at 24 and 48 h following the initial exercise bout. Blood was sampled before exercise (PRE), immediately postexercise (IP), and 30 min, 24 h, and 48 h postexercise (30P, 24P, and 48P, respectively). Circulating TNF-α was assayed, and TNFR1 expression on CD14+ monocytes was measured by flow cytometry. The exercise protocol
significantly elevated TNF-alpha in only PL and CWI IP. Mean percent changes show that TNF-α significantly increased from PRE to IP for only PL and CWI groups, whereas the percent change of TNF-alpha for HMB-FA and HMB-FA-CWI was not significant. TNFR1 expression was elevated in PL and CWI at 30P compared with PRE, whereas both HMB-FA-treated groups did not increase significantly. In conclusion, HMB-FA attenuated circulating TNF-alpha IP and TNFR1 expression during recovery compared with PL and CWI. HMB-FA supplementation may attenuate the initial immune response to intense exercise, which may reduce recovery time following intense exercise [13530].

Laboratory testing

For the quantification of beta-hydroxybutyrate (BHB) and beta-hydroxy-beta-methylbutyrate (HMB) in human whole blood, a method using hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC-MS/MS) was developed, which does not require chemical modification of the analytes. Samples were deproteinised by a mixture of methanol and acetonitrile, and the extracts were cleaned-up using both polymeric strong cation exchange and strong anion exchange sorbents. The analytes and their structural isomers were separated using a column with a zwitterionic stationary phase. Isotope dilution of both analytes was used for quantitative analysis. Separation of BHB from isobaric interferences was achieved through chromatography. The relative intra-laboratory reproducibility standard deviations were better than 10 percent for blood samples at concentration levels of 10-20 microM BHB and 1 microM HMB and better than 5 percent at concentration levels 10 times higher. The mean true extraction recoveries were close to 100 percent. The trueness expressed as the relative bias of test results was within ±5 % at concentration levels of 10-1000 microM BHB and 1-20 microM HMB. The lower limits of quantification were estimated to be 3 microM for BHB and 0.4 microM for HMB. A simple and highly sensitive and selective HILIC-MS/MS method was developed that is suitable for the quantification of BHB and HMB in whole blood [13531].

For the quantification of beta-hydroxybutyrate (BHB) and beta-hydroxy-beta-methylbutyrate (HMB) in human whole blood, a method using hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC-MS/MS) was developed, which does not require chemical modification of the analytes. Samples were deproteinised by a mixture of methanol and acetonitrile, and the extracts were cleaned-up using both polymeric strong cation exchange and strong anion exchange sorbents. The analytes and their structural isomers were separated using a column with a zwitterionic stationary phase. Isotope dilution of both analytes was used for quantitative analysis. Separation of BHB from isobaric interferences was achieved through chromatography. The relative intra-laboratory reproducibility standard deviations were better than 10 percent for blood samples at concentration levels of 10-20 microM BHB and 1 microM HMB and better than 5 percent at concentration levels 10 times higher. The mean true extraction recoveries were close to 100 percent. The trueness expressed as the relative bias of test results was within ±5 percent at concentration levels of 10-1000 microM BHB and 1-20 microM HMB. The lower limits of quantification were estimated to be 3 microM for BHB and 0.4 microM for HMB [13532].

Experimental

To investigate the efficacy and underlying mechanisms of beta-hydroxy-beta-methylbutyrate (HMB) on body composition, muscle mass and physical performance under catabolic versus normal training conditions. Mice were divided into four groups (n=10/group): 1) ALT=ad libitum+trained (1h/d for 3d/wk); 2) ALTH=ALT+HMB (0.5g/kg BW/d); 3) C=calorie restricted (-30 %)+trained (6h/d, 6d/wk); and 4) CH=C+HMB. Repeated in vivo assessments included
body composition, grip strength and sensorimotor coordination before and after the experimental protocol, while in vitro analyses included muscle wet weights, expression of selected genes and proteins regulating muscle mass, and myofiber cross-sectional area. ALTH had greater lean mass than ALT and sensorimotor function increased in ALTH, but decreased in ALT under normal training conditions. Grip strength decreased only in C, but was maintained in CH. Gastrocnemius mass and myofiber CSA were greater in CH than C following catabolic conditions. Gastrocnemius atrogin-1 mRNA expression was elevated in C but not in CH compared to all other groups whereas atrogin-1 protein levels showed no significant changes. It was concluded that HMB improves body composition and sensorimotor function during normal training and attenuates muscle mass and strength loss during catabolic conditions [13533].

**Summarised aspects of HMB in sports**

High intensity resistance training is essential for athletes seeking to add strength and hypertrophy. However, high intensity resistance training that results in skeletal muscle damage may take a number of days to recover from; in this case, overall training frequency may be reduced. HMB appears to speed recovery from high intensity exercise. These effects on skeletal muscle damage appear to be reliant on the timing of HMB relative to exercise, the form of HMB, the length of time HMB was supplemented prior to exercise, the dosage taken, as well as the training status of the population of interest. In particular, the supplement should be taken at 1-2 grams 30-60 minutes prior to exercise if consuming HMB-FA, and 60-120 minutes prior to exercise if consuming HMB-Ca. Finally, it is likely that HMB will work ideally if consumed at a dosage of 3 grams for two weeks prior to a high intensity bout that induces muscle damage [13526].

**1,4-Butanediol (1,4-BD)**

1,4-Butanediol (1,4-BD) is a gamma-hydroxybutyrate (GHB) pro-drug, with multiple commercial uses, and a drug of abuse. Although there are case reports of a withdrawal syndrome following 1,4-BD use, no studies have evaluated the physical dependence potential of 1,4-BD and characterized the time course of withdrawal. Vehicle and then 1,4-BD were administered continuously 24 h/day via intragastric catheters in male baboons (Papio anubis, n=3). Dosing was initiated at 100 mg/kg and increased by 100 mg/kg/day to 400 mg/kg. After a stabilization period, doses of 500 and then 600 mg/kg/day were each maintained for 3-4 weeks. Plasma levels of 1,4-BD and GHB were determined for each dose condition. Physical dependence was assessed via administration of a GABA-B antagonist (precipitated withdrawal test) during administration of the 600 mg/kg dose and via abrupt termination of chronic 1,4-BD administration (spontaneous withdrawal test). Outcome measures included the number of food pellets earned, performance on a fine-motor task, observed behaviors, and plasma levels of GHB and 1,4-BD. Following maintenance of 1,4-BD 600 mg/kg for 3 weeks, the number of food pellets earned was significantly decreased. At the end of chronic 1,4-BD dosing, the levels of GHB in plasma ranged from 1290 to 2300 μmol/L and levels of 1,4-BD in plasma ranged from 13.1 to 37.9 μmol/L. Signs of physical dependence were observed following precipitated and spontaneous withdrawal tests. Seizures were not observed. These data indicate chronic 1,4-BD produced physical dependence in baboons and the withdrawal syndrome can be characterized as mild to intermediate [13537].

**gamma-Oryzanol and ferulic acid**

1491
Gamma-oryzanol, a mixture of a plant sterol and ferulic acid ester first isolated from rice bran oil in the early 1950s, has since been found to be in the lipid fraction of many other plants, and can be identified in various vegetable oils and products. The phytosterol base, structurally similar to cholesterol, has been promoted as having cholesterol-lowering and testosterone-enhancing activities, but these have not been substantiated in humans. Like other plant sterols, gamma-oryzanol is poorly absorbed from the gastrointestinal tract. However, ferulic acid is well absorbed and has been proposed to be the active agent in gamma-oryzanol, with activities including antioxidant properties. As a result, ferulic acid has been isolated and marketed as a separate supplement. Despite a lack of evidence or consistent explanation of mechanisms underpinning claimed benefits, gamma-oryzanol and ferulic acid have been marketed to, and used by, body builders and strength-training athletes in the hope of boosting strength, increasing muscle gain, reducing body fat, speeding recovery and reducing postexercise soreness. These supplements also claim to promote endorphin release. Only three studies, of which only one was published in a peer-reviewed publication, have tested these supplement claims on athletic performance. A double-blind, placebo-controlled study of 22 recreationally weight-trained male college students involved a 9-week resistance exercise training protocol, combined with a daily intake of 500 mg of gamma-oryzanol or placebo. Improvements in muscle strength and vertical jump power, increased body mass and decreased skinfold thickness were observed in both groups. A significant decrease in resting testosterone and cortisol concentrations was also observed at the end of the testing period in both groups, with no other alterations in hormone, lipid or blood parameters. These findings support the benefits of a training programme but do not find additional benefits from gamma-oryzanol ingestion in terms of functional gains or alterations of various hormone concentrations. An abstract describes a double-blind crossover study of six highly trained male distance runners who were supplemented with either placebo or 50 mg of ferulate daily for 3 weeks. Although workouts increased blood concentrations of cortisol, testosterone and β-endorphins, there were no differences in the response between ferulate and placebo trials, with the exception of an increase in postexercise beta-endorphin concentrations during some sessions in the final week of intensified training. A further study reported in abstract form described a multicentre double-blind, placebo-controlled trial in which weight lifters received either a placebo or ferulate treatment (15 mg twice daily) for 8 weeks. Using small numbers of participants, the authors reported a significant increase in body weight and shoulder press strength in the supplemented group (n=6) compared with the placebo group (n=4), but no differences in leg and chest strength. In summary, the effects of supplementation with gamma-oryzanol and ferulic acid on athletic performance have not been well studied, and there is no current evidence to support their use in sport [10527].

Gamma-Oryzanol, a mixture of a plant sterol and ferulic acid ester, also marketed to body builders and strength training athletes, was used in hopes of boosting strength, increasing muscle gain, reducing body fat, speeding recovery and reducing postexercise soreness. It was first isolated from rice bran oil in the early 1950s, has since been found to be in the lipid fraction of many other plants, and can be identified in various vegetable oils and products. The phytosterol base, structurally similar to cholesterol, has been promoted as having cholesterol-lowering and testosterone-enhancing activities, but these have not been substantiated in humans. Like other plant sterols, gamma-oryzanol is poorly absorbed from the gastrointestinal tract. However, ferulic acid is well absorbed and has been proposed to be the active agent in gamma-oryzanol, with activities including antioxidant properties. As a result, ferulic acid has been isolated and marketed as a separate supplement. Despite a lack of evidence or consistent explanation of mechanisms underpinning claimed benefits, gamma-oryzanol and ferulic acid have been marketed to, and used by, body builders and strength-training athletes in the hope of boosting strength, increasing muscle gain, reducing body fat, speeding recovery and reducing postexercise soreness. These supplements also
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Gamma-oryzanol, a mixture of ferulic acid esters of sterol and triterpene alcohols occurs in rice (Oryza stavia) bran oil at a level of 1-2 percent. Gamma oryzanol is used in the belief that they may elicit anabolic effects via increased testosterone production. Although limited, the available research does not support an ergogenic effect of gamma-oryzanol supplementation in humans. Compared to a placebo group, weight-trained males who consumed 500 mg/day of gamma-oryzanol for 9 weeks of resistance exercise training experienced no significant differences in circulating concentrations of hormones (testosterone, human growth hormone, insulin), nor was there any improvement in muscular power or strength [06297].
ALCOHOL

The use of alcohol is often intimately associated with sport. As well as providing a source of energy, alcohol (ethanol) has metabolic, cardiovascular, thermoregulatory, and neuromuscular actions that may affect exercise performance. Strength is minimally affected, and performance impairments depend on the dose of alcohol and subject habituation to alcohol intake, exercise duration, environmental conditions, and other factors. Central nervous system function is impaired at high doses, resulting in decrements in cognitive function and motor skill, as well as behavioral changes that may have adverse effects on performance. Effects may persist for hours after intoxication [06223].

Alcohol is not an essential part of the human diet, but various alcohols, of which the only one of quantitative significance is ethanol, are regularly consumed by a large part of the world’s population. In addition to being a significant source of energy, providing about 7 kcal (29 kJ) per gram, ethanol has a number of effects that have implications for athletic performance. There are reasons to believe that acute alcohol intake may impair performance of endurance exercise because of effects on metabolic, cardiovascular, and thermoregulatory function. Performance of skilled tasks may be impaired because of effects on reaction time, fine motor control, levels of arousal, and judgment. All of these elements are important components of sports performance, but there are relatively few well-controlled studies of performance itself. The limited amount of experimental evidence is at least in part because institutional ethics review boards are reluctant to condone the administration of high doses of ethanol to volunteers. There have been rather few studies in this area in recent years, and there is an urgent need for further studies, particularly investigations involving measurement of sport-specific performance. In the past few years there appear to have been more review articles published on the effects of alcohol on sporting performance than original research papers. There is evidence that at least some groups of athletes consume more alcohol than nonathletes, but separating fact from anecdote can be difficult. Reports that include only average intakes can be misleading, as binge drinking is commonly found in some team sports, and some athletes will abstain from alcohol in training but will drink copious amounts after competition [06223].

Alcohol consumption control policies at U.S. intercollegiate sports events, and their association with student drinking, were assessed using data from a 2001 nationally representative survey of students and administrators (n=7,261 students, n=117 colleges). Alcohol was available to sports event attendees through in-stadium sales, tailgating parties, and allowing spectators to bring in alcohol. Policies varied by college, with fewer restrictions at large public schools with NCAA Division I athletics. Permitting alcohol at tailgate parties was associated with more students drinking at sports events. Future research should evaluate whether enacting policy restrictions can reduce drinking and related problems at intercollegiate sports events [10504].

The potential influence of alcohol consumption on endogenous steroids has already been described in the literature. In those studies the ethanol level after ingestion was monitored using its concentration in blood, urine or saliva. Corresponding methods are not commonly used in anti-doping laboratories. Ethylglucuronide (EtG), which can be easily detected by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), appears to be a more suitable parameter for this purpose. It is slowly excreted into the urine and indicates alcohol intake for a much longer period than blood or urinary alcohol and it is therefore routinely used for legal purposes as an alcohol consumption marker. In pharmacokinetic studies that aimed to establish calculation models after ethanol intake, the formation of EtG
was observed to coincide with elevated urinary testosterone/epitestosterone (T/E) ratios. Similarly, large amounts of EtG were correlated with abnormal steroid profiles found in routine doping samples. In this pilot study, several cases with significantly elevated T/E ratios were associated with urinary EtG concentrations higher than 50 microg/mL. These findings confirmed recent intake of ethanol in considerable amounts and suggest a connection to changes in specific steroid profile parameters. Owing to the ease with which procedures to determine EtG can be carried out, and the potential for such procedures to be introduced into screening schemes, the inclusion of this marker in the final evaluation of suspicious outliers in T/E ratio longitudinal studies would seem to be very useful [09301].

One study investigated the relationships among sports-specific factors, perceived peer drinking, and alcohol-related behaviors among adolescents, examining sex differences in the relationship between perceived peer drinking and alcohol-related behaviors. A questionnaire assessing demographics, sports-specific factors, perceived peer drinking, and alcohol-related behaviors was administered among 378 adolescents who were mostly male (76 %) and non-Hispanic black (70 %). Varsity sports participants reported significantly higher levels of perceived peer drinking compared to those who participated in sports at other levels (0.64, 95 % confidence interval 0.28 to 0.99). Participants in both sports offering team- and individual-level competition reported greater perceived peer drinking, compared to those who only participated in individual sports. Perceived peer drinking was associated with alcohol-related behaviors and there were no significant differences between males and females in this relationship. Suggestions for future research include examining factors contributing to the low prevalence of drinking behaviors, and investigating factors related to sports that impact perceived peer drinking and alcohol-related behaviors [09302].

One study investigated the effects of acute moderate alcohol intake on muscular performance during recovery from eccentric exercise-induced muscle damage. Eleven healthy males performed 300 maximal eccentric contractions of the quadriceps muscles of one leg on an isokinetic dynamometer. They then consumed a beverage containing 1g/kg bodyweight ethanol (as vodka and orange juice). On another occasion they performed an equivalent bout of eccentric exercise on the contralateral leg after which they consumed an isocaloric quantity of orange juice. Measurement of maximal isokinetic (concentric and eccentric) and isometric torque produced across the knee, plasma creatine kinase (CK) concentrations and muscle soreness were made before and at 36 and 60 h following each exercise bout. All measures of muscle performance were significantly reduced at 36 and 60h post-exercise compared to pre-exercise measures. The greatest decreases in peak strength were observed at 36 h with losses of 12, 28 and 19 percent occurring for orange juice isometric, concentric, and eccentric contractions, respectively. However, peak strength loss was significantly greater in alcohol group with the same performance measures decreasing by 34 percent, 40 percent and 34 percent, respectively. Post-exercise plasma creatine kinase activity and ratings of muscle soreness were not different between conditions. These results indicate that consumption of even moderate amounts of alcohol following eccentric-based exercise magnifies the normally observed losses in dynamic and static strength. Therefore, to minimise exercise related losses in muscle function and expedite recovery, participants in sports involving eccentric muscle work should avoid alcohol-containing beverages in the post-event period [09303].

One study was conducted to examine the effect of consuming a dilute alcohol solution (weak beer) on urine production in euhydrated and hypohydrated individuals. Twelve males completed an intermittent cycle protocol in hot, humid conditions to dehydrate by 1.9 ± 0.3 percent body mass in the evening. Twice they were then fed and rehydrated, while on two other occasions they were fed the same meal but remained hypohydrated. The following morning they were given 1 l of beer to drink. On two occasions the beer was alcohol-free,
while on the other two occasions the same beer contained 4 percent ethanol. Participants remained in the laboratory for monitoring over the subsequent 4 h. Blood and urine samples were taken prior to dehydration, prior to drink administration and once every hour of the monitoring period. No difference existed in the volume of urine produced between the alcohol and non-alcohol beer when hypohydrated, but there was a significant difference when euhydrated. Unsurprisingly, more urine was produced on both euhydrated trials than either of the hypohydrated trials. Blood alcohol concentration was elevated 1 h after drinking on the alcohol trials. Serum osmolality was higher 1 h after drinking on both the alcohol trials than on their non-alcohol, equivalent hydration trials. These results suggest that the diuretic action of alcohol is blunted when the body is hypohydrated [10394].

Sports participation, while offering numerous developmental benefits for adolescents, has been associated with alcohol use in prior research. However, the relationship between sports participation and alcohol use among adolescents remains unclear, particularly how research design elements impact evidence of this relationship. It was reviewed the evidence regarding sports participation and alcohol use among adolescents, with a focus on examining the potential impact of research design elements on this evidence. Studies were assessed for eligibility and coded based on research design elements including: study design, sampling method, sample size, and measures of sports participation and alcohol use. Fifty-four studies were assessed for eligibility, 29 of which were included in the review. Nearly two-thirds used a cross-sectional design and a random sampling method, with sample sizes ranging from 178 to 50,168 adolescents (median = 1,769). Sixteen studies used a categorical measure of sports participation, while 7 applied an index-type measure and 6 employed some other measure of sports participation. Most studies assessed alcohol-related behaviors (n=18) through categorical measures, while only 6 applied frequency only measures of alcohol use, 1 study applied quantity only measures, and 3 studies used quantity and frequency measures. It was concluded that sports participation has been defined and measured in various ways, most of which do not differentiate between interscholastic and community-based contexts, confounding this relationship. Stronger measures of both sports participation and alcohol use need to be applied in future studies to advance our understanding of this relationship among youths [11224].

Moderate, acute alcohol consumption after eccentric exercise has been shown to magnify the muscular weakness that is typically associated with exercise-induced muscle damage (EIMD). As it is not known whether this effect is dose-dependent, the aim of this study was to investigate the effect of a low dose of alcohol on EIMD-related losses in muscular performance. Ten healthy males performed 300 maximal eccentric contractions of the quadriceps muscles of one leg on an isokinetic dynamometer. They then consumed either a beverage containing 0.5 g of alcohol per kg bodyweight (as vodka and orange juice) or an isocaloric, isovolumetric non-alcoholic beverage. At least 2 weeks later, they performed an equivalent bout of eccentric exercise on the contralateral leg after which they consumed the other beverage. Measurement of peak and average peak isokinetic (concentric and eccentric) and isometric torque produced by the quadriceps was made before and 36 and 60 h post-exercise. Significant decreases in all measures of muscular performance were observed over time under both conditions; however, no difference between treatments was evident at any of the measured time points. Therefore, consumption of a low dose of alcohol after damaging exercise appears to have no effect on the loss of force associated with strenuous eccentric exercise [11225].

Alcohol expectancies have been associated with drinking behaviors among college students. Few studies, however, have focused on researcher-labeled "positive" and "negative" expectancies as well as the valuations (i.e. desirability) of these expectancies. Moreover, research on the correlates of heavy drinking among female college athletes remain relatively
sparse, despite the prevalence of elevated alcohol use in this population. It was examined the associations of expectancies and valuations with frequency of heavy drinking and context-specific drinking behaviors. The sample consisted of 145 female college athletes (mean age 20 years) who completed self-report surveys and indicated alcohol use in the past 30 days. Regression analyses indicated that favorable valuations of negative expectancies were related to heavy drinking, and that valuations accounted for significant proportions of variance in the model. Elevated endorsement of negative expectancies was also associated with the perceived likelihood of heavy use in convivial and personal-social drinking contexts, and favorable valuations of these expectancies accounted for significant variance in these models. These findings highlight the relevance of negative expectancies and valuations with respect to heavy drinking and context-specific drinking behaviors among female college athletes. The perception of “negative” effects of alcohol as “positive” could help explain the high rates of problematic drinking among female athletes [07205].

The authors examined alcohol use among students involved in recreational sports. To the authors' knowledge, this was the first study of alcohol use in which researchers separate recreational sports participants from intercollegiate athletes and examine them as a separate group of interest. The authors generated a random sample of 494 students from the undergraduate population at a 4-year university. They used a Web-based survey to collect data and stratified and weighted the sample by residence status to more accurately reflect the population. Students who participated in recreational sports used alcohol at a greater frequency and intensity than did those who were not involved in recreational sports across a variety of measures, even alongside other variables, including race, sex, and Greek membership. It was concluded on the basis of these findings, that alcohol consumption among those participating in recreational sports appears to be an area worthy of consideration in future research [07206].

Community-based sporting clubs in Australia are often sites of unregulated, problematic and unsafe drinking. The Good Sports program, initiated in Victoria in 2001, offers such clubs a model of incremental change designed to eliminate harmful drinking practices and establish safer norms of alcohol use. The Good Sports model was developed in situ over a period of 5 years and is currently operating in 1600 sporting clubs across Australia. It has strong face validity and is supported by sporting organizations and key stakeholders including public health, law enforcement, road safety, and local government. The article outlined the model, explains early evaluation results, and identifies challenges for the future [07207].

One study examined alcohol use among college students, focusing on variation in binge drinking based on involvement in athletics. Prior research indicates that college students who participate in athletics are more likely to report binge drinking than are students who are not involved in athletics. However, existing research has not offered an explanation why college athletes are at a greater risk for binge drinking. Using data from the 1999 Harvard School of Public Health College Alcohol Study, a national study examining substance use and other health risk behaviors of college students in the United States, the current research examines social norms as a possible source of the elevated levels of binge drinking among college athletes. Findings indicate that athletes are more likely to report binge drinking, in part, because they view alcohol use as being more normative [07208].

The aim of one study was to explore the associations of physical activity with smoking and alcohol consumption. It examined whether these associations are due to people participating in organized sports (the sport hypothesis), and/or reflect the concentration of drinking and smoking in manual occupational groups (the occupation hypothesis). Data from the 2003 Health Survey for England (n=11,617) were analyzed from a multilevel perspective. Four models were specified to examine the variation of heavy drinking, smoking, sports activity,
and occupational activity across different sociodemographic groups; and four sets of analyses further explored the associations of sports and occupational activity with heavy drinking and smoking. Some support was found for both the sport and occupation hypothesis. Sports activity and heavy drinking were more prevalent among sportsclub members, and occupational activity and heavy drinking were more prevalent among manual occupational groups. Sportsclub membership accounted for some of the association between sports activity and heavy drinking; and occupational position partly accounted for the association between occupational activity and heavy drinking. The occupation hypothesis is the more likely explanation for the association between physical activity and smoking. The study shows that it is worthwhile to distinguish between different types of physical activity; and that multiple processes underlie the clustering of health behaviors [07209].

The relationship between perceived alcohol use among peers and personal alcohol use was assessed in a sample of collegiate athletes. Data were collected on 109 intercollegiate athletes during the competitive season and 119 athletes during the off-season at a large, state university in the Northeastern United States. Participants were asked to estimate the normative alcohol use of four reference groups (closest athlete friend, closest nonathlete friend, typical athlete, and typical nonathlete). Results of both in-season and off-season analyses indicated that athletes estimated that others consumed more drinks per week than they did, and perceptions of these social norms predicted personal use. Although the typical athlete norm emerged as the strongest predictor of personal alcohol use, the relative strength of the relationships between individual behavior and the athlete and nonathlete norms varied according to seasonal status. Results have implications for the content and timing of prevention and intervention programs aimed at reducing high-risk alcohol use among intercollegiate athletes [07210].

To examine the relationship between athlete drinking motives and hazardous drinking across differing levels of sporting participation (club vs elite-provincial vs elite-international). Data from 1214 New Zealand sportspeople was collected. It was assessed hazardous drinking with the WHO's AUDIT questionnaire and sportspeople's psychosocial reasons for drinking with the ADS. Level of sporting participation (club/social, provincial/state, or international/olympic level) was also assessed. Hazardous drinking behaviours differed across levels of sporting participation, with elite-provincial sportspeople showing the highest level of hazardous drinking, club/social sportspeople the next highest and elite-international sportspeople the lowest. Sportspeople who placed a greater emphasis on drinking as a reward for participating in their sports tended to display more hazardous drinking behaviours, but other ADS motives differed over level of sporting participation. Elite-provincial sportspeople and elite-international sportspeople placed more emphasis on drinking as a way to cope with the stresses of participating in their sports. A relationship between team/group motives and AUDIT scores was fully mediated by positive reinforcement motives, and partially mediated by stress-related coping motives. These findings have implications for alcohol education programs targeted at sportspeople and sport administration, and may help improve the efficacy and focus of intervention programs [07211].

Chronic alcohol abuse has adverse effects on skeletal muscle, and reduced muscle strength is frequently seen in chronic alcoholics. In this study the acute effects of moderate alcohol intoxication on motor performance was evaluated in 19 non-alcoholic healthy subjects (10 women, 9 men). A randomised double-blinded placebo controlled design was applied to subjects receiving alcohol in juice and pure juice at two separate test periods. Isokinetic and isometric muscle strength and endurance were determined before, during, 24 and 48 h after the ingestion of alcohol in juice and juice (placebo). To detect a reduced activation of the central motor pathways superimposed external electrical stimulations during voluntary contractions were applied. Creatine kinase (CK) was measured to detect any alcohol-
induced changes in sarcolemmal integrity. No change was seen in isokinetic as well as in isometric muscle performance during or following the alcohol intoxication as compared to the non-alcoholic condition. Also, no central activation failure was observed. No significant difference in CK increment was observed comparing the alcoholic- and non-alcoholic condition. In conclusion, a single episode of moderate alcohol intoxication (1.4 g/L) does not impair motor performance, and no accelerated exercise-induced muscle damage is seen.

The objective of one study is to describe energy drink consumption and health behaviors among college students attending a predominantly minority university. Undergraduate and graduate students attending a private, minority-serving university were invited to participate in an online survey between 2009 and 2010. Out of 2,500 students, 407 participated yielding a response of 16 percent. Analysis assessed energy drink consumption as well as participation in sport activities and high-risk behaviors. Energy drink consumption is significantly related with drinking alcohol to inebriation and driving and to riding with a drunk driver. Athletes were more likely to engage in drinking alcohol to inebriation and driving. Energy drink consumption is a common practice among racial minority university students. Tailored health promotion strategies and interventions are needed to address misconceptions of energy drink and alcohol mixing.

Excessive alcohol consumption is responsible for considerable harm from chronic disease and injury. Within most developed countries, members of sporting clubs participate in at-risk alcohol consumption at levels above that of communities generally. There has been limited research investigating the predictors of at-risk alcohol consumption in sporting settings, particularly at the non-elite level. The purpose of this study was to examine the association between the alcohol management practices and characteristics of community football clubs and at-risk alcohol consumption by club members. A cross-sectional survey of community football club management representatives and members was conducted. Logistic regression analysis (adjusting for clustering by club) was used to determine the association between the alcohol management practices (including alcohol management policy, alcohol-related sponsorship, availability of low- and non-alcoholic drinks, and alcohol-related promotions, awards and prizes) and characteristics (football code, size and location) of sporting clubs and at-risk alcohol consumption by club members. Members of clubs that served alcohol to intoxicated people (OR 2.23) conducted “happy hour” promotions (OR 2.84) or provided alcohol-only awards and prizes (OR 1.80) were at significantly greater odds of consuming alcohol at risky levels than members of clubs that did not have such alcohol management practices. At-risk alcohol consumption was also more likely among members of clubs with less than 150 players compared with larger clubs (OR 1.45) and amongst members of particular football codes. The findings of the study suggest a need and opportunity for the implementation of alcohol harm reduction strategies targeting specific alcohol management practices at community football clubs.

Little is known about how adolescents’ peer relations might alter whether sport participation is associated with alcohol use. Consistent with social learning theory, we found that sport participation was protective against alcohol use if these peers had low alcohol use, but athletes were likely to use alcohol if their sport friends and teammates had high alcohol use. Interestingly, those with no or low sport participation seemed to emulate the alcohol use of their non-sport friends, whereas adolescents in a high number of sports had elevated alcohol use regardless of their non-sport friends’ alcohol use.

Heavy episodic drinking (HED) (consumption of five or more drinks on the same occasion) among adolescents is related to several problems and partaking in sport or physical activities.
has been suggested as an option to prevent or reduce alcohol consumption among this population. The aim of this study was to investigate the relationship between soccer practice and heavy episodic drinking among high school students from Brazil. Data were obtained from a cross-sectional study among a representative sample of public and private high school students from all Brazilian state capitals (n=19,132). Only students aged from 14 to 18 who reported having taken part in soccer practice, other team sports or non-practicing sports in the last month were included. Characteristics of sport practice (frequency and motivation) and HED in the last month (type of drink; where and with whom they drank; frequency of HED) were also considered. Regression models were controlled for sociodemographic variables. For all groups studied most of the students reported drinking beer, with friends and at nightclubs or bars. Soccer practice was associated to HED when compared to non-practicing sports and to other team sports. Compared to other team sports, playing soccer for pleasure or profession, but not for keep fit or health reasons, were more associated to HED. Frequency of soccer practice from 1 to 5 days per month and 20 or more days per month, but not from 6 to 19 days per month, were also more associated to HED. It was concluded that the relationship between soccer and HED appears to be particularly stronger than in other team sports among adolescents in Brazil. Induced sociability of team sports practice cannot be assumed as the main reason for HED among soccer players. Possibly these results reflect the importance of a strong cultural association between soccer and beer in Brazil and these findings should be integrated to future prevention or intervention programs [13542].

Alcohol (ethanol; referred to as alcohol or EtOH) is and has been one the most commonly consumed and abused drugs for a substantial period in human history. Alcohol is a dependence-producing drug which affects a host of organ systems and one that increases the risk of morbidity and mortality from different diseases when abused. Indeed, some authors have suggested that alcohol is harmful similar to drugs such as heroin or cocaine and that excessive alcohol consumption is a serious world-wide health risk. Although the detrimental effects of alcohol on human physiology are well known, even elite athletes consume alcohol. When looking at the effects of alcohol on overall health, it is, however, important to distinguish between chronic, moderate alcohol consumption versus alcohol abuse. Alcohol consumption and sport have been inextricably linked since ancient times when alcohol was considered the elixir of life. To some extent that may be true, given that a substantial body of epidemiological evidence shows that moderate ingestion of alcohol may reduce the risk of cardiovascular disease. The link between alcohol consumption and mortality is subject to a J-shaped curve i.e. improved longevity with moderate consumption with increasing intake resulting in greater mortality risk. Indeed, dietary guidelines from the American Heart Association recommend moderation of alcohol intake as it has been associated with a lower risk of cardiovascular events [13008].

Alcohol use is fairly widespread among the athletic population with 88 percent of intercollegiate American athletes reporting the use of alcohol. It is also noteworthy that many athletes consume alcohol prior to sports events. However, it is important to note that scientific evidence suggests that the consumption of alcohol has some detrimental effects on exercise performance. It is fairly obvious that it is unlikely for competitive athletes to be alcohol abusers and most performance studies have focused on the acute ergolytic effects of EtOH consumption. The chronic studies merely reinforce the point that EtOH is profoundly ergolytic in the long term setting. They also serve to reinforce that chronic EtOH use can be toxic to cardiac and skeletal muscle [13008].
Epidemiology

Sport participation can play an important and positive role in the health and development of children and youth. One area that has recently been receiving greater attention is the role that sport participation might play in preventing drug and alcohol use among youth. The current study is a systematic review of 17 longitudinal studies examining the relationship between sport participation and alcohol and drug use among adolescents. Results indicated that sport participation is associated with alcohol use, with 82 percent of the included studies (14/17) showing a significant positive relationship. Sport participation, however, appears to be related to reduced illicit drug use, especially use of non-cannabis related drugs. Eighty percent of the studies found sport participation associated with decreased illicit drug use, while 50 percent of the studies found negative association between sport participation and marijuana use. Further investigation revealed that participation in sports reduced the risk of overall illicit drug use, but particularly during high school; suggesting that this may be a critical period to reduce or prevent the use of drugs through sport. Future research must better understand what conditions are necessary for sport participation to have beneficial outcomes in terms of preventing alcohol and/or illicit drug use. This has been absent in the extent literature and will be central to intervention efforts in this area [13543].

Motives and attitudes for alcohol use by athletes

One study aimed to explore the mediating effects of conscientiousness and alexithymia in the relationship between parental attachment style and alcohol use in a large sample of athletic young people. Participants included 434 French sport sciences students. Alcohol use, parental attachment style, conscientiousness and alexithymia were assessed. The hypotheses were tested by using regression and bootstrapping mediation analyses. Maternal insecure attachment style is positively associated with alcohol use. The current study highlights a multiple pathway in this relationship. The results reveal the mediating effect of low conscientiousness and alexithymia between maternal insecure attachment and alcohol use. Athletes' alcohol use seems to be the result of a complex association of underlying psychological factors [13544].

Intercollegiate athletes report greater alcohol consumption and more alcohol-related problems than their non-athlete peers. Although college athletes share many of the same problems faced by non-athletes, there are some consequences that are unique to athletes. Studies have demonstrated that alcohol negatively affects athletic performance including increased dehydration, impeded muscle recovery, and increased risk for injury. Beyond risk factors for alcohol misuse that may affect college students in general, research has begun to examine risk factors that are unique to collegiate athletes. For example, research has found that off-season status, the leadership role, and athlete-specific drinking motives are associated with increased alcohol use. Given these findings, it is possible that other athlete-specific variables influence alcohol misuse. One such variable may be sport achievement orientation. The purpose of one study was to examine the relationship between sport achievement orientation and alcohol outcomes. Given previous research regarding seasonal status and gender, these variables were examined as moderators. Varsity athletes (n=263) completed the Sport Orientation Questionnaire, which assesses sport-related achievement orientation on three scales (Competitiveness, Win Orientation, and Goal Orientation). In addition, participants completed measures of alcohol use and alcohol-related problems. Results indicated that Competitiveness, Win Orientation, and Goal Orientation were all significantly associated with alcohol use, but not alcohol-related problems. Moreover, these
relationships were moderated by seasonal status and gender. These interactions, clinical implications, and limitations are discussed [13545].

Mechanism of action

Chronic alcohol abuse has significant detrimental effects on the human cardiac muscle and one of the putative mechanisms via which alcohol may induce cardiac dysfunction is through the induction of increased oxidative stress. Interestingly, exercise training blunted the oxidative damage observed in a rat model of chronic alcohol consumption. The authors suggest that exercise training results in an up-regulation of cardiac antioxidants which may in turn reduce the deleterious effects of chronic alcohol induced oxidative stress. Acute alcohol use can also have effects on cardiovascular determinants of exercise performance. It was examined the effects of acute alcohol administration on left ventricular contractility using echocardiography and found that alcohol had a significant depressant effect on the myocardium. Specifically, acute alcohol consumption resulted in a decreased end-systolic pressure-dimension slope and reduced velocity of myocardial fiber shortening. Alcohol has significant effects on skeletal muscle substrate utilization during exercise. Specifically, it has been demonstrated that alcohol consumption decreases glucose and amino acid utilization, which can have adverse effects on energy supply to exercising muscle. Ethanol consumption induces hypoglycemia and decreases glucose appearance in plasma by decreasing hepatic gluconeogenesis. Ethanol administration has been shown to worsen skeletal muscle determinants of exercise performance such as muscle capillary density and muscle fiber cross-sectional area. It was shown in vitro that alcohol can inhibit sarcolemmal calcium channel actions thereby potentially impair excitation-contraction coupling and diminishing muscular performance. Muscle capillary density is closely related to the oxidative capacity of skeletal muscle. Greater capillary density also allows for a greater surface area for gas exchange and delivery of metabolic substrates. Long term alcohol consumption is associated with the development of alcoholic myopathy which is characterized by a reduction in skeletal muscle capillarity. Exercise training, however, appears to attenuate these adverse changes [13008].

Epidemiological data suggest that moderate alcohol consumption is associated with many salutary changes in blood coagulation and fibrinolysis. However, compelling experimental evidence is lacking and often conflicting. Alcohol can also lead to significant post-exercise perturbations in levels of clotting factors. Moderate post-exercise alcohol consumption resulted in significantly elevated levels of Factor VIII at 5 and 22 hours during the post-exercise milieu. Both circulating catecholamine and vasopressin levels have been implicated in up-regulation of Factor VIII. These factors in turn, have been implicated in the pathogenesis of atherosclerosis in prospective studies. Alcohol and exercise may interact with each other to induce disorders in platelet aggregation which are associated with an elevated risk of cardiovascular and cerebrovascular events. Alcohol intoxication has been shown to be linked to cerebrovascular infarctions in a few case-control studies. However, the exact pathological mechanisms of the same are currently unknown. Alcohol consumption following athletic participation is commonly observed and may be associated with disorders in platelet aggregation. It was demonstrated that alcohol ingestion following exercise was associated with a marked increase in platelet count 1-hour following exercise. Platelet aggregation induced by adenosine diphosphate was found to be reduced when exercise was followed by alcohol consumption. Thus, it appears that ingestion of a moderate quantity of alcohol is associated with delayed recovery of platelet aggregation. It is important to note however, that the performance impact of ethanol consumption mediated post-exercise coagulopathy is unknown. Acute alcohol consumption is associated with the deterioration of psychomotor skills. A significant difference exists in injury rates between drinkers and non-
drinkers in athletic populations. Athletes that consume alcohol at least once a week have almost a 2-fold higher risk of injury compared to non-drinkers and this elevated injury rate holds true for the majority of sports examined [13008].

Alcohol may also interfere with the body’s ability to recover from injury. It was also examined the effects of 1 g/kg body weight alcohol consumption on recovery from eccentric exercise-induced muscle injury. The authors measured peak and average peak isokinetic and isometric torque produced by the quadriceps. Alcohol consumption was associated with significantly greater decreases in torque production (40-44 %) 36 hours into recovery. The authors concluded that the consumption of a moderate amount of alcohol after damaging exercise magnified the loss of muscle force production potential. Finally, there is some evidence to suggest that chronic alcohol consumption may result in a positive energy balance and a potentially obesogenic state. It has also been reported that chronic alcohol consumption did not result in a reduction in lipid intake and that a dietary regimen that provided a large fraction of energy in the form of alcohol increased the risk for a positive energy balance in a free-living state. It appears that alcohol may have a fat-sparing effect similar to that of carbohydrates and may cause fat gain. It has also been suggested that alcohol could result in excess body fat gain especially in the upper body. There is some evidence to suggest that obese individuals may be more susceptible to weight gain and the hyperlipidemic effects of alcohol consumption as compared to lean individuals. This is in contrast to epidemiological studies that report a negative association between alcohol consumption and adiposity. This may be explained by the induction of unregulated futile metabolic cycles that appear to significantly aid in the disposal of excess calories. In general, it appears that the effects of alcohol on body weight are controversial and it is likely that moderate consumption of alcohol that replaces calories from carbohydrates and fat is unlikely to result in weight gain [13008].

**Studies on alcohol and performance**

Many of the studies on the effects of alcohol on athletic performance suffer from one of more defects. Subject numbers are often small, and the lack of a definite outcome may be the result of underpowered studies. The doses of alcohol that have been used in many studies are small relative to those reported to be consumed by athletes. The reluctance of investigators to induce inebriation in their subjects, and the reluctance of ethics committees to sanction such studies because of safety concerns, are understandable, but this nonetheless limits the application of the studies to the real world of sport. It is not always clear that subjects have been fully familiarized with experimental protocols prior to measurements of performance, especially when tasks involving complex skills have been used. These factors may lead to performance changes that are of significance to the athlete being dismissed as being of no consequence [06223].

Alcohol has direct and demonstrable effects on athletic performance which may be due to its cardiovascular effects. It has been demonstrated significant ergolytic effects in short and middle distance runners. The adverse effects were most prominent in events that were more dependent upon aerobic capacity (i.e. 800 m and 1500 m). However there were no adverse effects observed in the 100 m run. Similarly, it was demonstrated a significant impairment in 60-minute treadmill time trial performance in trained athletes following alcohol ingestion. Heart rate and VO\textsubscript{2} were significantly elevated in subjects after alcohol ingestion and only 1 out of 4 subjects could complete the run. This may be due, in part, to the significant hypoglycemia that the subjects experienced at the 60 minute time point. Acute alcohol consumption may also result in small but significant reductions in sustained power output. It
was also demonstrated that acute ethanol ingestion resulted in a about 4 percent reduction in average cycling power output during a 60 minute time-trial. However, the detrimental effect of alcohol on aerobic performance seems to be dose dependent with a threshold of 20 mmol/L, upon which the effect becomes significant. Consumption of alcohol 24 hours prior to exercise has also been shown to reduce aerobic performance by 11 percent. Some studies have failed to show reductions in exercise performance following alcohol consumption. However, this may be due to limitations in their experimental design as well as type of exercise used. For instance, the lack of ergolytic effects on exercise performance during bicycle ergometer testing may be due to the fact that using a stationary bicycle ergometer does not place significant motor coordination demands as compared to running [13008].

Alcohol can also impair recovery following exercise. It has been shown that alcohol can impair glycogen resynthesis after prolonged cycling. More importantly, alcohol seems to interfere with protein synthesis, most likely by suppressing the mTOR pathway, which is critical to facilitate repair and hypertrophy following strength training. Although alcohol seems to have an overall ergolytic effect on exercise performance, a well-known athlete reported consumed alcohol before a ski downhill competition. This is potentially a dangerous precedent especially since alcohol has significant effects on executive functions such as judgment and decision making while also having significant adverse effects on motor control and coordination. This has to be considered in sports requiring a high level of boldness (downhill skiing, downhill mountain biking) and may have implications for pre-participation testing. In addition, the effects of an alcohol-induced hangover are poorly quantified and as such are relatively unknown and subject to further investigation in humans [13008].

The American College of Sports Medicine (ACSM) concludes in its position stand that alcohol consumption adversely affects psychomotor skills and exercise performance while resulting in minimal reductions in maximal oxygen consumption. The ACSM also recommends that if an athlete must consume alcohol, that they should refrain from alcohol consumption for at least 48 hours prior to competition. Chronic alcohol abuse is associated with significant impairments in cardiac and skeletal muscle. It also slows post-exercise recovery by inhibiting protein synthesis. Thus, alcohol is a uniformly ergolytic agent that has significant detrimental effects on exercise performance and that use of the same during competitive activity should be minimized for athlete safety and so as to maximize athletic performance [13008].

<table>
<thead>
<tr>
<th>Acute effect</th>
<th>Effect on performance</th>
<th>Alcohol dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced left ventricular contractility</td>
<td>Increased left ventricular dimensions and worsened left ventricular dysfunction. Negative effects on cardiac output</td>
<td>1.15 g/kg body weight</td>
</tr>
<tr>
<td>Decreased performance possibly due to reduced myocardial contractility and reduced lung ventilation</td>
<td>Increased 800 m-1500 m run time</td>
<td>0.05-0.1 mg/mL blood alcohol concentration</td>
</tr>
<tr>
<td>Hypoglycemia at 60-minute time-point</td>
<td>Reduced 60-min, treadmill time-trial performance</td>
<td>25 mL in 150 mL grapefruit juice</td>
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Practical effects of energy drinks on alcohol priming
While several researchers have proposed a causal relationship between alcohol mixed with energy drink (AmED) consumption and subsequent alcohol intake, there is a dearth of research exploring the potential mechanisms underpinning this association. Marczinski and colleagues (in press) report the results of a double-blind, placebo-controlled, between-groups study assessing whether an initial AmED dose primes an increased motivation to drink relative to alcohol alone. Participants (n=80) received either alcohol (0.91 ml/kg vodka), energy drink (ED; 1.82 ml/kg Red Bull®), AmED, or a placebo beverage and then self-reported their motivation to drink via the Desire-for-Drug scale. Subjective ratings of "desire more alcohol" were significantly higher than predrink in the placebo, alcohol, and AmED conditions, with this effect apparent at more time points in the AmED condition. While it was concluded that EDs may increase alcohol priming, between-condition analyses revealed that ratings did not differ significantly in AmED and alcohol conditions, with moderate magnitude treatment effects at most, and ratings of desire generally closer to 0 (absence of desire) than 100 (very much desire) [13546].

Effects of ethanol on glycogen metabolism

Resynthesis of the glycogen stores in liver and muscle is one of the key goals of athletes after intensive training or competition, and it is well recognized that ethanol has a variety of effects on carbohydrate metabolism in skeletal muscle and liver. Much of the available evidence comes from older studies using animal models, showing that synthesis of glycogen in both liver and oxidative skeletal muscle is impaired in the presence of even relatively low levels of ethanol, though there seems to be no effect on type 2 muscle fibers. There is also evidence of impaired hepatic glucose output in the presence of even low doses of ethanol. This may be of particular concern during prolonged, moderate-intensity exercise when glucose output from the liver is an important source of energy. It has been reported the effects of alcohol intake on muscle glycogen storage in humans during recovery from a prolonged cycling bout that resulted in a substantial reduction of carbohydrate stores. Subjects undertook three different diets following their glycogen-depleting exercise: a high-carbohydrate diet intended to optimize recovery, an alcohol displacement diet (reduced carbohydrate, in which about 210 g of dietary carbohydrate was replaced by about 120 g alcohol), and an alcohol plus carbohydrate diet (about 120 g alcohol added to the high-carbohydrate diet). Muscle glycogen storage was significantly reduced (by almost 50 % at 8 hours and about 16 % at 24 hours) on the alcohol displacement diet when the amount of carbohydrate provided by the diet was less than optimal. When the high carbohydrate diet was eaten, however, there was no clear evidence that alcohol intake caused a reduction in muscle glycogen storage; there was a small, not statistically significant reduction at 8 hours and no effect at all at 24 hours. It should be noted that there was a large variability of the responses of different subjects, and it may well be that some individuals will be unable to effectively replenish their glycogen reserves between daily training sessions if substantial amounts of alcohol are consumed. Even if there is not a direct metabolic effect of ethanol on glycogen storage when dietary carbohydrate intake is high, it is likely that athletes who consume large amounts of alcohol during the recovery period after training or competition will have a reduced carbohydrate intake, either as a result of a decreased total (nonalcohol) energy intake or because of a failure to follow the recommended eating strategies at this time [06223].

Hydration and thermoregulatory function

The diuretic action of ethanol is well recognized. This effect as been quantified to an excess
urine production of about 10 mL for each gram of ethanol ingested. It was also reported that the diuretic action was greatly attenuated, or eliminated altogether, in individuals who were already hypohydrated. Subsequent studies showed that alcohol acts via suppression of the release of antidiuretic hormone from the pituitary gland. However, alcohol has a negligible diuretic effect when consumed in dilute solution following a moderate level of hypohydration induced by exercise in the heat. There appears to be no difference in recovery from dehydration whether the rehydration beverage is alcohol-free or contains up to 2 percent alcohol, but drinks containing 4 percent alcohol tend to delay the recovery process by promoting urine loss. Based on this it is apparent that concentrated alcohol solutions will result in net negative fluid balance; a 25-mL measure of spirits (40 % ethanol) contains 10 mL of alcohol and 15 mL of water, resulting in a urine output of about 100 mL and net negative water balance of 85 mL. Ingestion of large volumes of dilute alcohol will result in a water diuresis, but should promote restoration of fluid balance after sweat loss provided that there is also an intake of sodium, which is essential for restoration of euhydration [06223].

The 1982 American College of Sports Medicine Position Stand on the use of alcohol in sports identified perturbations of thermoregulatory mechanisms, especially in the cold, as one of the reasons to abstain from alcohol prior to exercise. Small doses of ethanol given to human volunteers at rest in the absence of a thermal stress have very little effect on body temperature, but large doses administered before exercise at low ambient temperatures result in increased peripheral vasodilatation and a marked fall in core temperature. In combination with the concomitant fall in blood glucose concentration that is normally observed in this situation, there is clear potential for an adverse effect on performance. It was shown that ingestion of alcohol (2.5 mL/kg) prior to prolonged (3 hours) exercise in the cold resulted in increased heat loss, though this effect was somewhat attenuated by co-ingestion of glucose. In animal studies, administration of alcohol to animals exposed to ambient temperatures both above and below the thermoneutral zone, has shown that alcohol acts to impair adaptation to both heat and cold [06223].

In an animal study, rats were given 0, 4, 8, 12, or 16 percent ethanol as the sole source of drinking water for 14 days. Time to fatigue in treadmill running in the heat (35°C) of rats drinking 4 percent ethanol was similar to that of rats consuming water (32 vs 33 min, respectively), but running time of rats drinking 16 percent ethanol was reduced [06223].

**Effects of acute alcohol consumption on neuromuscular function.**

Voluntary and electrically stimulated muscular performance was examined to identify the effects of acute alcohol consumption on neuromuscular function in the presence and absence of exercise-induced muscle damage (EIMD). After initial neuromuscular performance measures were made, 12 subjects completed a bout of eccentric exercise (EX) using the quadriceps muscles of 1 leg while the remaining 11 subjects did not exercise (NX). Subjects then consumed either an alcoholic beverage containing 1 g/kg body weight (ALC) or a nonalcoholic beverage (OJ). On another occasion the contralateral leg of both groups was tested and those in the EX group performed an equivalent bout of eccentric exercise after which the other beverage was consumed. Measurements of neuromuscular function were made pre-exercise and 36 and 60 h post-beverage consumption. Creatine kinase (CK) was measured pre-exercise and at 12, 36, and 60 h. Significantly greater decrements in maximal voluntary isometric contraction were observed with EX ALC at 36 and 60 h compared with EX OJ, and no change was seen in the NX group. Significant decreases in voluntary activation were observed at 36 h and 60 h with EX ALC only. Elevations in CK were observed at all posteccentric exercise time points under both EX OJ and ALC. No
change in electromyography or low-frequency fatigue was observed under either treatment in either group. These results suggest that decreased neural drive appears to contribute to alcohol's effect on the magnitude of EIMD-related decrements in voluntary force generation [12344].

**Effects of acute alcohol consumption on recovery from a match**

In one study, it was investigated the effects of acute post-exercise alcohol consumption on measures of physical performance, creatine kinase, and immunoendocrine function in the 48 h following a rugby game simulation. Ten male senior rugby union players completed a rugby game simulation after which they consumed either 1 g of alcohol per kilogram of body mass or a non-alcoholic control beverage. Agility, 15 m sprint, countermovement jump, and scrumming performance were assessed pre-simulation and 24 and 48 h post-simulation. White blood cell count, testosterone, cortisol, and creatine kinase were measured before the simulation and 30 min, 12, 24, 36, and 48 h after the simulation. One week after the first trial, participants completed the second simulation after which the other beverage was consumed. The acute consumption of alcohol after a rugby game simulation negatively affected countermovement jump performance in the days following the simulation. No differences between treatments were observed for the other criterion measures made in this study. In conclusion, after 80 min of a simulated rugby game, the consumption of 1 g of alcohol per kg body mass negatively impacts lower body vertical power output. However, performance of tasks requiring repeated maximal muscular effort is not affected [12345].

One study compared the effects of "normal" post-game behaviour with recommended behaviour on physical performance in the days after a rugby union game. Additionally, the habitual drinking habits of rugby players were identified. After a rugby game, 26 players were split by team into a customary behaviour group (CB), who carried out their usual post-game behaviour, or recommended behaviour group (RB), whose diet and activity was controlled in the hours after the game. Counter movement jump, lower-body strength, repeated sprint ability, CK and hydration status were measured prior to and in the days after the game. Twenty-four hour behaviour recall questionnaires were completed throughout the trial period. The Alcohol Use Disorders Identification Test (AUDIT) was also administered to participants. Compared to baseline values, large volumes of alcohol and a loss in sleep was reported by the CB group in the hours after the game. Measures of performance and hydration status were unchanged over time and no difference was evident between groups. Total AUDIT scores for all participants were 17.75. CK was elevated in the days following the game. It was concluded that physical performance was not affected by participation in a game of senior club rugby, irrespective of post-game behaviour and possible muscle damage. AUDIT scores indicate that club rugby players may be at risk of serious alcohol related harm, with post-game binge drinking likely to be a major contributor [13548].

**Effects on brain's white matter**

Chronic alcohol abuse is related to numerous deleterious neurobiological consequences, including loss of gray matter, damage to white matter (WM), and impairment of cognitive and motor functions. Aerobic exercise has been demonstrated to slow cognitive decline and decrease the negative neural changes resulting from normal aging and from several diseases. It is possible that exercise may also prevent or repair alcohol-related neurological damage. One study tested the hypothesis that aerobic exercise protects WM in anterior and dorsal areas of the brain from damage related to heavy alcohol use. Sixty individuals underwent a diffusion tensor imaging session and completed measures of alcohol
consumption, loss of control over drinking, and aerobic exercise participation. Analyses examined the relationship of exercise, alcohol, and their interaction to fractional anisotropy (FA) in the superior longitudinal fasciculus (SLF), external capsule (EC), superior and anterior corona radiata, and fornix. The relationship of aerobic exercise and alcohol consumption to self-reported loss of control over drinking were also examined. A significant interaction was observed between alcohol consumption and aerobic exercise participation on FA in the SLF and EC. In the models examining loss of control over drinking, a significant interaction between aerobic exercise and alcohol consumption was observed, such that alcohol consumption was associated with loss of control more strongly for low exercisers than high exercisers. These results indicate that the association between heavy alcohol consumption and WM damage in the EC and SLF and the association between alcohol consumption and loss of control over drinking are greater among individuals who do not exercise regularly. These results are consistent with the notion that exercise may protect WM integrity from alcohol-related damage [13547].

The aftermath of alcohol use

There is limited and conflicting evidence on the effects of postalcohol consumption and hangover on functional capacity, but there is sufficient evidence of adverse effects the day after a heavy drinking session for such activities to be discouraged. It was shown reductions in aerobic exercise performance of rugby players the day after an evening bout of drinking involving an intake of 1 to 38 units of alcohol (equivalent to 10-380 mL pure alcohol), though anaerobic performance was unaffected. The negative effect on aerobic performance was apparent at even the smallest dose of alcohol. For obvious reasons, there are few studies of the effects of high alcohol intakes on soccer-specific performance, and there appear to be no studies on well-trained football players. In spite of substantial efforts on the part of the alcoholic drinks industry, the causes of the symptoms of hangover are not well understood, but are thought to include dehydration, acid-base disturbances, disruption of cytokine and prostaglandin pathways, and alterations in glucose metabolism via effects on circulating insulin and glucagon levels. There are also disturbances of cardiovascular function during the hangover phase, including increased heart rate, decreased left ventricular performance, and increased blood pressure [06223].

Effects of alcohol on injury and incapacity

The ingestion of alcohol is likely to have a number of behavioral and other effects that may influence the risk of injury and the recovery process after injury. There are many reported instances of athletes in various sports competing while under the influence of alcohol, even though prior alcohol consumption appears to increase the risk of sports-related injury. The mechanisms by which this association may be mediated are not entirely clear, but the increased risk of injury, and the increased severity of injuries that do occur, may be a consequence of increased risk-taking behaviors as alcohol removes some of the restraints that normally control behavior. Increased levels of aggression are also often displayed by those under the influence of alcohol. Intoxication during competition is probably rather rare, at least at the higher levels of sport, though it is not completely absent. It is perhaps not so unusual, though, for athletes training the morning after a high alcohol intake the previous night to be still under the influence of alcohol, and several high-profile players from the various football codes have publicly admitted to alcohol addiction. Athletes often experience some degree of muscle damage, either of intrinsic or extrinsic origin, after hard training or competition. This is usually in the form of minor damage that results in some degree of pain
and disability that may persist for hours or days. Because alcohol can act as a peripheral vasodilator, it is often stated that alcohol intake should be avoided after any exercise that may have resulted in muscle damage. There appears, however, to be no experimental evidence to support these anecdotal observations. It remains prudent, however, to avoid intoxication, as this is likely to result in inappropriate behaviors that may exacerbate existing muscle damage and delay the recovery process [06223].

**Influence of sex**

Studies indicate greater sexual risk-taking behaviors and alcohol use in student-athletes compared with nonathletes, particularly in college samples. Although research has documented an association between drinking and risky sex, studies have not examined the role of sex motives in predicting risky sex in athletes. The purpose of one study was to extend previous research on athletes’ risk-taking behaviors by examining incoming college student-athletes and nonathletes’ alcohol consumption, risky sexual behavior, and sex motives. Participants included 2,123 (59% female) incoming college students attending a northwest university, 221 of whom reported intercollegiate athletic participation during their upcoming year. Hierarchical multiple regression analyses were conducted to examine associations between sex motives and risky sexual behaviors using a cross-sectional design. Results indicated greater weekly alcohol consumption, frequency of drinking before or during sex, and number of sexual partners in athletes compared with nonathletes. Athletes also reported greater levels of enhancement motives for sex and lower levels of intimacy motives than nonathletes, although no differences were found for coping motives. Significant interactions indicated that, for athletes, greater levels of enhancement sex motives predicted a greater number of sexual partners and more frequent drinking before or during sex, and greater levels of intimacy motives predicted less frequent drinking before or during sex. It was concluded that student-athletes are at risk for problematic outcomes associated with risky sex, including drinking before or during sex and having sex with multiple partners. Prevention efforts targeted at incoming college student-athletes should consider the role of sex motives [07213].

**Impact of alcohol on steroid profiles**

The impact of ethanol consumption on steroid profiles was investigated. In a comprehensive study with 21 male and 15 female volunteers, alterations in steroid profile parameters were correlated with urinary ethanol-glucuronide and ethanol-sulfate concentrations, and threshold values of 48 mg/mL and 16 mg/mL for men and women, respectively, were suggested. When exceeded, an influence on urinary steroid profiles due to ethanol-induced suppression of steroid biotransformation processes should be considered during data interpretation [12017].

**Cardioprotective effects**

It is well-known that the moderate use of alcohol has a cardioprotective effect, especially as regards the process of atherothrombosis. This is mainly due to an increase in levels of HDL cholesterol and apolipoproteins A-I and A-II, and a decrease in levels of LDL cholesterol, as well as its favourable action on fibrinolytic activity. However, the long-term use of large quantities of alcohol leads to cardiovascular complications, such as arterial hypertension, dilated cardiomyopathy, stroke, arrhythmias, and coronary artery spasm, because of its sympathomimetic action [12126].
Carbohydrate deficient transferrin (CDT)

The determination of carbohydrate deficient transferrin (CDT) concentration is primarily used in social security studies as a proof of regular alcohol consumption exceeding the amount of 60 grams per day. One study was performed to investigate into how carbohydrate deficient transferrin CDT values in serum are affected by the so-called food supplements and chemicals included in doping lists. The investigation was carried out in 15 bodybuilders of two sport clubs and in 10 boxers. All sportsmen were males. In both groups serum carbohydrate deficient transferrin (CDT %), median red blood cell volume and (MCV) gamma-glutamyl-transpeptidase (GGT) values were measured. The authors found a significant difference between the two groups only in carbohydrate deficient transferrin CDT% that was the CDT% value in bodybuilders was twice as high as in boxers. Thus, not all the details of the specificity of carbohydrate deficient transferrin (CDT) concentration are known, however, the remarkably high sensitivity of the method makes it suitable and probably economically effective as a pre-screening tool in doping tests [12341].

In football

The use of alcohol is often intimately associated with sport, and the association is particularly strong in football. As well as providing a source of energy, alcohol (ethanol) has metabolic, cardiovascular, thermoregulatory, and neuromuscular actions that may affect exercise performance. Its actions on the central nervous system, however, result in decrements in skill and behavioural changes that may have adverse effects on performance. There is also evidence of dose-dependent decrements in aerobic capacity. Although the mechanisms are not well understood, the aftermath of alcohol use (hangover) may also adversely affect performance for many hours after intoxication. Alcohol intoxication may adversely affect the player's dietary choices by displacing carbohydrate from the diet at a time when restoration of glycogen stores should be a priority [06224].

Consumption in US college sports

The purpose of one study was to examine the drinking patterns of club and intramural college athletes and compare their alcohol consumption, perceived norms around the excessive use of alcohol, experience of negative consequences, and employment of protective strategies with those of campus varsity athletes. A total of 442 undergraduate students attending a private, suburban institution in the Northeast participated in the American College Health Association National College Health Assessment-II Web survey in spring 2011. Thirty-five students identified themselves as varsity athletes, 76 identified as club sport athletes, and 196 students identified themselves as intramural athletes. Survey responses were analyzed using Statistical Package for the Social Sciences. The Pearson's correlation coefficient and test for independence were applied to identify significant relationships between athlete status and identified variables related to alcohol use. Results: Results indicated that there were significant correlations between athlete status and all variables, to varying degrees. These findings have implications for campus health promotion professionals and athletics program coordinators seeking to address high-risk alcohol use among college athletes [12346].
**Sponsorship**

Sports sponsorship is a significant marketing tool. As such, it can promote products that pose risks to health (e.g., high fat and high sugar foods) or it can promote health-supporting products (e.g., sporting equipment and services). However, there is a lack of data on the proportion of sponsorship associated with "unhealthy" and "healthy" products and no methodology for systematically assessing it. This research aimed to explore this proportion with an Internet survey of sports sponsorship in the New Zealand setting. A search methodology was developed to identify Internet-based evidence of sports sponsorship at the national level and at the regional and club level in one specific region (Wellington). The top eight sports for 5-17-year-olds were selected and products and services of sponsors were classified in terms of potential public health impact (using a conservative approach). Sponsorship of these popular sports was common at the national, regional and club levels (640 sponsors listed on 107 websites overall). Sports sponsorship associated with sponsors' products classified as "unhealthy" (e.g., food high in fat and sugar, gambling and alcohol) were over twice as common as sponsorship associated with sponsors' products classified as "healthy" (33% vs 16%). "Gambling" was the most common specific type of sponsorship (19%) followed by alcohol (11%). There were significantly more "alcohol" sponsors for rugby, compared to all the other sports collectively (rate ratio (RR) = 2.47), and for top male sports compared to female (RR = 1.83). Also there was significantly more "unhealthy food" sponsorship for touch rugby and for "junior" teams/clubs compared to other sports collectively (RR = 6.54 and RR = 14.72). A validation study gave an inter-rater reliability for number of sponsors of 95 percent (n= 87 sponsors), and an inter-rater reliability of classification and categorisation of 100 percent. The study found that the sponsorship of popular sports for young people is dominated by "unhealthy" sponsorship (i.e. predominantly gambling, alcohol and unhealthy food) relative to "healthy" sponsorship. Governments may need to consider regulations that limit unhealthy sponsorship and/or adopt alternative funding mechanisms for supporting popular sports [06225].

There is accumulating evidence supporting a link between alcohol industry sponsorship and alcohol-related problems in both community and elite-level sports. Little is known, however, about the current status of such sponsorship, particularly of community sport. One study aimed to assess associations between alcohol industry sponsorship and different community football clubs in Australia. The study involved 101 community football clubs across New South Wales, Australia. One representative from each club took part in a cross-sectional telephone survey designed to assess club (football code, number of players, socioeconomic and geographic descriptors) and alcohol industry sponsorship (money, equipment, free alcohol or discounted alcohol) characteristics. Chi-square analysis was used to test associations between club characteristics, and any alcohol industry sponsorship; and type of sponsorship. Eighty-eight per cent of clubs reported receiving sponsorship from the alcohol industry, and most clubs (82%) were sponsored by a licensed premises. There were no significant associations between club characteristics and source of alcohol industry sponsorship. However, small clubs were found to be significantly more likely to receive free or discounted alcohol sponsorship than larger clubs. This exploratory study suggests a significant presence of alcohol industry sponsorship among community football clubs in Australia [12342].

Although there is evidence that alcohol sponsorship in sport is related to greater drinking, there is no empirical research on whether alcohol sponsorship is associated with alcohol-related harms. It was examined whether there is an association between receipt of alcohol industry sponsorship, and attendance at alcohol sponsor's drinking establishments (e.g. bars), and alcohol-related aggression and antisocial behaviour in university students who
play sport. University sports-people (n=652) completed surveys (response rate >80 %) assessing receipt of alcohol industry sponsorship, attendance at sponsor's establishments and confounders [i.e. age, gender, sport type, location and alcohol consumption measured by Alcohol Use Disorders Identification Test-alcohol consumption (AUDIT-C) scores]. Participants also completed measures assessing displays and receipt of aggressive and antisocial behaviours (e.g. assaults, unwanted sexual advance, vandalism). Logistic regression models including confounders and reported attendance at alcohol sponsor's establishments showed that sportspeople receiving alcohol industry sponsorship were more likely to have been the victim of aggression (adjusted odds ratio 2.62, 95 % confidence interval 1.22 to 5.64). Attending an alcohol sponsor's establishment was not associated with higher rates of other aggressive or antisocial behaviour. However, significant associations where found between AUDIT-C scores and having displayed and received aggression, and having damaged or had property damaged. Male sportspeople were more likely to have displayed and received aggressive and antisocial behavior. Higher AUDIT-C scores, gender and receipt of alcohol industry sponsorship were associated with alcohol-related aggression/antisocial behaviours in university sportspeople. Sport administrators should consider action to reduce the harms associated with excessive alcohol consumption and alcohol industry sponsorship in sport [12343].

Prevention

To examine 12-year changes in alcohol use and cigarette smoking in response to community-based prevention activities among Icelandic adolescents a study used a quasi-experimental, non-randomized control group design to compare outcomes in 4 Icelandic communities (n=3117) that participated in community-based substance use prevention activities designed to increase levels of parental monitoring and adolescent engagement in healthy leisure-time activities and a matched group of 7 comparison communities (n=1,907). Annual, nationwide, population-based cross-sectional surveys of the prevalence of adolescent substance use were conducted among cohorts of Icelandic adolescents, aged 14-15 years (n=5,024), in all communities from 1997 to 2009. Parental monitoring and adolescent participation in organized sports increased in communities that adopted the intervention program compared to communities that did not, whereas unmonitored idle hours and attendance at unsupervised parties decreased. Over time, alcohol use and being intoxicated during the last 30 days decreased significantly more in the intervention than control communities. Community-based prevention designed to strengthen parental monitoring and participation in organized sports may confer some protection against adolescent substance use [10395].

Alcohol control strategies at large sports events

To assess the implementation and effectiveness of strategies and actions to eliminate and/or reduce alcohol-related problems at large sports and entertainment events in New Zealand it was conducted site visits and monitoring observations at venues before, during and after a variety of large events between 2009 and 2010. Thirteen events were attended at nine different venues. Events included rugby, rugby league and cricket matches, motor racing, rowing, horse racing, an outdoor music festival, and food and wine festivals. Most large events appeared to pass with few or no alcohol-related problems. The exceptions were one of the horse-race meetings, a rugby league match and one food and wine festival. Common contexts at events where alcohol-related problems were seen included: inadequate alcohol control and management by security staff; the ability to purchase four alcoholic drinks (rather than two) at a time; inexperienced bar staff untrained in responsible alcohol service; no or
little promotion of low and non-alcoholic drinks; and a lack of monitoring and enforcement of the law on intoxication. An important approach to prevent and reduce alcohol-related problems at large spots and entertainment events is the use of specific alcohol-control strategies. The management of alcohol consumption is a major part of event management that must be planned with harm-minimisation strategies well in advance of the event itself. If strategies and actions are not properly implemented to manage the sale and supply of alcohol at large events, there is significant risk of alcohol-related problems and harm resulting from them [12347].
NICOTINE

Nicotine, 3-(1-methyl-2-pyrrolidinyl)pyridine, is a potent activator of the sympathetic nervous system, and in healthy humans increases heart rate and blood pressure, cardiac stroke volume and output and coronary blood flow. Nicotine also causes cutaneous vasoconstriction associated with a decrease in skin temperature, systemic venoconstriction and increased muscle blood flow. Circulating levels of noradrenaline and adrenaline increase after nicotine consumption, as do concentrations of free fatty acids, glycerol and lactate. Although the cardiovascular and metabolic effects of nicotine are pronounced, it is likely that during moderate-intensity or prolonged exercise when sympathetic output is high, the peripheral effects of nicotine may be attenuated. Nicotine is widely reported to increase alertness, improve co-ordination and enhance cognitive performance; however, to our knowledge there have been no attempts to replicate these findings in relation to exercise endurance. The purpose of one study was to determine the effects nicotine might have on cycling endurance, perception of exertion and a range of physiological variables. With local ethics committee approval and having obtained informed consent, 12 healthy, non-smoking men (22 ± 3 years) cycled to exhaustion at 18 degrees C and 65 percent of their peak aerobic power, wearing either a 7 mg transdermal nicotine patch (NIC) or a colour-matched placebo (PLA) in a randomized cross-over design; water was available ad libitum. Subjects were exercising at approximately 75 percent of their maximal O₂ uptake with no differences in cadence between trials. Ten out of 12 subjects cycled for longer with NIC administration, and this resulted in a significant improvement in performance. No differences were observed for perceived exertion, heart rate or ventilation. There were no differences in concentrations of plasma glucose, lactate or circulating fatty acids. In the absence of any effect on peripheral markers, it was concluded that nicotine prolongs endurance by a central mechanism. Possible modes of action are suggested [06192].

Of the well-known stimulants, amphetamine and cocaine use is marginal compared to caffeine and nicotine, which are considered as the most widely consumed psychostimulants in the world, with more than 80 percent of the world population consuming caffeine and/or nicotine on a daily basis. There are research on nicotine has concentrated on the pharmacodynamics of the drug and improving smoking cessation, with no published studies having examined the effects of nicotine on exercise endurance/capacity. Nicotine administration during light physical activity has been shown to increase heart rate and blood pressure with no effect on ventilation, as well as having no effect on perceived exertion, however, protocols included smokers, sedentary subjects and work rates of cycling between 30 and 150 W for a maximum of 15 min. It has also been observed a significant increase in heart rate and a decrease in stroke volume together with an increase in blood lactate at rest and during treadmill exercise at 60 and 85 percent maximal O₂ uptake with oral smokeless tobacco (OST), compared to a placebo. The authors concluded that OST-induced increases in plasma nicotine concentrations augment anaerobic energy production and suggest a nicotine-induced sympathetic stimulation of the heart [06192].

Smoking and administration of nicotine are often associated with increased alertness, improved coordination and positive mood changes. It is likely that cortical arousal is a result of nicotine directly stimulating cholinergic neurotransmission in the basal forebrain. Nicotine has also been shown to increase dopamine release in the striatum and nucleus accumbens, the reward centre of the brain, and this effect is mediated by nicotinic acetylcholine receptors. It is therefore likely that administration of nicotine may enhance motivation during prolonged exercise owing to an increased release of dopamine, possibly involving similar pathways to those suggested for the central actions of caffeine [06192].
Despite the widespread use of nicotine and general acceptance that it promotes many of the characteristics provided by other stimulants, such as caffeine, cocaine and amphetamines, there has been no quantitative research about its benefits on endurance capacity during moderate and prolonged exercise, where the progressive development of central fatigue is believed to be a contributing factor in maintaining motivation. Whilst the present observation of a about 17 percent improvement in exercise duration may be the first demonstration of the efficacy of nicotine, others have shown improvements of this magnitude at a similar intensity for caffeine as well as amphetamine sulphate ingestion, which delays fatigue and improves time to exhaustion during short, intense bouts of exercise of < 10 min. It is likely that, as for caffeine, the maximal benefits of nicotine will be seen with exercise of 70-80 percent maximal aerobic capacity; depletion of muscle glycogen is likely to be the limiting factor during exercise below this intensity, whilst at higher intensities, cardiovascular and respiratory limitations, combined with peripheral muscle fatigue, will predominate [06192].

Consumption of nicotine in the form of smokeless tobacco (snus, snuff, chewing tobacco) or nicotine-containing medication (gum, patch) may benefit sport practice. Indeed, use of snus seems to be a growing trend and investigating nicotine consumption amongst professional athletes is of major interest to sport authorities. Thus, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the detection and quantification of nicotine and its principal metabolites cotinine, trans-3-hydroxycotinine, nicotine-N'-oxide and cotinine-N-oxide in urine was developed. Sample preparation was performed by liquid-liquid extraction followed by hydrophilic interaction chromatography-tandem mass spectrometry (HILIC-MS/MS) operated in electrospray positive ionization (ESI) mode with selective reaction monitoring (SRM) data acquisition. The method was validated and calibration curves were linear over the selected concentration ranges of 10-10,000 ng/mL for nicotine, cotinine, trans-3-hydroxycotinine and 10-5000 ng/mL for nicotine-N'-oxide and cotinine-N-oxide, with calculated coefficients of determination (R²) greater than 0.95. The lower limit of quantification (LLOQ) for all analytes was 10 ng/mL. Repeatability and intermediate precision were ≤9.4 and ≤9.9%, respectively. In order to measure the prevalence of nicotine exposure during the 2009 Ice Hockey World Championships, 72 samples were collected and analyzed after the minimum of 3 months storage period and complete removal of identification means as required by the 2009 International Standards for Laboratories (ISL). Nicotine and/or metabolites were detected in every urine sample, while concentration measurements indicated an exposure within the last 3 days for eight specimens out of ten. Concentrations of nicotine, cotinine, trans-3-hydroxycotinine, nicotine-N'-oxide and cotinine-N-oxide were found to range between 11 and 19,750, 13 and 10,475, 10 and 8217, 11 and 3396, and 13 and 1640 ng/mL, respectively. When proposing conservative concentration limits for nicotine consumption prior and/or during the games (50 ng/mL for nicotine, cotinine and trans-3-hydroxycotinine and 25 ng/mL for nicotine-N'-oxide and cotinine-N-oxide), about half of the hockey players were qualified as consumers. These findings significantly support the likelihood of extensive smokeless nicotine consumption. However, since such conclusions can only be hypothesized, the potential use of smokeless tobacco as a doping agent in ice hockey requires further investigation [10505].

Tobacco consumption is a global epidemic responsible for a vast burden of disease. With pharmacological properties sought-after by consumers and responsible for addiction issues, nicotine is the main reason of this phenomenon. Accordingly, smokeless tobacco products are of growing popularity in sport owing to potential performance enhancing properties and absence of adverse effects on the respiratory system. Nevertheless, nicotine does not appear on the 2011 World Anti-Doping Agency (WADA) Prohibited List or Monitoring Program by lack of a comprehensive large-scale prevalence survey. Thus, this work describes a one-year monitoring study on urine specimens from professional athletes of
different disciplines covering 2010 and 2011. A method for the detection and quantification of nicotine, its major metabolites (cotinine, trans-3-hydroxycotinine, nicotine-N'-oxide and cotinine-N-oxide) and minor tobacco alkaloids (anabasine, anatabine and nornicotine) was developed, relying on ultra-high pressure liquid chromatography coupled to triple quadrupole mass spectrometry (UHPLC-TQ-MS/MS). A simple and fast dilute-and-shoot sample treatment was performed, followed by hydrophilic interaction chromatography-tandem mass spectrometry (HILIC-MS/MS) operated in positive electrospray ionization (ESI) mode with multiple reaction monitoring (MRM) data acquisition. After method validation, assessing the prevalence of nicotine consumption in sport involved analysis of 2185 urine samples, accounting for 43 different sports. Concentrations distribution of major nicotine metabolites, minor nicotine metabolites and tobacco alkaloids ranged from 10 (LLOQ) to 32,223, 6670 and 538 ng/mL, respectively. Compounds of interest were detected in trace levels in 23 percent of urine specimens, with concentration levels corresponding to an exposure within the last three days for 18 percent of samples. Likewise, hypothesizing conservative concentration limits for active nicotine consumption prior and/or during sport practice (50ng/mL for nicotine, cotinine and trans-3-hydroxycotinine and 25 ng/mL for nicotine-N'-oxide, cotinine-N-oxide, anabasine, anatabine and nornicotine) revealed a prevalence of 15 percent amongst athletes. While this number may appear lower than the worldwide smoking prevalence of around 25 percent, focusing the study on selected sports highlighted more alarming findings. Indeed, active nicotine consumption in ice hockey, skiing, biathlon, bobsleigh, skating, football, basketball, volleyball, rugby, American football, wrestling and gymnastics was found to range between 19 and 56 percent. Therefore, considering the adverse effects of smoking on the respiratory tract and numerous health threats detrimental to sport practice at top level, likelihood of smokeless tobacco consumption for performance enhancement is greatly supported [11226].

Smokeless tobacco (ST) use is common in some sports. Nicotine is a psychomotor stimulant and this raises the issue of whether such use constitutes that of a performance-enhancing drug. Most of the data come from baseball players. Both adolescent and adult baseball players use ST. Among major league players, 45 percent have been reported to use ST. The amount of nicotine extracted from ST ranges between 20 and 45 percent and bioavailability is 40-60 percent of the extracted amount. Plasma levels peak after 30 minutes, with values which could reach an average of 20-30 ng/mL at the end of the day (similar to moderate to heavy smoking). ST users are thus exposed to a large amount of nicotine over a longer period than from smoking. Nicotine has been found in some studies to enhance information processing and attentional processes in humans. The robustness of this effect and whether in smokers it represents more than normalization of impairment experienced as a result of nicotine withdrawal has been debated. In baseball ST users there are only limited data. It has been assessed muscular force, reaction time and choice reaction time in athlete ST users (under abstinence) versus non-users. ST users showed a stronger force, a slower choice reaction time but no difference in simple reaction time. Another study compared field performance between ST users versus non-users by using common baseball statistics parameters. Indeed, higher values for parameters investigated are an expression of a better integration between cognitive and motor processes, such as perception, vigilance, divided attention, decision-making and executive outcome, in order to meet demanding game situations. These key moments of the match are also characterized by high alertness and arousal states, as well as by a higher stress condition. Also modest enhancement induced by ST use may make a difference in performance that could affect the outcome of a highly competitive match, where teams and players appear to be well balanced. Thus, while the limited set of available data suggests that some psychometric values are not changed in baseball ST users under abstinence, no data are available from the field, or even from the laboratory, under actual ST exposure. The question still remains: are those subjects who are taking ST benefiting from legal doping? At present, there is limited translation or modeling of
complex psychomotor performance – as in sports – into the laboratory. Although specific models have been developed to study sport performance in the laboratory, valid experimental paradigms are still lacking for investigations on the effects of addictive substances on complex psychomotor performance. A possible experimental approach is to begin by correlating the statistical data available on baseball performances in the United States with information on ST use of leading players. The results of such correlations may support experimental placebo-controlled studies on any enhancing effects of low- or high-nicotine ST. This is a difficult area, but it makes sense to begin the debate now and start collecting relevant data [07203].

Aerobic exercise can acutely reduce cigarette cravings during periods of nicotine deprivation. The primary aim of this study was to assess the differential effects of light and vigorous intensity aerobic exercise on cigarette cravings, subjective and physiological reactivity to smoking cues, and affect after overnight nicotine deprivation. A secondary aim was to examine cortisol change as a mediator of the effects of exercise on smoking motivation. 162 (55 female, 107 male) overnight nicotine-deprived smokers were randomized to one of three exercise conditions: light intensity, vigorous intensity, or a passive control condition. After each condition, participants engaged in a standardized cue reactivity assessment. Self-reported urges to smoke, affect, and salivary cortisol were assessed at baseline (i.e., before each condition), immediately after each condition, and after the cue reactivity assessment. Light and vigorous exercise significantly decreased urges to smoke and increased positive affect, relative to the control condition. In addition, those in the vigorous exercise condition demonstrated suppressed appetitive reactivity to smoking cues, as indexed by the startle eyeblink reflex. Although exercise intensity was associated with expected changes in cortisol concentration, these effects were not related to changes in craving or cue reactivity. Both light and vigorous exercise can reduce general cravings to smoke, whereas vigorous exercise appears especially well-suited for reducing appetitive reactions to cues that may precede smoking. Results did not support exercise-induced cortisol release as a mechanism for these effects [13549].

Nicotine or 3-(1-methyl-2-pyrrolidinyl)pyridine is a naturally occurring alkaloid and one of the most widely used psychostimulants in the world. Cigarettes are the most common source of nicotine. Smoked tobacco contains additional harmful constituents and chemicals, which have detrimental effects on the respiratory system. In summary, nicotine seems to have ergogenic potential. Athletes appear to benefit from activation of the sympathoadrenal system with increased catecholamine release and subsequent increases in muscle blood flow and lipolysis. One component of nicotine action seems to act via a central mechanism (by nicotine receptor activation and/or dopaminergic pathways. There is evidence for the abuse of nicotine by athletes. Although the sale of snuff is illegal within the European Union, anecdotal observations by coaches and research from Scandinavia shows a high prevalence of snus use among athletes [13008].

Mechanism of action

In general, nicotine has a psychostimulatory effect on the CNS at low doses via enhancing the actions of norepinephrine and dopamine in the brain. At higher doses, however, nicotine enhances the effect of serotonin and opiate activity, exerting a calming and depressing effect. Nicotine-induced stimulation of the sympathetic nervous system leads to increased heart rate and blood pressure, cardiac stroke volume and output and coronary blood flow. Although the results are conflicting and some authors report increases in cutaneous blood flow and skin temperature, others report a decrease in cutaneous blood flow and subsequent decline in skin temperature associated with nicotine consumption. These differences in
cutaneous blood flow are possibly related to differences in nicotine administration. Both snus and nicotine gums enable nicotine to diffuse across the mucous membranes and are taken up by the bloodstream or, when inhaled, diffuses across the alveolar membrane of the alveoli, and enters the bloodstream. Although the amount of nicotine inhaled is lower than with conventional cigarettes, the use of electronic cigarettes is becoming more and more popular. However, the amount taken up by smokeless tobacco users tends to be much greater than by smoking. Once in the bloodstream, nicotine is quickly (within seconds) delivered to the brain, where it interacts with neural nicotinic acetylcholine receptors (nAChRs). It is metabolized by the liver cytochrome P450 enzyme system and has a half-life of approximately 2 hours. Upon binding of ACh or its exogenous ligand nicotine, the ion channel is opened and causes an influx of sodium and calcium (Ca\(^{2+}\)). This local increase in intracellular Ca\(^{2+}\) can alter cellular functions. A mechanism termed Ca\(^{2+}\)-induced Ca\(^{2+}\) release can further boost intracellular calcium upon activation of nAChR. In vitro experiments using human neutrophils showed a dose-dependent rise in intracellular Ca\(^{2+}\) levels of 700% over baseline at a concentration of 10\(^{-2}\) M nicotine. In numerous pathways, Ca\(^{2+}\) acts as an intracellular messenger, setting the stage for nAChRs as potent candidates to influence a variety of Ca\(^{2+}\)-dependent neuronal processes, such as neurotransmitter release, synaptic plasticity or gene transcription [13008].

**Effect on performance**

While it is clear that smoking can lead to the development of respiratory, cardiovascular, and skin diseases as well as a number of tobacco-related cancers there are other forms of application such as the use of alternative smokeless tobacco (snuff), which is gaining popularity among athletes as it bypasses the respiratory system. Snuff and cigarette consumers show similar peak blood nicotine levels after use with a tendency for higher cotinine levels in the former. Nicotine activates the sympathoadrenal system, which leads to increased heart rate, contractility, vasoconstriction and a rise in blood pressure and the level of circulating catecholamines during light exercise. Nicotine also increases muscle blood flow and lipolysis due to enhanced circulating levels of norepinephrine and epinephrine as well as direct action on nicotinic cholinergic receptors in adipose tissue. The effects exerted by nicotine may be beneficial in a wide variety of sports and it is suggested that nicotine is abused by athletes. A cumulative exposure to nicotine metabolites were found in 26-56 percent of urine samples that were subjected to screening for tobacco alkaloids. After correcting for exposure to second-hand smoke, 15 percent of the athletes were considered active nicotine consumers. Among athletes, this is high considering the WHO's 25 percent worldwide estimate of smoking prevalence. It can be hypothesized that the metabolites stem mostly from smokeless tobacco due to the adverse effects of conventional cigarettes for athletes, which most severely affects athletes engaging in endurance type sports [13008].

A large number of human and animal studies have found nicotine-induced improvements in several aspects of cognitive function, including learning and memory, reaction time, and fine motor abilities. Studies addressing the question of a direct performance enhancing effect of nicotine are rare. Sports most affected include ice hockey, skiing, biathlon, bobsleigh, skating, football, basketball, volleyball, rugby, American football, wrestling and gymnastics. These sports seem to gain performance benefits from the stimulating effect of nicotine as evident from the use of other prohibited stimulants according to the Anti-Doping Database. It was found a 17 percent improvement in time to exhaustion after nicotine patch application compared to a placebo without affecting cardiovascular and respiratory parameters or substrate metabolism. In this sense, nicotine seems to exert similar effects as caffeine by delaying the development of central fatigue as impaired central drive is an important factor contributing to fatigue during exercise. To date, no improvement on anaerobic performance
(Wingate test) has been reported. It is important to note that, compared to rest, exercise can lead to increased drug absorption from transdermal nicotine patches, possibly due to exercise-induced increase in peripheral blood flow at the site of the transdermal patch. It was reported increased plasma nicotine levels and toxicity due to increased drug absorption during physical exercise. To prevent undesirable side effects, health professionals, trainers and coaches should therefore be aware of proper transdermal patch use, particularly while exercising. Athletes are encouraged to inform their physician about their exercise routine before beginning transdermal patch use. Athletes should further be familiar with signs and symptoms of drug toxicity related to the medication contained in the transdermal patch and consult their physician if signs or symptoms arise. Reducing exercise intensity and duration for the first 1-2 weeks until potential side effects are known might also help to minimize toxicity. To reduce increased exercise-induced drug absorption, athletes are encouraged to avoid exercising in extreme environmental and temperature conditions, wear appropriate breathable sports garments and drink plenty of fluids to prevent dehydration [13008].

Additionally, although nicotine may have ergogenic potential, it is also highly addictive, reportedly as addictive as heroin and cocaine. Therefore, detrimental effects on motor performance can be altered after a short abstinence duration. However, it was noted that motor performance declines in heavy smokers after a short period of abstinence appears, this decline being similar to the motor symptoms of Parkinsonism. The abstinence symptoms are ameliorated by cigarette smoking. It is important to consider the concerning addictive potential with following deterioration of motor performance upon abstinence. Interestingly, however, it was noted that moderate and vigorous exercise led to significant reductions of the desire to smoke among abstaining smokers, possibly via reductions in cortisol. A recent meta-analysis showed that exercise has the potential to acutely reduce cigarette cravings and could therefore be a promising strategy to attenuate withdrawal symptoms in smokers. It is also important to mention that the vasoconstriction mediated by nicotine could limit exercise performance in a hot environment. As skin blood flow increases during exercise to transfer heat impaired nicotine-induced skin blood flow may be ergolytic. A recent meta-analysis clearly suggests significant effects of nicotine on fine motor abilities, including attention and memory. Participants of the studies included in the meta-analysis were mainly nonsmokers, therefore avoiding confounding of nicotine withdrawal. Considering the importance of cognition in sport, such an optimization of neurobiological function in our view seems to be beneficial for a variety of sports such as sport games or track and field. Finally, nicotine’s effect on increased pain tolerance might be of advantage in a wide variety of sports [13008].

**Detection**

Nicotine use can be detected in urine samples based on its major metabolites (cotinine, trans-3-hydroxycotinine, nicotine-N0-oxide and cotinine-N-oxide) and tobacco alkaloids (anabasine, anatabine and nornicotine) which can be used as an indicator of a person’s exposure to nicotine [13008].
NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDS)

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely consumed among athletes worldwide in relation to muscle injury and soreness. This review aims to provide an overview of studies investigating their effects on skeletal muscle, in particular the repair processes in injured muscle. Muscle injury occurs in diverse situations and the nature of muscle injuries varies significantly, complicating extrapolations between experimental models and “real life.” Classical muscle strain injuries occur at the interface between the muscle fibers and connective tissue, most often in the myotendinous junction, whereas contusion or overload injury can damage both myofibers and intramuscular connective tissue. The role of NSAIDs in muscle repair is complicated by differences in injury models used, variables evaluated, and time point(s) selected for evaluations. While the temporal pattern of the influence of NSAIDs on muscle repair is difficult to settle on, it appears that a potential beneficial effect of NSAIDs in the early phase after injury is not maintained in the long term, or is even negated by a long-term repair deficit. At the cellular level, evidence exists for a negative influence of NSAIDs on the muscle stem cell population (satellite cells). At a structural level, it is known that muscle connective tissue undergoes significant remodeling during muscle regeneration, but the potential of NSAID exposure to alter this response in humans needs investigation [12329].

Superstitions and rituals are commonplace in sports, and range from simple activities such as each player touching a special inanimate object before entering the field of play to more extreme behaviors such as not washing a uniform or wearing the same underwear during a winning streak. These practices are relatively harmless and may reduce pre-competition anxiety, but a concerning ritual that has recently developed in sports is the prophylactic use of non-steroidal anti-inflammatory drugs (NSAIDs) [09298].

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in sports medicine. NSAIDs have known anti-inflammatory, analgesic, antipyretic, and antithrombotic effects, although their in-vivo effects in treating musculoskeletal injuries in humans remain largely unknown. NSAIDs analgesic action is not significantly greater than paracetamol for musculoskeletal injury but they have a higher risk profile, with side-effects including asthma exacerbation, gastrointestinal and renal side-effects, hypertension, and other cardiovascular disease. The authors recommend an approach to NSAID use in sports medicine whereby simple analgesia is preferentially used when analgesia is the primary desired outcome. However, based both on the current pathophysiological understanding of most injury presentations, and the frequency that inflammation may actually be a component of the injury complex, it is premature to suppose that NSAIDs are not useful to the physician managing sports injuries. The prescribing of NSAIDs should be cautious, and both situation and pathology specific. Both dose and duration minimization should be prioritized and combined with simple principles of protection, rest, ice, compression, elevation (PRICE) which should allow NSAID-sparing. NSAID use should always be coupled with appropriate physical rehabilitation. NSAIDs are probably most useful for treating nerve and soft-tissue impingments, inflammatory arthropathies, and tenosynovitis. There are not generally indicated for isolated chronic tendinopathy, or for fractures. The use of NSAIDs in treating muscle injury is controversial. Conditions where NSAID use requires more careful assessment include; ligament injury, joint injury, osteoarthritis, haematoma, and post-operatively [09296].

Unaccustomed exercise leads to satellite cell proliferation and increased skeletal muscle protein turnover. Several growth factors and cytokines may be involved in the adaptive
responses. Non-steroidal anti-inflammatory drugs (NSAIDs) negatively affect muscle regeneration and adaptation in animal models, and inhibit the exercise-induced satellite cell proliferation and protein synthesis in humans. However, the cellular mechanisms eliciting these responses remain unknown. Eight healthy male volunteers performed 200 maximal eccentric contractions with each leg. To block prostaglandin synthesis locally in the skeletal muscle, indomethacin (NSAID) was infused for 7.5 h via microdialysis catheters into m. vastus lateralis of one leg. Protein synthesis was determined by the incorporation of 1,2-13C2-leucine into muscle protein from 24 to 28 h post-exercise. Furthermore, mRNA expression of selected genes was measured in muscle biopsies (5 h and 8 days post-exercise) by real-time reverse transcriptase PCR. Myofibrillar and collagen protein synthesis were unaffected by the local NSAID infusion. Five hours post-exercise, the mRNA expression of cyclooxygenase-2 (COX2) was sixfold higher in the NSAID leg compared with the unblocked leg. The expression of growth factors and matrix-related genes were unaffected by NSAID. Although NSAIDs inhibit the exercise-induced satellite cell proliferation, it was observed only limited effects on gene expression, and on post-exercise protein synthesis [11513].

Although athletes are young and generally healthy, they use a variety of non-doping classified medicines to treat injuries, cure illnesses and obtain a competitive edge. Athletes and sports medicine physicians try to optimize the treatment of symptoms related to extreme training during an elite athlete's active career. According to several studies, the use of antiasthmatic medication is more frequent among elite athletes than in the general population. Recent studies show that athletes use also NSAIDs and oral antibacterials more commonly than age-matched controls, especially athletes competing in speed and power sports. Inappropriately high doses and concomitant use of several different NSAIDs has been observed. All medicines have adverse effects that may have deleterious effects on elite athletes' performance. Thus, any unnecessary medication use should be minimized in elite athletes. Inhaled beta2-agonists may cause tachycardia and muscle tremor, which are especially harmful in events requiring accuracy and a steady hand. In experimental animal models of acute injury, especially selective cyclo-oxygenase-2 inhibitors have been shown to be detrimental to tissue-level repair. They have been shown to impair mechanical strength return following acute injury to bone, ligament and tendon. This may have clinical implications for future injury susceptibility. However, it should be noted that the current animal studies have limited translation to the clinical setting. Adverse effects related to the CNS and gastrointestinal adverse reactions are commonly reported in connection with NSAID use also in elite athletes. In addition to the potential for adverse effects, recent studies have shown that NSAID use may negatively regulate muscle growth by inhibiting protein synthesis [08366].

The World Anti-Doping Agency does not prohibit the use of nonsteroidal anti-inflammatory drugs (NSAIDs) because these agents are not performance enhancing, and their analgesic and anti-inflammatory effects are at best performance enabling. Consequently, athletes have relatively unrestricted access to NSAIDs, which are readily available as over-the-counter drugs. However, concern has been raised on athletes’ prophylactic use of these agents. Data from many sporting fields have consistently demonstrated that many individuals self-administer NSAIDs prior to athletic participation to prevent pain and inflammation before it occurs. However, scientific evidence for this approach is currently lacking, and athletes should be aware of the potential risks in using NSAIDs as a prophylactic agent. These agents are not benign, and can produce significant side effects, including gastrointestinal and cardiovascular conditions, as well as musculoskeletal and renal side effects. The latter side effects appear paradoxical to the rationale for prophylactic use of NSAIDs [10209].

Sports medicine physicians often treat athletes in pain with non-steroidal anti-inflammatory drugs (NSAIDs). However, there is a lack of high-quality evidence to guide NSAID use. Their
adverse effects have clinical relevance, and their possible negative consequences on the long-term healing process are slowly becoming more obvious. One article provides some practical management guidelines for the use of NSAIDs, developed to help sports medicine physicians deal with frequent sports-related injuries. NSAIDs are not recommended for muscle injuries, bone fractures (also stress fractures) or chronic tendinopathy. In all cases, if chosen, NSAID treatments should always be kept as short as possible and should take into account the specific type of injury, the level of dysfunction and pain [10210].

To examine the prescribing habits of NSAIDs among pediatric medical and surgical practitioners, and to examine concerns and barriers to their use a sample of 1289 pediatricians, pediatric rheumatologists, sports medicine physicians, pediatric surgeons and pediatric orthopedic surgeons in the United States and Canada were sent an email link to a 22-question web-based survey. 338 surveys (28 %) were completed, 84 were undeliverable. Of all respondents, 164 (50 %) had never prescribed a selective cyclooxygenase-2 (COX-2) NSAID. The most common reasons for ever prescribing an NSAID were musculoskeletal pain, soft-tissue injury, fever, arthritis, fracture, and headache. Compared to traditional NSAIDs, selective COX-2 NSAIDs were believed to be as safe (42 %) or safer (24 %); have equal (52 %) to greater efficacy (20 %) for pain; have equal (59 %) to greater efficacy (15 %) for inflammation; and have equal (39 %) to improved (44 %) tolerability. Pediatric rheumatologists reported significantly more frequent abdominal pain (81 % vs 23 %), epistaxis (13 % vs 2 %), easy bruising (64 % vs 8 %), headaches (21 % vs 1 %) and fatigue (12 % vs 1 %) for traditional NSAIDs than for selective COX-2 NSAIDs. Prescribing habits of NSAIDs have changed since the voluntary withdrawal of rofecoxib and valdecoxib; 3 percent of pediatric rheumatologists reported giving fewer traditional NSAID prescriptions, and while 57 percent reported giving fewer selective COX-2 NSAIDs, 26 percent reported that they no longer prescribed these medications [10211].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used in sports medicine to reduce time of incapacity. To describe the frequency of NSAIDs use by athletes in the XV Pan-American Games all athletes who were tested by the anti-doping control filled a form. The voluntarily declared medications were recorded and categorised according to sport modality, gender, region and control situation according to the World Anti-Doping Agency. Among the 1261 athletes tested (231 out-competition (OC) and 1030 in-competition (IC); 733 men and 528 women), 63 percent reported use of drugs, NSAIDs being the most frequently (64% of users) used medications. The use of medications was not significantly different between sexes or among different regions of the world. The number of users of only one type of NSAID was higher than those who used more than one type of NSAIDs or a combination with analgesics (335 vs 168 cases). IC reports presented higher use of NSAIDs than OC. Athletes tested by the anti-doping control of the XV Pan-American Games reported a high frequency of NSAIDs use. The frequent utilisation in competition suggests that these medications might be used as ergogenic aid [11230].

Sports medicine physicians often treat athletes in pain with non-steroidal anti-inflammatory drugs (NSAIDs). However, there is a lack of high-quality evidence to guide NSAID use. Their adverse effects have clinical relevance, and their possible negative consequences on the long-term healing process are slowly becoming more obvious. This article provides some practical management guidelines for the use of NSAIDs, developed to help sports medicine physicians deal with frequent sports-related injuries. It was not recommend their use for muscle injuries, bone fractures (also stress fractures) or chronic tendinopathy. In all cases, if chosen, NSAID treatments should always be kept as short as possible and should take into account the specific type of injury, the level of dysfunction and pain [06220].

Nonsteroidal antiinflammatory drugs (NSAIDs) are frequently used in the treatment of acute
athletic injuries, often for analgesic purposes as the evidence to support enhanced healing is limited. However, the current evidence on NSAID use in athletic injury is slowly growing. On the basis of animal models and limited human studies, some practical management guidelines can be drawn to assist the sports physician. Specifically, NSAIDs are not recommended in the treatment of completed fractures, stress fractures at higher risk of nonunion, or in the setting of chronic muscle injury. The only exception may be very short-term use (e.g. a few days) for analgesic purposes only. Judicious use of NSAIDs may be more appropriate in the management of acute ligament sprains, muscle strains, tendinitis, and eccentric muscle injury. However, length of treatment should always be kept as short as possible, with consideration of the specific type of injury, level of dysfunction, and pain.

Non-steroidal anti-inflammatory drugs (NSAIDs) act by inhibiting cyclo-oxygenase-1 (COX-1) and COX-2 enzymes, which are involved in prostaglandin synthesis, resulting in their analgesic, anti-inflammatory, and antipyretic effects. Arachidonic acid is metabolised by the cyclo-oxygenase isoenzymes COX-1 and COX-2 to intermediate prostaglandins (PGG2, then PGH2), which are then converted to other prostaglandins that are mediators of pain, inflammation, and fever, and are gastroprotective. Thromboxane A2 causes platelet aggregation, and prostacyclin produces vasodilation. By blocking COX enzymes, NSAIDs inhibit the synthesis and thus the effects of prostaglandins, prostacyclin, and thromboxane A2. The clinical effects of NSAIDs depend largely on their selectivity for these enzymes. Aspirin works by irreversibly inhibiting COX-1 mediated synthesis of thromboxane A2, and is used to inhibit platelet aggregation. Although the boundary is blurred, there are two broad groups of NSAIDs: the older, traditional, nonselective NSAIDs that inhibit both COX-1 and COX-2 and the newer, selective COX-2 inhibitors that predominantly inhibit COX-2. The non-selective NSAID aspirin is used primarily for its antiplatelet effect, thus reducing the risk of myocardial re-infarction and stroke.

Non-selective (NSAIDs) and selective (COX-2) nonsteroidal anti-inflammatory drugs are commonly used for their analgesic and anti-inflammatory effects. Their role after orthopaedic surgery has been infrequently described and remains controversial because of unclear effects on soft tissue and bone healing. One study critically reviewed the available literature to describe the effects of NSAIDs and COX-2 inhibitors on soft tissue and bone healing. A Medline search was performed using NSAIDs or COX-2 inhibitors and tissue healing. The combined search yielded 637 articles. Following exclusion, 44 articles were deemed relevant with 9 articles on soft tissue healing and 35 articles on bone healing. The available evidence is based primarily on animal data (39 studies), with considerable variation in study methods. In regard to soft tissue healing, there is insufficient evidence of a detrimental effect when using either NSAIDs or COX-2 inhibitors at standard doses for ≤2 weeks. For soft tissue to bone healing, a limited number of studies demonstrate impairment in healing. However, with respect to bone healing, indomethacin appears to have a clear detrimental effect, with less substantial evidence for other NSAIDs. It was concluded that short-term, low-dose use of NSAIDs and COX-2 inhibitors does not appear to have a detrimental effect following soft tissue injury, but is inhibitory in cases involving bony healing. However, additional well-controlled human studies are necessary to draw more definitive conclusions regarding their role. Clinically, the prudent use of anti-inflammatory medications following sports medicine injuries and surgeries appears to be a reasonable option in clinical practice unless bone healing is required.

The indications and effectiveness of the various NSAIDs differs, although there is little difference in their mean efficacy, and although a review of individual patient data indicates that patients vary in their responses to different NSAIDs. Effectiveness may vary with different conditions.
- For acute pain and dysmenorrhoea, meta-analyses indicate that both classes of NSAIDs are highly effective analgesics compared with placebo.
- Both groups of NSAIDs play a useful but adjunctive anti-inflammatory role in rheumatoid arthritis, although disease control with antirheumatic drugs such as methotrexate and biological agents is critical.
- Both groups of NSAIDs are often used for osteoarthritis and low back pain; their effectiveness is generally small.

**Mechanism of action**

Mechanism of action is the first aspect of NSAIDs that should be considered prior to prescribing. Traditional NSAIDs act in the body by nonselectively blocking cyclooxygenase (COX). COX's primary mechanism of action is to catalyze the transformation of arachidonic acids to prostaglandins. There are two main forms, COX-1 and COX-2, each producing different prostaglandins. COX-1 is present at all times in the human body and is responsible for basal functions such as protection of the gastric mucosa and platelet aggregation. COX-2 is an inducible enzyme created and up-regulated during times of injury. Traditional NSAIDs inhibit both COX-1 and COX-2, leading to decreased inflammatory response, but the decrease in gastric mucosa protection also increases the risk of gastrointestinal irritation. COX-2–specific inhibitors were developed with the goal of suppressing the inflammation produced by up-regulation of COX-2 after injury while sparing the gastrointestinal tract. This is accomplished by not inhibiting prostaglandin secretion in the gastric wall mucosa. Unfortunately, that concept did not take into account the effects on prostacyclin produced in vascular endothelial cells. Without prostacyclin, thromboxane A2 (mediated by COX-1) is unopposed, creating a potential shift in the tissue balance away from prostacyclin's vasodilatory and antiplatelet effects and toward more deleterious vasoconstriction and platelet aggregation. This shift's end result theoretically promotes thrombosis; more than one COX-2 inhibitor has been removed from the market following an increase in cardiovascular events [07216].

**Ketoprofen**

Ketoprofen (KP), a nonsteroidal anti-inflammatory drug (NSAID), possesses analgesic, antipyretic and anti-inflammatory properties. Oral KP is widely used in musculoskeletal pain and inflammation in muscles and joints, including arthritis pain, osteoarthritis, stiffness of the joints, soft tissue rheumatism, and sports injuries. In common with all NSAIDs, oral KP has been associated with systemic adverse events and in particular gastrointestinal disorders. Topical application of the active ingredient is locally effective and at the same time minimises the risk of systemic adverse events. Pharmacokinetic studies show that serum levels of the active ingredient following topical KP 2.5 percent gel are less than 1 percent of those reported after oral dosing, thereby providing good levels of pain relief without the systemic adverse events normally associated with oral NSAIDs. In comparative studies, topical KP 2.5 percent gel twice daily showed clinical benefits in patients with a range of musculoskeletal conditions. KP 2.5 percent gel is generally well tolerated but the treated skin area should not be exposed to direct sunlight, including solarium (sunbeds), during the treatment and for 2 weeks afterwards as topical photosensitization has been reported [11369].

**Topical NSAID**
Use of topical NSAIDs to treat acute musculoskeletal conditions is widely accepted in some parts of the world, but not in others. Their main attraction is their potential to provide pain relief without associated systemic adverse events. To review the evidence from randomised, double-blind, controlled trials on the efficacy and safety of topically applied NSAIDs in acute pain it was searched MEDLINE, EMBASE, The Cochrane Library, and the own in-house database to December 2009. It was also sought unpublished studies by asking personal contacts and searching on-line clinical trial registers and manufacturers web sites. It was included randomised, double-blind, active or placebo (inert carrier)-controlled trials in which treatments were administered to adult patients with acute pain resulting from strains, sprains or sports or overuse-type injuries (twisted ankle, for instance). There had to be at least 10 participants in each treatment arm, with application of treatment at least once daily. Two review authors independently assessed trial quality and validity, and extracted data. Numbers of participants achieving each outcome were used to calculate relative risk and numbers needed to treat (NNT) or harm (NNH) compared to placebo or other active treatment. Forty-seven studies were included; most compared topical NSAIDs in the form of a gel, spray, or cream with a similar placebo, with 3455 participants in the overall analysis of efficacy. For all topical NSAIDs combined, compared with placebo, the number needed to treat to benefit (NNT) for clinical success, equivalent to 50% pain relief, was 4.5 (3.9 to 5.3) for treatment periods of 6 to 14 days. Topical diclofenac, ibuprofen, ketoprofen, and piroxicam were of similar efficacy, but indomethacin and benzydamine were not significantly better than placebo. Local skin reactions were generally mild and transient, and did not differ from placebo. There were very few systemic adverse events or withdrawals due to adverse events. There were insufficient data to reliably compare individual topical NSAIDs with each other or the same oral NSAID. Topical NSAIDs can provide good levels of pain relief, without the systemic adverse events associated with oral NSAIDs, when used to treat acute musculoskeletal conditions [06222].

Topical medications, including NSAIDs, hold a lot of promise by delivering medicines known to be effective, only without the associated risks when administered orally. Topical NSAID preparations have in some instances good evidence to support their use. A prospective, double-blind, randomized controlled trial of once-daily topical ketoprofen patch was performed in France and found a statistically significant decrease in pain for the topical ketoprofen group versus the placebo group. Treatment with topical NSAIDs can keep plasma levels low (thereby limiting systemic adverse events) while allowing penetration into soft tissues of an injured extremity [07216].

Creams, solutions, gels, sprays, and patches of non-selective NSAIDs are widely used for their local effects, low systemic absorption, and considerable safety. For acute soft-tissue injuries (sprains, minor injuries, etc), systematic reviews have shown that topical products are more effective than placebo products. For effects on osteoarthritis, the most comprehensive data have been obtained with diclofenac. Although the results of clinical trials are variable, systematic reviews indicate that the available gel formulations of diclofenac provide greater relief of pain than placebo, though the improvement is small [13559].

How safe are NSAIDs?

Gastrointestinal

Harm from both types of NSAID is a major problem in osteoarthritis because of their prolonged use in the treatment of this disease. The absolute risk of adverse effects increases substantially in patients over 70 years old and with duration of use and size of dose, and is
affected by individual risk factors. A major adverse effect of the non-selective NSAIDs is serious injury to the upper gastrointestinal tract (perforations, ulcers, bleeding). The COX-2 selective inhibitors are also associated with upper gastrointestinal toxicity, but large controlled and observational studies and meta-analyses indicate this is significantly less than with non-selective NSAIDs. Modelling indicates that adding a proton pump inhibitor to an NSAID reduces the rate of upper gastrointestinal adverse effects. Dyspepsia is common with both types of NSAID, although a meta-analysis indicates that the risk is slightly smaller (12%) with the selective COX-2 inhibitors [13559].

Cardiac

Overall, both NSAID classes, with the apparent exception of the non-selective NSAID naproxen, are associated with a significantly increased risk of myocardial infarction and coronary death. From a recent, very large meta-analysis of individual data from randomised trials, this translated to three more major vascular events (mainly major coronary events) occurring in 1000 patients taking a selective COX-2 inhibitor or diclofenac for one year than seen with placebo, one of these extra events being fatal. Thus, the higher the background risk, the higher the absolute risk will be on exposure to one of these drugs. These conclusions are supported by a cost effectiveness analysis of data from three large controlled clinical studies on NSAIDs. Naproxen seems to be the safest NSAID, with a similar rate of myocardial infarction as with no treatment. Of the non-selective NSAIDs, there is broad agreement that diclofenac seems to have the greatest risk of myocardial infarction, similar to COX-2 selective drugs [13559].

Renal and hypertension

As renal prostaglandins and prostacyclin are synthesised by both COX-1 and COX-2 both classes of NSAIDs increase the risk of further renal impairment and of worsening cardiac failure. All NSAIDs cause a dose dependent increase in blood pressure. The mean increase is around 2-3 mm Hg in systolic blood pressure, but it can be dramatic and may be greater in patients with hypertension or those taking angiotensin converting enzyme inhibitors, angiotensin II receptor blockers, or diuretics [13559].

Asthma

A systematic review showed that taking aspirin reduces forced expiratory volume in one second (FEV1) in about 20 percent of adults and 5 percent of children with asthma. There is almost total cross reactivity with the non-selective NSAIDs. However, asthma has not been produced by selective COX-2 inhibitors in double blind challenge tests on asthmatic patients sensitive to aspirin [13559].

Pregnancy

A large epidemiological study has found that both classes of NSAID may lead to abortion in the first trimester, with an odds ratio of 2.43 (95% confidence interval 2.1 to 2.8) compared with women not taking NSAIDs. Both classes of NSAID may also delay labour and lead to premature closure of the ductus arteriosus, while non-selective NSAIDs may increase blood loss at delivery. Pregnant patients should not take NSAIDs with misoprostol, which is a prostaglandin analogue used to prevent NSAID associated ulcers and may also induce miscarriage [13559].

Comparison with other analgesics
**Paracetamol**
For chronic conditions, such as osteoarthritis and back pain, paracetamol is generally recommended as first line treatment even though meta-analyses indicate that both groups of NSAID are, on average, superior in reducing pain. The reason for the preference of paracetamol is its superior tolerability compared with both classes of NSAID. Paracetamol has little gastrointestinal toxicity, minimal effect on blood pressure and renal function, no reported increase in the rate of myocardial infarction, and no effect on the antiplatelet effect of aspirin [13559].

**Opioids**
These drugs should not be used as first line treatment of milder pain because of their adverse effects such as constipation, drowsiness, and cognitive blunting. Use of opioids in elderly patients is also associated with a high incidence of falls and fractures [13559].

**In ultramarathon runners**
Despite increased 161-km ultramarathon participation in recent years, little is known about those who pursue such an activity. This study surveyed entrants in two of the largest 161-km trail ultramarathon runs in North America to explore demographic characteristics and issues that affected race performance. All entries of the 2009 Western States Endurance Run and the Vermont 100 Endurance Race were invited to complete a postrace questionnaire. There were 500 respondents among the 701 race entries (71% response). Finish time was found to have a significant negative association with training volume and was generally directly associated with body mass index. Among nonfinishers, the primary reason for dropping out was nausea and/or vomiting (23%). Finishers compared with nonfinishers were more likely to report blisters (40% vs 17%), muscle pain (37% vs 20%), and exhaustion (23% vs 14%) as adversely affecting race performance, but nausea and/or vomiting was similar between groups (37% vs 40%). Nausea and/or vomiting was no more common among those using nonsteroidal anti-inflammatory drugs (NSAIDs), those participating in the event with higher ambient temperatures, those with a lower training volume, or those with less experience at finishing 161-km races. Overall use of NSAIDs was high, and significantly greater among finishers (61%) than nonfinishers (46%). From this study, it was conclude that primary performance-limiting issues in 161-km ultramarathons include nausea and/or vomiting, blisters, and muscle pain, and there is a disturbingly high use of NSAIDs in these events [11231].

**Use in handicapped athletes**
Non-steroidal anti-inflammatory drugs (NSAIDS) are frequently used in sports medicine to reduce time of incapacity. To describe the frequency of NSAIDS use by athletes in the XVth Pan-American Games all athletes who were tested by the anti-doping control filled a form. The voluntarily declared medications were recorded and categorized according to sport modality, gender, region, and control situation according to the World Anti-doping Agency. Among the 1,261 athletes tested (231 out competition and 1030 in competition; 733 men and 528 women), 63 percent reported use of drugs, NSAIDS being the most frequently (64% of users) used medications. The use of medications was not significantly difference between genders or among different regions of the world. The number of users of only 1 type of NSAID was higher than those who used more than one type of NSAIDS or a combination with analgesics (335 cases vs 168 cases). In competition reports presented higher use of
NSAIDS than out competition. Athletes tested by the anti-doping control of the XVth Pan-American Games reported a high frequency of NSAIDS use. The frequent utilization in competition suggests that these medications might be used as ergogenic aid [09299].

Side effect

Nicolau Syndrome (also known as Embolia cutis medicamentosa and livedo-like dermatitis) is a rare but severe localized adverse drug reaction to a range of intra-muscular preparations. It manifests as acute pain, cutaneous, subcutaneous and intra-muscular inflammation and necrosis immediately following an injection, with potentially devastating sequelae. It was described the syndrome in a 21-year-old national level race walk athlete following an intramuscular diclofenac injection [09297].

Recommendations

Sports medicine physicians often treat athletes in pain with non-steroidal anti-inflammatory drugs (NSAIDs). However, there is a lack of high-quality evidence to guide NSAID use. Their adverse effects have clinical relevance, and their possible negative consequences on the long-term healing process are slowly becoming more obvious. One article provided some practical management guidelines for the use of NSAIDs, developed to help sports medicine physicians deal with frequent sports-related injuries. It was not recommend their use for muscle injuries, bone fractures (also stress fractures) or chronic tendinopathy. In all cases, if chosen, NSAID treatments should always be kept as short as possible and should take into account the specific type of injury, the level of dysfunction and pain [10213].

Non-steroidal anti-inflammatory drugs (NSAID) are commonly utilised in sports medicine. NSAID have known anti-inflammatory, analgesic, antipyretic and antithrombotic effects, although their in-vivo effects in treating musculoskeletal injuries in humans remain largely unknown. NSAID analgesic action does not appear to be significantly greater than paracetamol for musculoskeletal injury but they have a higher risk profile, with side-effects including asthma exacerbation, gastrointestinal and renal side-effects, hypertension and other cardiovascular diseases. Evidence-based working groups on pain management recommend using regular paracetamol as first-line treatment for acute and chronic musculoskeletal pain. Furthermore, intramuscular NSAID have additional risks with fluctuant drug levels, infection and muscle necrosis. NSAID works via cyclooxygenase (COX) inhibition, thus blocking prostaglandin production from arachadonic acid. Prostaglandin inhibition will decrease the cascading inflammatory response, but will also lead to increased leucotriene production through the “overflow” pathway for arachadonic acid. COX-2 inhibitors are a subclass of NSAID that specifically block the COX-2 enzyme, and subsequently have fewer gastrointestinal side-effects and renal side-effects but at a cost of an increased risk of cardiovascular side-effects. NSAID in sports medicine practice are delivered as topical, oral, intramuscular or, less commonly, intravenous preparations. Daily NSAID use in the general population is 1-4 percent and in elite athletes at Olympic games or during Fédération Internationale de Football Association World Cup football tournaments, the reported use of NSAID is as high as 25-35 percent. Given their availability, the use of NSAID to treat sporting injury in the general population is probably similar to that seen in elite sporting populations. As medical practitioners, sports physicians are obliged by the Hippocratic oath to “first do no harm”, and this discussion paper will attempt to address the utilisation of NSAID use in musculoskeletal injury pragmatically. Recent well-publicised adverse events surrounding COX-2 anti-inflammatories, in combination with an increased understanding of soft-tissue

1528
injury pathophysiology, have resulted in NSAID use in sports medicine being challenged. Early functional mobilisation is a key component to the rehabilitation and early return to sporting activity following many injuries, and pain management is integral to this. In addition to their well-documented anti-inflammatory action, NSAID are known to be moderately potent analgesics, and therefore when prescribing, one must consider whether they are being used for analgesic, anti-inflammatory or a combined benefit. Regarding muscle strain injury, concerns of increased bleeding from the anti-platelet effect of COX-1 inhibition of thromboxane A2 had traditionally led to NSAID use being a relative contraindication in the early phases, but with the advent of specific COX-2 inhibitor formulations, this became less relevant. Inflammation initiates macrophage action, with subsequent phagocytosis of necrotic tissue and stimulation of new capillary formation, vital aspects of muscle regeneration. In contrast to muscle strain injuries, the benefits of using NSAID in eccentrically induced muscle soreness is well established. The use of NSAID patches have recently been shown to be beneficial in the early management of contusion type injuries, and the risk of myositis ossificans has been shown to be reduced post-surgically by the expedient use of NSAID. NSAID are thus not contraindicated in muscle injury per se, but should be used with consideration of the specific pathology being treated. The majority of literature surrounding tendinopathy is specific to chronic tendinopathy. The histopathology of tendinopathy is typically degenerative, with an absence of inflammatory cells or biochemical markers of inflammation. The pain of tendinopathy thus not associated with bursitis, tenosynovitis or other inflammatory comorbidity could be rationally treated with paracetamol rather than NSAID. Whereas tendinopathies with an associated co-morbid inflammatory component may benefit from NSAID, it is also not uncommon for team physicians at the elite level to see the first presentation of a tendinopathy within 1-2 days of pain onset. In this situation, it may not be unreasonable to attempt to treat any suspected tendon or paratendon inflammatory-mediated symptoms with NSAID. COX-2 inhibitors have been shown to impair healing in ligament and bone, and probably should not be used to treat acute joint injuries. While several studies have shown enhanced functional recovery after ankle sprain when NSAID were employed in addition to standard rehabilitation techniques, it is suggested that while the decreased pain may allow an earlier return to army training, ultimately they have increased instability and a decreased range of motion. This may suggest that NSAID are having both an analgesic and antisynovitic benefit, rather than any effect on ligament healing. However, to uniformly exclude NSAID from the treatment armoury of ankle sprains would appear premature, based on the knowledge available, and when the predominant functional limitation may be synovitis. Inflammatory conditions such as rheumatoid arthritis clearly benefit from NSAID, and many of the co-morbidities of such conditions, in particular inflammatory bursitis, intra-articular synovitis and tenosynovitis, are common sporting presentations to the sports physician. A trial of oral NSAID before more aggressive interventions is considered acceptable practice and should not be excluded. It was recommended [09300]

- considering regular paracetamol as first-line treatment for acute and chronic musculoskeletal pain, due to comparable analgesic efficacy with NSAID but a lower side-effect profile
- NSAID are useful in treating inflammatory pathologies such as tenosynovitis and soft-tissue impingement, but not conditions such as chronic tendinopathies and fractures
- NSAID in treating muscle injury remains controversial

Medical ethics

NSAIDs are a commonly used class of drugs available both OTC and by prescription. NSAIDs are taken for a myriad of reasons, including treatment of pain, soft tissue swelling, fever, and sprains/strains, especially overuse strains. Given the easy OTC access, many
people assume that there is little risk involved in taking these medications. Although NSAIDs have shown promise in treating painful inflammatory conditions such as rheumatoid arthritis, beneficial effects of NSAIDs in common musculoskeletal injuries are not clearly established in the medical literature. The decision to use these medications should consider several issues regarding safe and appropriate use [07216].

Before prescribing NSAIDs, it also is important to consider the natural healing process when no extrinsic factors are in play and how these medicines may interfere with that process. Injuries that trigger the inflammatory cascade allow for signaling to increase blood flow to the area and recruitment of inflammatory cells that are an important part of the healing response. Although this process occurs in a predictable fashion, certain stages require a brisk inflammatory response to be successful and to prepare the injured tissue for the final reconstruction of elements to produce complete healing. NSAID inhibition of the early inflammatory response theoretically could impact or alter natural healing [07216].
AICAR (5-AMINO-4-IMIDAZOLECARBOXYAMIDE RIBONUCLEOSIDE)

AICAR (5-amino-4-imidazolecarboxyamide ribonucleoside) arguably provides performance-enhancing properties even in the absence of physical exercise and, therefore, the substance is banned in elite sports since 2009. Due to the natural presence of AICAR in human blood and urine, uncovering the misuse by direct qualitative analysis is not possible. Entering the circulation, the riboside is immediately incorporated into red blood cells (RBCs) and transformed into the corresponding ribotide (5'-monophosphate) form. Within the present study, an analytical method was developed to determine AICAR-ribotide concentrations in RBC concentrates by means of liquid chromatography-tandem mass spectrometry. The method was validated enabling quantitative result interpretation considering the parameters specificity, precision (intra- and interday), linearity, recovery, accuracy (LOD/LOQ), stability and ion suppression. By analysing 99 RBC samples of young athletes, normal physiological levels of AICAR-ribotide were determined (10-500 ng/mL), and individual levels were found to be stable for several days. Employing in vitro incubation experiments with AICAR riboside in fresh whole blood samples, the ribotide concentrations were observed to increase significantly within 30 min from baseline to 1-10 microg/mL. These levels are considered conserved for the lifetime of the erythrocyte and, thus, the results of the in vitro model strongly support the hypothesis that measuring abnormally high AICAR-ribotide concentrations in RBC of elite athletes has the potential to uncover the misuse of this substance for a long period of time [13575].
ANTICONVULSANTS

Although the World Anti-Doping Code (WADC) permits anticonvulsants in general, harmonization of antidoping permits an international sport federation (IF) to prohibit specific medications within that IF. The anticonvulsants levetiracetam, tiagabine, and lamotrigine may pose ethical dilemmas and could be considered violations of antidoping codes. Lamotrigine, with antiglutamatergic and sodium channel properties, is FDA-approved for maintenance treatment of bipolar disorder, in addition to its use in the treatment of major depression, anxiety disorders, and schizophrenia. Tiagabine, a selective GABA reuptake inhibitor, has mood-stabilizing and anxiolytic properties. Levetiracetam, whose unique mechanism involves the modulators beta-carboline and zinc, has anxiolytic and mood-stabilizing properties. Anxiolytics, antidepressants, and antipsychotics are banned in archery; under strict liability, all three anticonvulsants violate WADC/IF for that specific sport and could result in disqualification unless therapeutic use exemptions (TUEs) are obtained. Ethical issues regarding the use of anticonvulsants by athletes and the need to obtain TUEs are addressed. The WADC with harmonized IF policies are meant to prevent doping by athletes, but not appropriate medical treatment. When anticonvulsants have other psychotropic properties, ethical issues arise. Athletes should list all medications taken with diagnoses, obtain TUEs as indicated, and contact the appropriate IF or Olympic organization to determine the status of the proposed medication (banned, restricted, nonbanned). Further, clinicians should be knowledgeable regarding these issues when treating athletes [07024].
The World Antidoping Agency (WADA) has introduced some changes in the 2012 prohibited list. Among the leading innovations to the rules are that both 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (peroxisome proliferator-activated receptor-delta [PPAR-delta]-5' adenosine monophosphate-activated protein kinase [AMPK] agonist) and GW1516 (PPAR-delta-agonist) are no longer categorized as gene doping substances in the new 2012 prohibited list but as metabolic modulators in the class "Hormone and metabolic modulators" [12333].

Since January 2009, the list of prohibited substances and methods of doping as established by the World Anti-Doping Agency includes new therapeutics such as the peroxisome-proliferator-activated receptor (PPAR)-delta agonist GW1516, which is categorized as a gene doping substance. GW1516 has completed phase II and IV clinical trials regarding dyslipidemia and the regulation of the lipoprotein transport in metabolic syndrome conditions; however, its potential to also improve athletic performance due to the upregulation of genes associated with oxidative metabolism and a modified substrate preference that shifted from carbohydrate to lipid consumption has led to a ban of this compound in elite sport. In a recent report, two presumably mono-oxygenated and bisoxygenated urinary metabolites of GW1516 were presented, which could serve as target analytes for doping control purposes after full characterization. Hence, in one study now, phase I metabolism was simulated by in vitro assays employing human liver microsomal fractions yielding the same oxygenation products, followed by chemical synthesis of the assumed structures of the two abundant metabolic reaction products. These allowed the identification and characterization of mono-oxygenated and bisoxygenated metabolites (sulfoxide and sulfone, respectively) as supported by high-resolution/high-accuracy mass spectrometry with higher-energy collision-induced dissociation, tandem mass spectrometry, and nuclear magnetic resonance spectroscopy. Since urine samples have been the preferred matrix for doping control purposes, a method to detect the new target GW1516 in sports drug testing samples was developed in accordance to conventional screening procedures based on enzymatic hydrolysis and liquid-liquid extraction followed by liquid chromatography, electrospray ionization, and tandem mass spectrometry. Validation was performed for specificity, limit of detection (0.1 ng/ml), recovery (72 %), intraday and interday precisions (8-15 %), and ion suppression/enhancement effects (<10 %) [10207].
Since urine samples have been the preferred matrix for doping control purposes, a method to detect the new target GW1516 in sports drug testing samples was developed in accordance to conventional screening procedures based on enzymatic hydrolysis and liquid-liquid extraction followed by liquid chromatography, electrospray ionization, and tandem mass spectrometry. Validation was performed for specificity, limit of detection (0.1 ng/ml), recovery (72 %), intraday and interday precisions (7.7-15.1 %), and ion suppression/enhancement effects (<10 %) [10208].

Peroxisome proliferator-activated receptor delta (PPARdelta) regulates expression of genes involved in lipid and carbohydrate metabolism. To examine the association of a functional +294T/C polymorphism of PPARD gene with human physical performance, it was studied the distribution of PPARD alleles and genotypes in a cohort of athletes (n=1256; stratified by specialty and skill level) and controls (n=610). It was found that the frequency of PPARD C allele (with higher transcriptional activity compared to T allele) in a group of endurance-oriented athletes (n=898) is significantly higher than in controls (18 % vs 12 %). Moreover, in the group of endurance-oriented athletes with cyclic activity it was revealed an increasing frequency of PPARD C allele with the rising of athletes' skill level. Thus, PPARD C allele is associated with predisposition to endurance performance [07217].

The elucidation of metabolic pathways and the detection of emerging therapeutics potentially enhancing athletic performance are of paramount importance to doping control authorities to protect the integrity of elite sports. A new drug candidate belonging to the family of the peroxisome proliferator-activated receptor-delta agonists termed GW1516 (also referred to as GW501516) has been prohibited by the World Anti-Doping Agency in 2009 due to its potential to artificially increase endurance. Consequently, sports drug testing laboratories need to establish detection methods enabling the identification of the intact substance and/or its metabolite(s) that unambiguously prove the presence or absence of the target substances in doping control specimens. Simulating human metabolic reactions using liver microsomal preparations, minute amounts of possible urinary metabolites were obtained that were characterized by mass spectrometry-based methods. Subsequently, the most abundant metabolic products were chemically synthesized and as well characterized by mass spectrometry and nuclear magnetic resonance spectroscopy. Finally, GW1516 and two oxidized metabolites were implemented in a routine doping control analytical assay based on liquid chromatography-(tandem) mass spectrometry (LC-MS/MS), which was tested for its fitness-for-purpose using spiked urine samples [13567].

Sensitive and robust bioassays able to detect nuclear receptor activation are very useful for veterinary and doping control, pharmaceutical industry and environmental scientists. Here, we used bioassays based on human leukemic monocyte lymphoma U937 and human liver hepatocellular carcinoma HepG2 cell lines to detect the ligand-induced activation of the peroxisome proliferator-activated receptor delta (PPAR-delta). Exposure of U937 cells to the PPAR-delta agonist GW501516 resulted in a marked increase in mRNA expression of the PPAR-delta target gene Angptl4 which was quantified by qRT-PCR analysis. Exposure of HepG2 cells transiently transfected with a PPAR-delta expression plasmid and a PPAR-response element-driven luciferase reporter plasmid to PPAR-delta agonists GW501516, GW610742 and L-165041 resulted in clear dose-response curves. Although the qRT-PCR resulted in higher fold inductions, the luciferase assay with transfected HepG2 cells is cheaper and quicker and about ten times more sensitive to GW501516 compared to analysis of Angptl4 mRNA expression in U937 cells by qRT-PCR. The HepG2-based luciferase assay was therefore used to screen GW501516-spiked supplements and feed and water samples. After liquid extraction and clean-up by solid phase extraction using a weak anion exchange column, extracts were screened in the HepG2 bioassay followed by confirmation with a newly developed UPLC-MS/MS method, using two transitions for each compound, i.e., for
Mitochondrial dysfunction has been linked to many diseases including metabolic diseases such as diabetes. Peroxisome proliferator-activated receptor gamma co-activator 1 (PGC-1) is a superfamily of transcriptional co-activators which are important precursors to mitochondrial biosynthesis found in most cells including skeletal muscle. The PGC-1 superfamily consists of three variants all of which are directly involved in controlling metabolic gene expression including those regulating fatty acid oxidation and mitochondrial proteins. In contrast to previous reviews on PGC-1, this mini-review summarizes the current knowledge of many known dietary stimulators of PGC-1 and the subsequent mitochondrial biosynthesis with associated metabolic benefit in skeletal muscle [13569].

Performing exercise in a glycogen depleted state increases skeletal muscle lipid utilization and the transcription of genes regulating mitochondrial beta-oxidation. Potential candidates for glycogen-mediated metabolic adaptation are the peroxisome proliferator activated receptor (PPAR) coactivator-1alpaha (PGC-1alpha) and the transcription factor/nuclear receptor PPAR-delta. It was therefore the aim of the present study to examine whether acute exercise with or without glycogen manipulation affects PGC-1alpha and PPAR-delta function in rodent skeletal muscle. Twenty female Wistar rats were randomly assigned to 5 experimental groups (n 4): control [CON]; normal glycogen control [NG-C]; normal glycogen exercise [NG-E]; low glycogen control [LG-C]; and low glycogen exercise [LG-E]. Gastrocnemius (GTN) muscles were collected immediately following exercise and analyzed for glycogen content, PPAR-delta activity via chromatin immunoprecipitation (ChIP) assays, AMPK alpha1/alpha2 kinase activity, and the localization of AMPK and PGC-1alpha. Exercise reduced muscle glycogen by 47 and 75 percent relative to CON in the NG-E and LG-E groups, respectively. Exercise that started with low glycogen (LG-E) finished with higher AMPK-alpha2 activity (147 %), nuclear AMPK-alpha2 and PGC-1alpha, but no difference in AMPK-alpha1 activity compared to CON. In addition, PPAR-delta binding to the CPT1 promoter was significantly increased only in the LG-E group. Finally, cell reporter studies in contracting C2C12 myotubes indicated that PPAR-delta activity following contraction is sensitive to glucose availability, providing mechanistic insight into the association between PPAR-delta and glycogen content/substrate availability. The present study is the first to examine PPAR-delta activity in skeletal muscle in response to an acute bout of endurance exercise. Our data would suggest that a factor associated with muscle contraction and/or glycogen depletion activates PPAR-delta and initiates AMPK translocation in skeletal muscle in response to exercise [13570].

Genetics

Peroxisome proliferator-activated receptor delta (PPAR-delta; encoded by the PPARD gene) plays a role in energy metabolism and mitochondrial function. It was investigated the distribution of PPARD rs2267668, rs2016520 and rs1053049 polymorphisms, individually and in haplotype, in a cohort of 660 elite athletes which was subdivided into four different groups based on the different metabolic demands of their respective sports and 704 healthy controls. PPARD rs2016529 and rs1053049 were individually significantly associated with overall elite athletic performance and also with athletes grouped as strength endurance. Furthermore, PPARD A/C/C haplotype (rs2267668/rs2016520/rs1053049) was significantly underrepresented in all athletes and each subgroup of athletes when compared with controls, suggesting that harboring this specific haplotype is unfavorable for becoming an
elite athlete. These results help to identify which genetic profiles may contribute to elite athletic performance, specifically the role of variants within the PPARD gene, and may be useful in talent identification or optimizing the response to training [13571].

Effect of lactate

The effects of exercise are not limited to muscle, and its ability to mitigate some chronic diseases is under study. A more complete understanding of how exercise impacts non-muscle tissues might facilitate design of clinical trials and exercise mimetics. Here, we focused on lactate's ability to mediate changes in liver and brain bioenergetic-associated parameters. In one group of experiments, C57BL/6 mice underwent 7 weeks of treadmill exercise sessions at intensities intended to exceed the lactate threshold. Over time, the mice dramatically increased their lactate threshold. To ensure that plasma lactate accumulated during the final week, the mice were run to exhaustion. In the liver, mRNA levels of gluconeogenesis-promoting genes increased. While peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1alpha) expression increased, there was a decrease in PGC-1beta expression, and overall gene expression changes favored respiratory chain down-regulation. In the brain, PGC-1alpha and PGC-1beta were unchanged, but PGC-1-related co-activator expression and mitochondrial DNA copy number increased. Brain tumor necrosis factor alpha expression fell, whereas vascular endothelial growth factor A expression rose. In another group of experiments, exogenously administered lactate was found to reproduce some but not all of these observed liver and brain changes. The data suggest that lactate, an exercise byproduct, could mediate some of the effects exercise has on the liver and the brain, and that lactate itself can act as a partial exercise mimetic [13572].

Pioglitazone

Peroxisome-proliferator-activated receptor (PPAR) delta and adenosine monophosphate (AMP)-activated protein kinases (AMPKs) regulate the metabolic and contractile characteristics of myofibres. PPAR proteins are nuclear receptors that function as transcription factors and regulate the expression of multiple genes. AMPK has been described as a master metabolic regulator which also controls gene expression through the direct phosphorylation of some nuclear proteins. Since it was discovered that both PPARdelta agonists (GW1516) and AMPK activators (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, known as AICAR) are very effective performance-enhancing substances in sedentary mice, the World Anti-doping Agency (WADA) included AICAR and GW1516 in the prohibited list of substances as metabolic modulators in the class “Hormone and metabolic modulators”. Thiazolidinediones are PPAR-gamma agonists that can induce similar biological effects to those of PPAR-delta and PPAR-delta-AMPK agonists. Thus in this study, the effects of pioglitazone on mitochondrial biogenesis and performance were evaluated. Blood glucose levels and the protein expression of the intermediates involved in the mitochondrial biogenesis pathway and the citrate synthase activity were determined in both gastrocnemius and soleus muscles. Maximal aerobic velocity (MAV), endurance capacity, and grip strength before and after the training period were also determined. The MAV endurance capacity and grip strength of trained animals significantly increased. It was found that the peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) and the nuclear respiratory factor-1 (NRF-1) protein content and citrate synthase activity significantly increased in the soleus muscle of trained animals. No effect of treatment was found.
Therefore in the study, pioglitazone administration did not affect mitochondrial biogenesis signaling pathway [13573].

**Interaction with beta-adrenergic blocker**

Interaction of peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1alpha) with other cellular signalling pathways plays an important role in training-induced mitochondrial adaptations. The purpose of one study was to examine whether pyrrolidine dithiocarbamate (PDTC), a nuclear factor-kappaB inhibitor and antioxidant, and the beta-adrenergic blocker propranolol would affect the PGC-1alpha-induced mitochondrial transcription factors, enzymes and proteins involved in energy metabolism and antioxidant defense in response to endurance training. Female Sprague-Dawley rats (aged 8 weeks) were randomly divided into two groups (n=24), one subjected to 8 weeks of treadmill training and one remaining sedentary. The data indicates that nuclear factor-kappaB-inhibitory and antioxidant properties of PDTC can attenuate PGC-1alpha-mediated mitochondrial adaptation to endurance training, whereas the beta-adrenergic pathway has little adverse effect [13574].
PHOSPHODIESTERASE-4 INHIBITORS

The recent discovery of resveratrol's capability to inhibit cAMP-specific phosphodiesterases (PDEs) and, as a consequence, to enhance particularly the activity of Sirt1 in animal models has reinforced the interest of preventive doping research organizations, especially in PDE4 inhibitors. Among these, the archetypical PDE4-inhibitor rolipram significantly increased the number of mitochondria in laboratory rodents, which further demonstrated a performance increase in a treadmill-test (time-to-exhaustion) of approximately 40%. Besides rolipram, a variety of new PDE4-inhibiting substances including cilomilast, roflumilast, and numerous additional new drug entities were described, with roflumilast being the first-in-class having received clinical approval for the treatment of chronic obstructive pulmonary disease (COPD). Due to the availability of these substances, and the fact that a misuse of such compounds in sport cannot be excluded, it deems relevant to probe for the prevalence of these compounds in sports drug testing programs. Known urinary phase-I metabolites of rolipram, roflumilast, and cilomilast were generated by in vitro incubations employing human liver microsomal preparations. The metabolites obtained were studied by liquid chromatography with high-resolution/high-accuracy tandem mass spectrometry (LC/MS/MS) and the reference product ion mass spectra of established and most relevant metabolites were utilized to provide the information necessary for comprehensive doping controls. The analytical procedure was based on conventional routine doping control assays employing enzymatic hydrolysis followed by liquid-liquid extraction and subsequent LC/MS/MS measurement. Structures of diagnostic product ions and dissociation pathways of target analytes were elucidated, providing the information required for implementation into an existing test method for routine sports drug testing. The established method allowed for detection limits for the intact drugs of 1-5 ng/mL, and further assay characteristics (intraday precision 1.5-13.7 percent, interday precision 7.3-18.6 percent, recovery 20-100 percent, ion suppression/enhancement, and specificity) were determined. In addition, proof-of-concept analyses concerning roflumilast were conducted with a urine sample obtained from a COPD patient under roflumilast treatment [13517].

Another class of substances gained attention as to its potential to increase athletic performance, namely phosphodiesterase-4 (PDE4) inhibitors. A recent report on the ability of PDE4 inhibiting molecules to increase mitochondrial function and physical stamina in mice raised the question if PDE4 inhibitors are covered by the Prohibited List and if these substances are considered as banned. While one representative (roflumilast) is approved as therapeutic agent in several countries, the archetypical analog rolipram and the next-generation PDE4-inhibitor cilomilast have not (yet) received full clinical approval. In order to provide the required tools for preventive and proactive doping controls, a detection assay for these compounds and main metabolites (as generated by in vitro approaches) was developed in 2013. Also here, established sports drug test methods employing enzymatic deconjugation of substances followed by LLE and LC-MS/MS were expanded to comprise the additional target compounds. In addition to the active drugs of rolipram, roflumilast, and cilomilast, their main metabolites (dealkylated, hydroxylated, or oxo-derivatives) were included in the method and detected at 1-5 ng/mL of urine [13009].
NUTRITIONAL SUPPLEMENTS: GENERAL ASPECTS

The Oxford English Dictionary definition of supplement is: “Something added to supply a deficiency”. Yet many supplements, or their individual ingredients, are nutrients or food chemicals for which the body does not have an estimated or theoretical requirement. Thus there are clearly other factors that underpin their use by athletes. Athletes choose to consume a supplement for a number of reasons, including:

- to prevent or treat a perceived nutrient deficiency, especially when requirements for a nutrient are increased by their exercise programme
- to provide a more convenient form of nutrients in situations where everyday foods aren’t practical – particularly to address nutritional needs/goals around an exercise session
- to provide a direct ergogenic (performance-enhancing) effect
- because they believe every top athlete is consuming it and they can’t afford to miss out

Nutrition significantly influences sports performance; however, the efficacy of any nutritional supplement or strategy should be carefully considered in relation to the event and the sex, training and nutritional status of the participant. The causes of fatigue, mechanism of action, safety and legality of the supplement, together with the scientific evidence from studies with an appropriate experimental design, should all be taken into account before incorporating into the training and/or competition diet. The efficacy of ingesting nutritional supplements immediately before and/or during endurance exercise (duration 45-180 min) was reviewed. The ingestion of CES (carbohydrate-electrolyte solutions) have been shown to improve both exercise capacity and performance, either due to the maintenance of euglycaemia throughout exercise or the sparing of muscle glycogen early on in exercise. The addition of caffeine to CES may improve endurance performance as a consequence of a reduced perception of effort. Research suggests that the addition of protein to CES may only be effective when a suboptimal amount of CHO (carbohydrate) is ingested during exercise (<60 g of CHO per hour); however, recovery of performance may be enhanced due to a reduction in subsequent muscle soreness and the promotion of muscle protein synthesis after exercise. The findings from studies investigating the effects of ingesting MCTs (medium-chain triacylglycerols) and BCAAs (branched-chain amino acids), either on their own or in combination with CES, on endurance performance have been equivocal and therefore would not be recommended. Any nutritional strategy should be practised in training before being used during a competition.

Physical training and proper nutrition are paramount for success in sport. A key tissue is skeletal muscle, as the metabolic pathways that produce energy or ATP allow the muscles to complete the many activities critical to success in sport. The energy-producing pathways must rapidly respond to the need for ATP during sport and produce energy at a faster rate or for a longer duration through training and proper nutrition which should translate into improved performance in sport activities. There is also continual interest in the possibility that nutritional supplements could further improve muscle metabolism and the provision of energy during sport. Most legal sports supplements do not improve performance following oral ingestion. However, three legal supplements that have received significant attention over the years include creatine, carnitine and sodium bicarbonate. The ingestion of large amounts of creatine for 4-6 days increases skeletal muscle creatine and phosphocreatine contents. The majority of the experimental evidence suggests that creatine supplementation can improve short-term exercise performance, especially in sports that require repeated short-term

1539
sprints. It may also augment the accretion of skeletal muscle when taken in combination with a resistance-exercise training programme. Supplementary carnitine has been touted to increase the uptake and oxidation of fat in the mitochondria. However, muscle carnitine levels are not augmented following oral carnitine supplementation and the majority of well-controlled studies have reported no effect of carnitine on enhancing fat oxidation, V0\textsubscript{2max} or prolonged endurance exercise performance. The ingestion of sodium bicarbonate before intense exercise decreases the blood hydrogen to potentially assist the efflux of hydrogen from the muscle and temper the metabolic acidosis associated with intense exercise. Many studies have reported performance increases in laboratory-based cycling tests and simulated running races in the field following sodium bicarbonate ingestion where the need for ATP from substrate phosphorylation is high. However, other studies have reported no benefit and the incidence of negative side effects is high [08368].

There are indications that performance enhancing supplement use positively correlates with the probability of prohibited performance enhancing substance use [369]. Supplement taking has also been used as a proxy for attitude toward prohibited substances [08370]. It has also been found that supplement users have the need or desire to assist their performance but wish to do so by legal means, or alternatively, supplement use is the first step [08371].

In the world of athletes’ nutrition, there is a suspicion that in practice, large doses of supplements in athletes are not taken for nutritional purposes. Very often, the effects of supplements are hormone-related, or supplements influence hormone secretion. Examples of possible links between “supplements or ergogenic compounds” and the endocrine and metabolic system may be given [08372].

Although some supplements do enhance athletic performance, many have no proven benefits and have serious adverse effects. Caffeine, creatine, and sodium bicarbonate have been shown to enhance performance in certain contexts and have few adverse effects. No performance benefit has been shown with amino acids, beta-hydroxy-beta-methylbutyrate, chromium, human growth hormone, and iron. Carbohydrate-electrolyte beverages have no serious adverse effects and can aid performance when used for fluid replacement [08374].

Athletes often use supplements believing that they can help them to achieve sport success. The nutrition way of Polish athletes seems to be inappropriate. Diet contained too much fat and delivered too less nutritive constituents to cover daily dietary intake. Analysis also demonstrated differences between estimated and determined content of nutritive constituents in the food studied [08375].

Practitioners who work with elite athletes know that the pressure and considerable rewards involved with success provide a high level of motivation to look for any safe and legal strategy that might enhance performance, even by small margins. Dietary supplements operate in this space, whether they promise a large performance boost or just create the fear that an athlete cannot afford to miss out on what everyone else is using. It is often tempting to overlook the lack of evidence to support the claims made about a supplement on the basis that the stakes are higher for elite athletes; therefore the cost:benefit ratio favours experimentation in the absence of clear proof. Over the past decade, however, we have become aware that the cost of getting it wrong has also escalated for elite athletes. A new hazard related to supplement use has emerged: inadvertent ingestion of substances that are banned under the antidoping codes in place in elite sport, but present in supplement products. In some cases, the level of the presence, or contamination, of banned substances in supplements presents a health hazard for all consumers. In some cases, the concentration may be too small to achieve any health or performance effect but large enough to record an infringement for athletes who submit to doping tests. Newspapers, the internet and Courts of
Arbitration in Sport now bear stories of dedicated athletes whose careers have been or are being jeopardised because of the ingestion of a banned substance via a dietary supplement. It is clear that there is a real risk that athletes who use dietary supplements may unknowingly ingest a banned substance that will cause them to record a positive doping outcome. There are cases in which a doping infringement can be traced back to supplement use and for which the athlete has undertaken some strategies to reduce this risk. For example, the athlete has received written advice from a supplement manufacturer that their produce does not contain banned substances, but following a positive doping test, a sealed container of the dietary supplement has been examined and found to contain the banned ingredient. Unfortunately, strict liability applies to these situations and even if athletes have been successful in having the terms of their ban from sport reduced, a doping infringement will still be recorded against their name. The loss of a career, livelihood and reputation are stakes that an athlete must take into account when using dietary supplements [11010].

Many athletes use dietary supplements as part of their regular training or competition routine, including about 85 percent of elite track and field athletes. Supplements commonly used include vitamins, minerals, protein, creatine, and various "ergogenic" compounds. These supplements are often used without a full understanding or evaluation of the potential benefits and risks associated with their use, and without consultation with a sports nutrition professional. A few supplements may be helpful to athletes in specific circumstances, especially where food intake or food choice is restricted. Vitamin and mineral supplements should be used only when a food-based solution is not available. Sports drinks, energy bars, and protein-carbohydrate shakes may all be useful and convenient at specific times. There are well-documented roles for creatine, caffeine, and alkalinizing agents in enhancing performance in high-intensity exercise, although much of the evidence does not relate to specific athletic events. There are potential costs associated with all dietary supplements, including the risk of a positive doping result as a consequence of the presence of prohibited substances that are not declared on the label [07218].

Performance enhancing drugs, ergogenic aids, and supplements come in many forms. The financial, personal, social, and health-related impact of these substances has wide and varied consequences. This article reviews common substances and practices used by athletes. It discusses the history, use, effects, and adverse effects of androgenic anabolic steroids, peptide hormones, growth factors, masking agents, diuretics, volume expanders, β-blockers, amphetamines, caffeine, other stimulants, and creatine. The evidence base behind the use, safety, and efficacy of these items as well as testing for these substances is discussed [13577].

An athlete taking a spiked supplement with intent to dope may claim that he/she did not realise the product contained a prohibited substance. It is difficult to know the intent: nevertheless, the athlete will benefit from the ergogenic effect of the prohibited substance and have an unfair advantage over their competitor. There are many cautionary tales of athletes taking energy boosting supplements before or during the games and subsequently being sanctioned. Many dietary supplements that promise to enhance performance either contain a prohibited substance or are an example of false advertising. The reality is that a significant percentage (5-20 %) of supplements contain prohibited substances, either by inadvertent contamination or deliberate adulteration, during the production process. This phenomenon has been demonstrated repeatedly and sporting federations as well as anti-doping organisations continue to impress this warning upon athletes. For example, several athletes have been recently sanctioned over the stimulant methylhexaneamine (MHA), explicitly prohibited since 2009. This was considered to be a dietary supplement from geranium oil, despite the fact that several studies, including a very recent one demonstrated that its presence in supplements was not from geranium oil but due to the addition of
synthetic MHA. Whether natural or synthetic, athletes need to avoid these types of products. Many athletes continue to take supplements to try and improve recovery from training or to gain a performance edge in competition or are advised that supplements are necessary for health maintenance. Dietary supplement use by high-level athletes is estimated at 65-95 percent. Supplement commercialisation is a multibillion dollar industry where many claims are made with little scientific evidence; regulation for purity or side effects is still lacking in many countries. Members of the athletes’ entourage often push substances without sufficiently understanding physiology or nutrition. There are a very limited number of dietary supplements which are permitted and considered ergogenic. Athletes need to focus on proper training, optimal recovery practices and wholesome nutrition regimens before they even consider supplements. Some education programmes appear to be resulting in decreased supplement use among Olympic athletes [13015].

**Governance around sports supplementation**

Supplementation has become an integral part of most high-performance sporting programmes. This is true around the world. Any supplementation programme in high-performance sport should be based on three pillars:

- Athlete safety
- Evidence-based science
- Compliance with the World Anti-Doping Agency (WADA) Code.

If these three pillars underpin a sporting organisation’s supplementation policy, the sporting organisation will minimize the risk of being implicated in a doping scandal. High-performance sporting organisations, even relatively small organisations, should have a “supplementation panel” comprising at least three of a sports nutritionist, the head of strength and conditioning, the athletic performance manager and the team/organisation doctor. This panel needs to decide the range of supplements that are going to be used by the organisation/team, understanding that the above “three pillars” must be satisfied when including any particular supplement in the programme. Supplementation protocol should be such that there can be no alteration to the supplements used without unanimous agreement of the panel. That is, no single member of the panel should be able to introduce a new supplement or strategy without the agreement of their peers [13027].

If an athlete truly believes he or she should take a dietary supplement, according to a WADA statement everything should be done to minimise the risk [13015]:

- Do not rely on advice from friends, fellow athletes or coaches but undergo a proper evaluation by a qualified physician and/or sports nutrition professional familiar with sport and anti-doping rules. It is quite likely that dietary supplements are not necessary and nutrient deficiencies may be corrected from food sources
- Avoid any product making claims of performance enhancement or any exaggerated claims or uses the words: “stimulant, energy or muscle booster, enhancer, legal or alternate steroid, extreme, blast, weight loss”. Even if no prohibited substance is listed on the label, the product may be spiked with one
- Herbal stimulants and prohormones are especially high risk. Use of the terms herbal or natural does not in any way mean that the product does not contain a prohibited substance
- Some companies offer guarantees of purity or are certified by other companies that do quality control. Verify the third party testing system reputation and remember there are no absolute guarantees
- Avoid any company that states their products are WADA approved. WADA or its accredited laboratories never test supplements or any products when not part of a doping control process. WADA cannot recommend any company or quality control system. In order to guarantee purity, each product batch would have to be tested for all prohibited substances
- Avoid products containing multiple ingredients as there is a higher risk of contamination. Vitamins and minerals (often classified as supplements) should be from reputable pharmaceutical companies and should not be mixed with other products
- Seek guidance from your anti-doping organisation about recent information on contaminated or dangerous products in your part of the world

Although athletes must exhibit utmost caution when using supplements due to risk of contamination, governmental authorities also have a duty to endeavour to ensure proper regulation and quality control within the supplement industry. Some initiatives are trying to improve this situation. According to Article 10, UNESCO International Convention against Doping in Sport, governments must encourage producers and distributors of dietary or nutritional supplements to establish marketing best practices, including accurate labelling, quality assurance and avoidance of false marketing. In a survey (UNESCO Conference of Parties 2011), only 43 percent of governments responded that they implemented extensive or substantial measures to address these issues. Furthermore, a more systematic approach is needed for analysing risks and benefits of dietary supplements. The real risks to health of ingesting potentially dangerous substances contained in poorly regulated supplements are occasionally lost in the discussion of inadvertent doping. Anti-doping regulations were developed over many years to promote fairness in sport and to protect the health of the athlete. Athletes are keen to improve their performance and nutrition may play an integral part in their overall plan. However, when athletes embark upon using performance-enhancing supplements, the risks often outweigh the benefits. Improved regulation of the dietary supplement industry would go a long way towards reducing the risks, but the onus remains on the athlete to make the right choices [13015].

**Role of sports medicine specialists**

The involvement of the sports medicine specialist doctor in the supplement panel is critical. The organisations that are under most scrutiny in Australia appear to have allowed supplementation to take place without the input or oversight of the team doctor. This highlights what was believed to be a widespread weakness in high-performance sport. Team doctors providing sports medicine services to high-performance teams often limit their involvement to matters of illness and injury, allowing supplementation policies and protocols to be controlled by others in the organisation. Such flawed systems must be eliminated from high-performance sport. Medical practitioners who are not prepared to maintain the currency of their own knowledge around supplementation and doping and further contribute to ethical supplementation practices should not be involved with high-performance sporting teams [13027].

**Legislation**

There is no clear distinction between the regulation of food, supplements and medicines in
South Africa. Consequently, grey areas exist in implementing the legislation, particularly in the supplement industry. The increase in supplement sales in South Africa can be attributed to aggressive marketing by manufacturers whose claims are not always supported by published peer-reviewed evidence. Such claims often go unchecked, resulting in consumers being mislead about the role of supplements. As a result of poor regulation, contaminants or adulterants in supplements may also cause insidious effects unrelated to the listed ingredients. To assess the regulations, legislation, and claims associated with nutritional supplement products in South Africa peer-reviewed literature and the relevant South African statutes were consulted. The National Health Act incorporates the Medicine Control Council, which is charged with ensuring the safety, quality and effectiveness of medicines, and related matters, including complementary/alternative medicines. The South African Institute for Drug-Free Sport and Amendment Act provides for testing athletes for using banned substances, but currently does not concern itself with monitoring nutritional supplements for contaminants or adulterants that may cause a positive drug test, which has implications for sports participants and also the health of the general population. The implementation of the Consumer Protection Act 68 of 2008 (CPA) could protect consumer rights if it is administered and resourced appropriately [11370].

Theoretical aspects

Diet can significantly influence athletic performance, but recent research developments have substantially changed our understanding of sport and exercise nutrition. Athletes adopt various nutritional strategies in training and competition in the pursuit of success. The aim of training is to promote changes in the structure and function of muscle and other tissues by selective modulation of protein synthesis and breakdown in response to the training stimulus. This process is affected by the availability of essential amino acids in the post-exercise period. Athletes have been encouraged to eat diets high in carbohydrate, but low-carbohydrate diets up-regulate the capacity of muscle for fat oxidation, potentially sparing the limited carbohydrate stores. Such diets, however, do not enhance endurance performance. It is not yet known whether the increased capacity for fat oxidation that results from training in a carbohydrate-deficient state can promote loss of body fat. Preventing excessive fluid deficits will maintain exercise capacity, and ensuring adequate hydration status can also reduce subjective perception of effort. This latter effect may be important in encouraging exercise participation and promoting adherence to exercise programmes. Dietary supplement use is popular in sport, and a few supplements may improve performance in specific exercise tasks. Athletes must be cautious, however, not to contravene the doping regulations. There is an increasing recognition of the role of the brain in determining exercise performance: various nutritional strategies have been proposed, but with limited success. Nutrition strategies developed for use by athletes can also be used to achieve functional benefits in other populations [12364].

Scientific nutrition strategy

It was investigated whether an athlete’s self-chosen nutrition strategy (A), compared with a scientifically determined one (S), led to an improved endurance performance in a laboratory time trial after an endurance exercise. S consisted of about 1000 mL/h fluid, in portions of 250 mL every 15 min, 0.5 g sodium/L, 60 g glucose/h, 30 g fructose/h, and 5 mg caffeine/kg body mass. Eighteen endurance-trained cyclists (16 male; 2 female) were tested using a randomized crossover-design at intervals of 2 weeks, following either A or S. After a warm-up, a maximal oxygen uptake test was performed. Following a 30-min break, a 2.5-h
endurance exercise on a bicycle ergometer was carried out at 70% maximal oxygen uptake. After 5 min of rest, a time trial of 64.37 km (40 miles) was completed. The ingested nutrition was recorded every 15 min. In S, the athletes completed the time trial faster (128 vs 136 min) and with a significantly higher power output (212 vs 184 W). The intake of fluid, energy (carbohydrate-, mono-, and disaccharide), and sodium was significantly higher in S compared with A during the endurance exercise. In the time trial, only sodium intake was significantly higher in S. It was concluded that a time trial performance after a 2.5-h endurance exercise in a laboratory setting was significantly improved following a scientific nutrition strategy [12365].

**Attempts to measure effects of supplements**

The Danish Fitness and Nutrition Council has examined the scientific literature to evaluate the performance and health-related aspects of consuming dietary supplements in the context of physical activity. Certain nutritional supplements such as creatine and caffeine have documented ergogenic effects in specific situations. However, for the moderately physically active adult and healthy individual, who already consumes an energy- and nutrient balanced diet, consuming any currently legal dietary supplement does not seem to confer additional benefits on performance or health [09309].

Several nutritional strategies have been used in cycling to improve performance. Carbohydrate feeding during exercise has been shown to be effective, but recent studies have suggested that recommendations may have to be adjusted to take into account recent findings. Protein co-ingested with carbohydrate during exercise has received a lot of recent interest, but the evidence is equivocal, at best. Thus, in the absence of a plausible mechanism, it is difficult to see how protein would increase endurance performance. There also has been a lot of interest in training with low glycogen to maximize training adaptations, but the longer-term effects upon performance are still unclear. Various supplements have been suggested to improve endurance performance, but most of these nutrition supplements lack the scientific support that would warrant the recommendation [09310].

The regular performance of resistance exercises and the habitual ingestion of adequate amounts of dietary protein from high-quality sources are two important ways for older persons to slow the progression of and treat sarcopenia, the age-related loss of skeletal muscle mass and function. Resistance training can help older people gain muscle strength, hypertrophy muscle, and increase whole body fat-free mass. It can also help frail elderly people improve balance and physical functioning capabilities. Inadequate protein intake will cause adverse metabolic and physiological accommodation responses that include the loss of fat-free mass and muscle strength and size. Findings from controlled feeding studies show that older persons retain the capacity to metabolically adjust to lower protein intakes by increasing the efficiency of nitrogen retention and amino acid utilization. However, they also suggest that the recommended dietary allowance of 0.8 g protein per kg and day might not be sufficient to prevent subtle accommodations and blunt desired changes in body composition and muscle size with resistance training. Most of the limited research suggests that resistance training-induced improvements in body composition, muscle strength and size, and physical functioning are not enhanced when older people who habitually consume adequate protein (modestly above the recommended daily intake) increase their protein intake by either increasing the ingestion of higher-protein foods or consuming protein-enriched nutritional supplements [08373].

*Measurements of enzyme levels*
Total creatin kinase (CK) and lactate dehydrogenase (LDH) serum levels depend on age, gender, race, muscle mass, physical activity, and climatic conditions. High creatine kinase serum levels in athletes following rest and without any further predisposing factors should prompt a full diagnostic workup, with special regards to signs of muscle weakness or other signs not always evident. In subjects who have silent myopathy, repeated intense prolonged exercise may produce negative effects, because given the continuous loss of muscle proteins, it does not induce the physiological muscle adaptations to physical training. Serum total LDH and specific isozyme activities change with the training status of the athlete. Variation in LDH isozymes profile might have a role in studying muscle response to training [08376].

**Molecular markers in dietary supplement research**

In laboratory and clinical studies of diet and dietary supplement interventions more overt clinical markers include imaging tests, biopsy samples, prostate-specific antigen kinetics, and urinary testing. Many molecular markers are currently available, including antiapoptotic and apoptotic proteins, cell adhesion molecules, cell cycle compounds, growth factors, angiogenic markers, and proliferative and inflammatory signals. Protein kinases and transcription factors should also be considered for diversity. Testing of numerous molecular markers has become critical in gaining preliminary insight into the potential impact of a novel diet and supplemental agents [11373].

**Analysis of actual content in supplements**

In recent years the availability of so-called legal highs over the Internet has hugely increased. There has been a recent explosion in the number of substances that can be purchased from the Internet marketed as legal highs. A survey of Internet availability published in April 2009 found 39 individual UK online legal-high retailers selling a total of 1308 products. These substances are sold as “not for human consumption” and for a variety of uses, such as research chemicals, plant food, and bath salts, but it is clear that they are intended for human consumption and that the intended market is as a replacement for either 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) or cannabis on the recreational drugs scene. In fact, recent figures suggest that there has been a decrease in the use of MDMA with the suggestion that this is not due to a decrease in recreational drug use but simply a switch to more readily available legal highs. The term “legal high” carries with it a perception of safety and acceptability and although many of the compounds advertised as being the active ingredient in the currently marketed products are not controlled, many of these have as yet not been tested on humans and the purity of the products is unknown. This is an unregulated area and is simply being driven by the market. 2009 and 2010 saw a significant increase in legislation in the UK to try and curb the availability and use of legal highs with a number of modification orders to the Misuse of Drugs Act. Analysis of 24 products claiming to be legally purchased from 18 UK-based websites obtained in a six-week period following the April 2010 ban of cathinones revealed that 63 percent of the products contained these controlled substances. Clearly this is unknown to the user and may well be unknown to the online retailer. As substances are controlled, new substances quickly appear and are marketed as being better than previous products. The synthesis of many of the active ingredients of these second-generation legal highs such as 5,6-methylenedioxy-2-aminoindane (MDAI), 5-iodo-2-aminoindane (5-IAI), and 6-(2-aminopropyl)benzofuran (6-APB) have been previously published; however, little is known about their effects on humans. Another problem highlighted is that substances labeled as the same product may in fact be structural isomers of the expected active ingredient which are unknown within the literature.
Numerous online legal-high retailers market a broad variety of products which are advertised as research chemicals, bath salts, or plant food although clearly intended for human consumption as recreational drug replacements. No guidelines exist as to what is sold and in what purity. Consumers are led to believe that purchased goods are entirely legal. In one study, several legal-high products were purchased and analyzed for their content. The powdered products were screened with attenuated total reflectance – Fourier Transform Infrared (ATR-FTIR) followed by gas chromatography-mass spectrometry (GC-MS) analysis of methanol extracts. Spectra were compared to reference standards and the NIST library. Results showed that 6 out of 7 products did not contain the advertised active ingredient. Moreover, five samples contained the controlled substances benzylpiperazine and 1-[3-(trifluoromethyl)phenyl]piperazine combined with caffeine. Seven samples were purchased online as legal highs and characterized by FTIR and GC-MS. The FTIR study revealed that 6 out of 7 samples did not contain the claimed drug but large quantities of caffeine. The presence of large amounts of caffeine was confirmed by GC-MS. Moreover, it was found that only one of the purchased samples contained MDAI as claimed. Five samples were a mixture of the controlled substances BZP and 3-TFMPP combined with caffeine. As BZP and 3-TFMPP are both controlled substances, users of these Internet products are in possession of illegal substances having purchased them assuming them to be legal. Another issue of concern, previously highlighted by others, is the lack of consistency between products of the same name. If purchasers use different suppliers for the same-named product, it is also highly likely that they could be using products with different active ingredients.

**Quality assurance of dietary supplements**

The use of dietary supplements is widespread in the general population, in athletes and recreational exercisers, and in military personnel. A wide array of supplements is available, but protein-containing products are consistently among the most popular, especially among those who engage in resistance training. There are significant risks associated with the use of unregulated dietary supplements. Risks include the absence of active ingredients, the presence of harmful substances (including microbiological agents and foreign objects), the presence of toxic agents, and the presence of potentially dangerous prescription-only pharmaceuticals. There is ample evidence of athletes who have failed doping tests because of the use of dietary supplements. There is also growing evidence of risks to health and of serious adverse events, including a small number of fatalities, as a result of supplement use. The risk associated with the use of protein powders produced by major manufacturers is probably low, and the risk can be further reduced by using only products that have been tested under one of the recognized supplement quality assurance programs that operate in various countries. Nevertheless, a small risk remains, and athletes, soldiers, and other consumers should conduct a cost-benefit analysis before using any dietary supplements.

**Perceptions of nutritional supplementation**

The purpose of one investigation was to examine the nutritional supplement intake of athletes from a state-based sports institute. Athletes (n=72) from seven sports (kayaking, field hockey, rowing, waterpolo, swimming, athletics and netball) completed a questionnaire detailing their daily usage and rationale therefore. The large majority (63/72) of surveyed athletes reported using nutritional supplements, with no difference between female and male athletes. Kayakers (6.0) consumed a higher number of nutritional supplements than swimmers (4.0), field hockey (1.5), rowing (2.4), waterpolo (2.3), athletics (2.5) and netball (1.7) athletes. The athletes believed that nutritional supplements are related to performance.
enhancements (65 %), positive doping results (63 %), and that heavy training increases supplement requirements (65 %). The cohort was equivocal as to their health risks (56 %) or their need with a balanced diet (53 %). The most popular supplements were minerals (46 %), vitamins (43 %), other (32 %), iron (31 %), caffeine (22 %), protein (17 %), protein-carbohydrate mix (14 %), creatine (13 %) and glucosamine (4 %). The majority of supplementing athletes (n=63) did not know their supplements active ingredient (62 %), side effects (57 %) or mechanism of action (54 %) and admitted to wanting additional information (57 %). Only half of the athletes knew the recommended supplement dosages (53 %). The performance enhancing perception may explain the large proportion of athletes that reported using nutritional supplements, despite over half of the athletes believing that supplements are not required with a balanced diet and can cause positive doping violations [10216].

Motives for use of supplements

One study examined incidence of sport-related injury, interest in supplements to treat injury, and sources of supplement information among 145 college athletes (89 males, 56 females). A survey was used to assess sport-related injuries, interest in three categories of supplements to treat injury, and sources of supplement information among college athletes who used athletic training room and weight training facilities. Sport-related injuries were experienced by 91 percent of athletes (93 % males, 88 % females). Overall, 17 percent of participants were interested in supplements to improve circulation, 34 percent for joint and soft tissue repair, and 22 percent to reduce inflammation. Significant sex differences were not found for any supplements in any categories evaluated. Males were more likely than females to rely on strength coaches (37 %, 20 %) for supplement information. Athletic trainers (71 % of athletes), coaches (60 %), and physicians (41 %) were the primary professionals, and the internet (79 %), magazines (68 %), and television (52 %) the most popular sources of media for supplement information. The majority of athletes experience injury during their college athletic career and 17 to 34 percent express an interest in supplements for injury treatment. Athletes would benefit from scientifically sound guidance to identify appropriate supplements for injury treatment and internet sites for supplement information. Future research should identify if athletes are more likely to increase supplement use when they are injured or if supplement use is more prevalent among athletes who are prone to injury [07228].

The widespread use of nutritional supplements among athletes is poorly understood. The prevalence of supplement intake and users' knowledge have been researched independently leading to useful, but disconnected, information on supplement use. The "UK Sport 2005 Drug Free Survey" data (n=874) were re-analysed. The use of selected supplements varied widely as follows: multivitamin (73 %), vitamin C (70 %), echinacea (31 %), iron (30 %), magnesium (11.0%) and ginseng (8.3%). Associations with motive were found in 8 of the 10 test pairs which were expected from literature precedents, however only weak associations exist. Of these, four were associated with avoidance of sickness, multivitamin, vitamin C, and Echinacea. The remaining 4 associations were: no time to prepare meals with ginseng and multivitamin, overcoming injuries with magnesium. These results suggest a lack of understanding regarding supplements and health maintenance, except for vitamin C and echinacea. Furthermore, supplement use is apparently independent of physicians/dieticians' advice, except for iron. This may suggest a widespread circumvention of expert advice in the growing area of supplement use and therefore should be addressed to underscore potential health risks [07229].

Use of supplements administered by a doctor to the Canadian Olympic athletes was measured by a questionnaire. The percentage of current users was 69 percent in the Atlanta
Games and 74 percent in the Sydney Games. Use of supplements was also measured by a self administered questionnaire distributed to the World Master Athletes in Germany. A total of 69 percent of the men used nutritional supplements. The use of supplements in the general US population of men was lower, around 47 percent in the National Health and Nutrition Examination Survey. This survey suggests that subjects reporting strenuous physical activity are more likely to be supplement consumers. The high use of supplements in professional athletes is not surprising, especially because they are recommended in order to reduce physical exercise-induced oxidative stress and tissue damage. Growing evidence indicates that reactive oxygen species are responsible for exercise-induced protein oxidation and contribute to muscle fatigue. In addition, high-level physical activity may lead to suppression of the immune system, which could be compensated for by supplementation with dietary micronutrients [07230].

Attitudes towards dietary supplements

The aim of one study was to examine the prevalence of (daily) dietary-supplement (DS) use among elite adolescent athletes and to differentiate use by different types of DS according to their function. Data were analyzed for associations between users of these DS types, sociodemographic, sport-specific characteristics, and opinion on the need for DS. In addition, sources of supply and information were examined. In the framework of the GOAL Study, 1,138 German elite adolescent athletes (14-18 years) answered questions about DS. The data were analyzed to identify groups at risk for using DS after a classification by supplemental function. Of the young athletes, 91.1% reported DS use during the previous month. (Daily) DS use was significantly associated with gender, kind of sport, and the weekly duration of sporting activity. Furthermore, some athletes were required to use DS by their sporting organization. DS use was more likely in these athletes than in those whose sporting organizations had no such requirement. Overall, DS with short- and long-term supplemental function were mostly associated with the use of magnesium. However, DS with medium-term muscle-building function played an important role among daily users. The main source of information about DS was coaches; main source of supply was parents. Professional education is urgently needed, as 9 out of 10 athletes used DS, and strong positive opinions toward the use of DS were present, particularly in the DS users [12374].

Stacking

In one summary, stacking was defined as the consumption of two or more supplements at the same time in an attempt to maximise results. This practice originated from anabolic steroid regimes of body builders and other resistance training athletes where products were combined, often to address the side effects or potentiate the actions of individual products. A more global view of supplement stacking in sports nutrition finds that supplement combinations can be deliberate or unintentional. Deliberate stacking aims to take advantage of positive physiological responses that arise from combining nutrients, foods or compounds (e.g. vitamin C enhancing iron absorption). Few studies have examined the possible benefits to stacking and have produced equivocal results despite theoretical support. Combining creatine with beta-alanine had a unique effect on delaying fatigue while stacking creatine with hydroxy-B-methylbutyrate produced additive effects on lean body mass. The combination of beta-alanine (intracellular) and bicarbonate (extracellular) buffers for events involving sustained high-intensity exercise that produces significant acidosis warrants comprehensive study. While stacking some supplements may provide additional benefits, some athletes may not be aware that taking multiple supplement products creates the potential for doubling up on some ingredients or co-ingesting ingredients that are best not combined. Inadvertent or unintentional stacking increases the risk of unintended negative
consequences or adverse reactions such as interference with nutrient absorption (e.g., combining caffeine and iron supplements). Overdosing on single ingredients from multiple sources can also occur. For example, toxic levels of zinc may be consumed from the combination of daily dietary intake (15+mg), one or more multivitamin mineral supplements (15+mg), protein shake (10 mg), recovery shake (10 mg), sports bar (15 mg) and immunity supplement (5+mg); this total of over 70 mg/day exceeds the upper safe limit of 45 mg. High zinc levels are assessed and monitored with a blood test, with high levels increasingly being reported in regular and frequent sports supplement use. Athletes should be aware of combining supplements and possible adverse reactions and have supplement use reviewed regularly by a registered sport nutritionist or sports dietitian [12300].

For injury prevention

The topic of exercise-induced skeletal muscle injury has received considerable attention in recent years. Likewise, strategies to minimise the injury resulting from heavy resistance exercise have been studied. Over the past 15 years, several investigations have been performed focused on the role of nutritional supplements to attenuate signs and symptoms of muscle injury. Of these, some have reported favourable results, while many others have reported no benefit of the selected nutrient. Despite these mixed findings, recommendations for the use of nutritional supplements for the purposes of attenuating muscle injury are rampant within the popular fitness media and athletic world, largely without scientific support. Those nutrients include the antioxidant vitamin C (ascorbic acid) and vitamin E (tocopherol), N-acetyl-cysteine, flavonoids, L-carnitine, astaxanthin, beta-hydroxy-beta-methylbutyrate, creatine monohydrate, essential fatty acids, branched-chain amino acids, bromelain, proteins and carbohydrates. A discussion of all published peer-reviewed articles in reference to these nutrients and their impact on resistance exercise-induced skeletal muscle injury is presented, in addition to a brief view into the potential mechanism of action for each nutrient. Based on the current state of knowledge, the following conclusions can be made with regard to nutritional supplements and their role in attenuating signs and symptoms of skeletal muscle injury occurring as a consequence of heavy resistance exercise: there appears to be a potential role for certain supplements (vitamin C, vitamin E, flavonoids, and L-carnitine); these supplements cannot effectively eliminate muscle injury, only attenuate certain signs and symptoms; it is presently unclear what the optimal dosage of these nutrients is (whether used alone or in combination); it is unclear what the optimal pretreatment period is; and the effectiveness is largely specific to non-resistance trained individuals. Ultimately, because so few studies have been conducted in this area, it is difficult to recommend with confidence the use of selected nutrients for the sole purpose of minimising signs and symptoms of resistance exercise-induced muscle injury, in particular with regard to resistance-trained individuals [07219].

Effects of energy conditioning on food preferences and choice

One study investigated the development of conditioned preferences for foods varying in energy content in human adults in a laboratory setting. In a within-subjects design, 44 participants consumed high and low energy yoghurt drinks (255 kcal and 57 kcal per 200 ml serving, respectively) first thing in the morning following 8 h of fasting, every day for two weeks, with 5 exposures to each yoghurt drink on alternate days. The high and low energy yoghurt drinks were paired with two coloured labels (blue or pink), with the pairings fully counter-balanced. Every day of the third (test) week, participants were given a free choice of either consuming the pink or the blue labelled yoghurt drink. Participants chose the high energy drink significantly more often over the low energy drink, suggesting a conditioned preference for a delayed (energy) reward. These findings provide further evidence for energy
based learning in human adults. This study also provides a new approach to the conditioning paradigm (cueing energy via a coloured label instead of flavour) and includes a new and important measure in this research area (preference instead of liking) [11372].

**Side effects of dietary supplements**

In recent years there has been a significant increase in the consumption of dietary energy supplements (DES) associated with the parallel advertising against obesity and favoring high physical performance. It was presented a case and outcome of a young patient who developed acute mixed liver injury (hepatocellular and cholestatic) after ingestion of various "over the counter" products to increase muscle mass and physical performance (NO Xplode®, creatine, L-carnitine, and Growth Factor ATN®). The diagnosis was based on the exclusion of other diseases and liver biopsy findings. The dietary supplement and herbal multivitamins industry is one with the highest growth rates in the market, with annual revenues amounting to billions and constantly lacking scientific or reproducible evidence about the efficacy and/or safety of the offered products. Furthermore, and contrary to popular belief, different forms of injury associated with these natural substances have been documented particularly in the liver, supporting the need of a more strict regulation [12375].

**Nutritional supplement screening**

Sweetened beverage and fast-food intake have been identified as important targets for obesity prevention. However, there are few brief dietary assessment tools available to evaluate these behaviors among adolescents. The objective of one research was to examine reliability and validity of a 22-item dietary screener assessing adolescent consumption of specific energy-containing and non-energy-containing beverages (nine items) and fast food (13 items). The screener was administered to adolescents (ages 11 to 18 years) recruited from the Minneapolis/St Paul, MN, metro region. One sample of adolescents completed test-retest reliability of the screener (n=33, primarily white adolescents). Another adolescent sample completed the screener along with three 24-hour dietary recalls to assess criterion validity (n=59 white adolescents). Test-retest assessments were completed approximately 7 to 14 days apart, and agreement between the two administrations of the screener was substantial, with most items yielding Spearman correlations and kappa statistics that were >0.60. When compared to the gold standard dietary recall data, findings indicate that the validity of the screener items assessing adolescents' intake of regular soda, sports drinks, milk, and water was fair. However, the differential assessment periods captured by the two methods (i.e. 1 month for the screener vs 3 days for the recalls) posed challenges in analysis and made it impossible to assess the validity of some screener items. Overall while these screener items largely represent reliable measures with fair validity, the findings highlight the challenges inherent in the validation of brief dietary assessment tools [09320].

**Appropriate regulations**

A large number of recreational and elite athletes use nutritional supplements in hopes of improving performance. These aids can be costly and potentially harmful, and the advertised ergogenic gains are often based on little or no scientific evidence. Due to the lack of regulation of the dietary supplement industry, an abundance of supplement products of dubious value, content, and quality are now available around the world. Many supplement products contain substances that are prohibited in sport or that have been associated with significant morbidity and mortality. For athletes, lack of knowledge or misinformation has
been established despite numerous sources of information being available, and the reasons for, and implications of, unsupervised and unrestricted supplement use require further attention. In addition to the necessity of an appropriate regulation of dietary supplements, nutritional education and scientifically sound guidance for athletes is required. Intervention and prevention efforts should be particularly targeted to adolescents [09321].

Quantitation of use of supplements in sports

To determine dietary supplementation practices and opinions, preferred means for dietary supplement (DS) education, and antidoping opinions among elite Canadian athletes varying in age and gender. A total of 582 high-performance athletes (314 male, 268 female) representing 27 sports with a mean age of 20 years completed a validated questionnaire assessing dietary supplement practices and opinions by recall. Sport categories included varsity, Canadian Sport Centre Calgary, and National Sport School. There was extensive DS use, with 88 percent of participants taking one or more dietary supplement (mean of 3 DS per user) during the previous 6 months. Overall, sport drinks (22 %), sport bars (14 %), multivitamins and minerals (14 %), protein supplements (9 %), and vitamin C (6 %) were most frequently reported. Older athletes were significantly more likely to report greater DS usage; to be advised by teammates, health food store retailers, and magazines; to prefer supplementation education via individual interviews; to claim awareness of anti-doping rules; and to perceive anti-doping compliance. Relative to gender, significant differences were observed for the types of dietary supplement reported; supplementation advisors; justifications for dietary supplement use; and awareness of anti-doping regulations. It was concluded that utilization of this validated and reliable questionnaire has the potential for broad use and provides insight into the factors that influence dietary supplement use in elite athletes [07227].

Athletes use dietary supplements in order to increase energy, maintain strength, enhance performance, maintain health and immune system and prevent nutritional deficiencies. A recent increase in DS use has been observed in various sports and especially among elite athletes. There are several studies estimating that supplement use among athletes is common and varies between 59 to 88 percent multivitamins, minerals, proteins and energy drinks being most common products being consumed. Most supplement users consume more than one product and the amount of supplements used varies between age groups, gender and different sports. A Norwegian study reported a great difference of supplement use between different sport groups: power sport athletes had the most frequent use of supplemental creatine, proteins/amino acids, vitamins and minerals while cross-country skiers had the most frequent intake of iron, vitamin C and fish oils. Athletes are willing to use many kinds of dietary supplements, although researches have not been able to prove that most supplements perform as claimed. In their recent statement, American Dietetic Association (ADA) lists ergogenic aids into four groups according to their safety and efficiency:

- those that perform as claimed
- those that may perform as claimed but for which there is insufficient evidence of efficacy at this time
- those that do not perform as claimed
- those that are dangerous, banned, or illegal and, therefore, should not be used.

Group one contains creatine, caffeine, sport drinks, gels and bars, sodium bicarbonate and proteins and amino acids. On the contrary, group three includes majority of the ergogenic
aids currently on the market including widely used ginseng and branched chain amino acids. When it comes to vitamin and mineral supplementation, according to ADA using them does not improve performance among individuals who consume nutritionally adequate diets. The aim of yet another study was to assess the frequency of use of dietary supplements (DS) among large sample of elite Finnish athletes and to describe possible changes in dietary supplement use between the years 2002 and 2009 in a prospective follow-up study conducted on Olympic athletes. The first survey was conducted on Olympic athletes in 2002 (n=446) and the follow-up study was conducted between 2008 and 2009 (n=372). In 2002, a total of 81 percent of the athletes used dietary supplements (a mean of 3 ± 3 DS per user) and in 2009, a total of 73 percent of the athletes (a mean of 3 ± 3 per DS user) used them. After adjusting for age-, gender- and sport type, the odds ratio for use of any dietary supplement was significantly less in 2009 as compared with 2002 results. Decrease in DS use was observed in all supplement subgroups (vitamins, minerals, nutritional supplements). Athletes in speed and power events and endurance events reported use of any dietary supplement significantly more often than team sport athletes both in 2002 and 2009. In year 2009, the frequency of all dietary supplement use increased when athlete's age increased and the increase was significant in older age groups: of the athletes under 21 years 63 percent, 21-24 years 83 percent and over 24 years 90 percent consumed nutritional supplements. Based in our study, there seems to be a lowering trend of dietary supplement use among elite Finnish athletes although differences between sport subgroups and age groups are considerable [11244].

Although dietary nutrient intake is often adequate, nutritional supplement use is common among elite athletes. However, high-dose supplements or the use of multiple supplements may exceed the recommended daily allowance (RDA) of particular nutrients or even result in a daily intake above tolerable upper limits (UL). One case report presented nutritional intake data and supplement use of a highly trained male swimmer competing at international level. Habitual energy and micronutrient intake were analysed by 3 daily dietary reports. Supplement use and dosage were assessed, and total amount of nutrient supply was calculated. Micronutrient intake was evaluated based on RDA and UL as presented by the European Scientific Committee on Food, and maximum permitted levels in supplements (MPL) are given. The athlete's diet provided adequate micronutrient content well above RDA except for vitamin D. Simultaneous use of ten different supplements was reported, resulting in excess intake above tolerable UL for folate, vitamin E and Zn. Additionally, daily supplement dosage was considerably above MPL for nine micronutrients consumed as artificial products. Risks and possible side effects of exceeding UL by the athlete are discussed. Athletes with high energy intake may be at risk of exceeding UL of particular nutrients if multiple supplements are added. Therefore, dietary counselling of athletes should include assessment of habitual diet and nutritional supplement intake. Educating athletes to balance their diets instead of taking supplements might be prudent to prevent health risks that may occur with long-term excess nutrient intake [11245].

Intake of dietary supplements is widespread among athletes in developed countries. This study evaluated the use of dietary supplements in athletes from a developing country. Dietary supplementation practices of 113 national-level athletes age 15-35 years in Sri Lanka were assessed. All athletes from track-and-field, badminton, football, swimming, cycling, and karate squads who consented to participate in the study were administered an anonymous questionnaire by an interviewer. Information on number of supplements taken, frequency of use, nature of product, rationale, sources of advice, and reasons for taking supplements was obtained. Most athletes (94 %) consumed dietary supplements. On average, 3.7 products/day were consumed. Footballers had significantly lower intake of supplements than other athletes (footballers 71 %, others 98 %). They also consumed fewer products per day (footballers 0.7, others 3.5). Popular supplements included multivitamins, vitamin E, calcium,
energy foods and drinks, and creatine. Multiple supplement use was common, with 29 percent athletes taking 4 products/day. The athletes sought advice on supplement use from sports doctors (45 %), team coaches (40 %), or friends (15 %). Most took supplements to improve performance (79 %), and 19 % claimed to take supplements to improve their overall health status [10217].

Survey data from 847 high-performing athletes in the UK were analysed using descriptive statistics. The survey, conducted by UK Sport, consisted of questions regarding knowledge of the prohibited substances, testing procedure, nutritional supplement use and perceptions of the doping problem. The proportion of supplement users and the relative use of each supplement were compared by age, gender and professional status. Among the 874 high-performing athletes in the UK sample, 59 percent reported the use of at least one nutritional supplement. Among supplement users, 83 percent used more than one and 12 percent reported use of more than five nutritional supplements. The average number of supplements used by athletes in the users’ sample was 3 (1674 instances with 520 athletes), indicating that supplements are used in combinations. Of the 9 supplements listed, multivitamins (73 %) and vitamin C (71 %) were used most, followed by creatine (36 %), whey protein (32 %), echinacea (31 %), iron (30 %) and caffeine (24 %). Less than 11 percent reported the use of magnesium or ginseng. Creatine use was typically associated with males regardless of status and across all ages, whereas iron was characteristically used by females. A “typical” supplement user is male, between 24 and 29 years of age, involved in professional sport and using a combination of supplements. Male professional players between age 30 and 34 years, and female non-professional athletes between 24 and 29 years of age also represented a considerable proportion of supplement users. Athletes older than 40 years of age were practically non-users. Concomitant use of supplements is characteristic of male users more than females [08377].

One study examined incidence of sport-related injury, interest in supplements to treat injury, and sources of supplement information among 145 college athletes (89 males, 56 females). A survey was used to assess sport-related injuries, interest in three categories of supplements to treat injury, and sources of supplement information among college athletes who used athletic training room and weight training facilities. Sport-related injuries were experienced by 91 percent of athletes (93 % males, 88 % females). Overall, 17 percent of participants were interested in supplements to improve circulation, 34 percent for joint and soft tissue repair, and 22 percent to reduce inflammation. Significant sex differences were not found for any supplements in any categories evaluated. Males were more likely than females to rely on strength coaches (37 %, vs 20 %) for supplement information. Athletic trainers (71 % of athletes), coaches (60 %), and physicians (41 %) were the primary professionals, and the internet (79 %), magazines (68 %), and television (52 %) the most popular sources of media for supplement information [08380].

A recent study estimates that 15.2 percent of American adults use nonprescription dietary supplements for weight loss. Sale of ephedrine- and ephedrine-alkaloid-containing products was prohibited by the Food and Drug Administration in February 2004 after research demonstrated an increased risk of arrhythmia, mortality and hypertension following use of products containing these sympathomimetics. Subsequently, nutritional supplement manufacturers have turned to other products to promote weight loss. In a case report a 28-year-old woman with no prior psychiatric history who was hospitalized secondary to an acute psychotic episode. The patient reported starting several weight-loss and nutritional sports supplements approximately one week prior to admission. The relationship between the onset of psychosis and the initiation of the dietary supplements strongly suggests a correlation exists [08381].
The purpose of one investigation was to examine the nutritional supplement intake of athletes from a state-based sports institute. Athletes (n=72) from seven sports (kayaking, field hockey, rowing, waterpolo, swimming, athletics and netball) completed a questionnaire detailing their daily usage and rationale therefore. The large majority (63/72) of surveyed athletes reported using nutritional supplements, with no difference between female and male athletes. Kayakers (6.0 ± 2.9) consumed a higher number of nutritional supplements than swimmers (4.0 ± 2.2), field hockey (1.5 ± 1.0), rowing (2.4 ± 1.4), waterpolo (2.3 ± 2.4), athletics (2.5 ± 1.9) and netball (1.7 ± 1.0) athletes. The athletes believed that nutritional supplements are related to performance enhancements (47/72), positive doping results (45/72), and that heavy training increases supplement requirements (47/72). The cohort was equivocal as to their health risks (40/72) or their need with a balanced diet (38/72). The most popular supplements were minerals (46 %), vitamins (43 %), other (32 %), iron (31 %), caffeine (22 %), protein (17 %), protein-carbohydrate mix (14 %), creatine (13 %) and glucosamine (4 %). The majority of supplementing athletes (n=63) did not know their supplements active ingredient (62 %), side effects (57 %) or mechanism of action (54 %) and admitted to wanting additional information (57 %). The performance enhancing perception may explain the large proportion of athletes that reported using nutritional supplements, despite over half of the athletes believing that supplements are not required with a balanced diet and can cause positive doping violations [09313].

Combination with anabolic steroids

The aim of one study was to describe the prevalence, trends and associated factors of dietary supplements and anabolic-androgenic steroids (AAS) use among Finnish adolescents. The sample comprised 30 511 adolescents aged 12-18 years, of which 22 519 (74 %) answered a questionnaire. It was also studied associations between 14 socioeconomic, health and health behavioural variables and dietary supplements and AAS use by logistic regression. The proportion of respondents using dietary supplements was 45 percent during the past year and it increased linearly by age. Vitamins (37 %) and herbal products (13 %) were the most common dietary supplements. In 1991, 9 percent of the boys aged 16-18 years reported protein use, while the frequency in 2005 was 17 percent which was a significant increase. Anabolic steroid use was uncommon; only 53 boys (0.5 %) and 20 girls (0.2 %) reported AAS use. The strongest factors associated with dietary supplements use in multivariate model were physical exercise outside sports clubs (odds 1.9), and in sports clubs (odds ratio 1.7). Recurrent drunkenness (odds ratio 5.8) and peer drug use in boys (odds ratio 2.1) were the risk factors for AAS use, whereas physical exercise outside sports clubs (odds ratio 0.3) was a protecting factor. Although the overall use of dietary supplements remained at the same level during the study period, there was a slight trend towards increasing use of vitamin and protein supplements. Dietary supplements use is associated with frequent sports participation and poorer than average health, while AAS use is associated with health-compromising behaviours [09314].

Germany

Little is known about the prevalence and motives of supplement use among elite young athletes who compete on national and international levels. Therefore, the current survey was performed to assess information regarding the past and present use of dietary supplements among 164 elite young athletes (17 ± 3 years of age). A 5-page questionnaire was designed to assess their past and present (last 4 weeks) use of vitamins, minerals, carbohydrate, protein, and fat supplements; sport drinks; and other ergogenic aids. Furthermore, information about motives, sources of advice, supplement sources, and supplement contamination was assessed. Eighty percent of all athletes reported using at least 1
supplement, and the prevalence of use was significantly higher in older athletes. Among supplement users, minerals, vitamins, sport drinks, energy drinks, and carbohydrates were most frequently consumed. Only a minority of the athletes declared that they used protein/amino acids, creatine, or other ergogenic aids. Major motives for supplement use were health related, whereas performance enhancement and recommendations by others were less frequently reported. Supplements were mainly obtained from parents or by athletes themselves and were mostly purchased in pharmacies, supermarkets, and health-food stores. Among all athletes, only 36 percent were aware of the problem of supplement contamination. The survey shows that supplement use is common and widespread among German elite young athletes. This stands in strong contrast to recommendations by leading sport organizations against supplement use by underage athletes [09316].

Spain

One article describes a study that evaluated the adequacy of 2 different menu settings in a group of elite adolescent Spanish soccer players. Five-day food intake was assessed on 2 occasions, while athletes were consuming a flexible "buffet-style" diet (B; n=33) and a fixed "menu-style" diet (M; n=29). For all principal meals of the day food weighing was performed, and snacks were recorded by self-report. M provided significantly higher total energy and carbohydrate intakes than B. Breakfast and snacks both provided more energy in M. Calories obtained from fat were excessive in both settings. Calcium and vitamin D were below recommendations in B but not in M. Fiber, magnesium, folate, vitamin A, and vitamin E intake fell below recommended values in both settings. M provided significantly greater quantities of magnesium and vitamins D and E. Both feeding options were far from optimal in satisfying current scientifically based recommendations for active adolescents [07234].

The abuse of all types of substance to improve sport performance and physical fitness has spread to regularly gym users. The aim of one study was to evaluate the intake of nutritional and dietary supplements in a group of 415 individuals (260 males and 155 females) from 4 gyms in Seville (Spain). The users completed a previously designed questionnaire whose content validity had been tested in a pilot study. Out of the total sample, 56 percent had consumed a supplement at some time. Among these, the objective was improvement of physical appearance in 57 percent, health care in 17 percent, and sports performance enhancement in 13 percent. The profile of the supplement consumer is a young man who has performed activities in gyms for some time, goes to the gym for several hours a week and is on some type of diet. The percentage of nutritional supplement users (56 %) is within values reported in other studies. The five supplements most frequently consumed by these individuals were (in decreasing order): proteins (28 %), L-carnitine (19 %), sport drinks (18 %), creatine (17 %) and vitamin complex (17 %) [09317].

Greece

The aim of one study was to monitor the nutritional status of 9 Greek national top-level swimmers during a competitive season of eight months. The swimmers were assessed through recording of food and supplement intake, blood sampling, and anthropometry at four landmarks: in the beginning of the season (baseline), after completing a phase of intensive and voluminous training (at 10 weeks), at a minor taper (19 weeks), and during the major taper (32 weeks). Energy and macronutrient intake did not change significantly over time, and only a few significant changes were found in micronutrient intakes. Low carbohydrate and high fat intakes (e.g. 36 and 42 % of total energy, respectively, in males), inadequate intake of some micronutrients, and improper use of supplements indicated suboptimal dietary habits. Blood hemoglobin fluctuated significantly during the season. No significant changes in parameters indicative of the iron stores (transferrin saturation and ferritin) were found,
although iron intake increased by supplementation with the onset of training. Serum markers of training stress were not significantly altered. In conclusion, Greek top-level swimmers should be guided toward a balanced diet and a rational use of supplements. Monitoring of dietary intakes during a competitive season is highly recommended [07233].

Italy

The aims of one study were to collect data on consumption of different food supplements in a sample of the adult Italian population and to characterize users by demographic, physical and health-related characteristics, lifestyle and behaviour. The study was conducted in 2008 in ten towns of Italy (two towns from each of the five macro-areas: Northwest, Northeast, Centre, South and Islands). Adults (n=10 000) aged ≥18 years were randomly selected and asked to fill in a self-administered questionnaire regarding their use of food supplements and the above variables. The effect of these variables on food supplement use was evaluated by univariate and multivariate logistic regression. Of the 1723 individuals who returned the questionnaire, 49 % were users of food supplements. A large proportion (54 %) of users used more than one category of food supplement: vitamin and/or mineral supplements were the most used (61 %), followed by supplements with botanicals and botanical extracts (28 %). The results obtained by logistic regression showed that gender, town size, education level, sports practice, regular use of wholemeal cereal-based foods and presence of a low stress level were determinants for the use of food supplements in the examined population. However, these determinants were not shared by all categories of supplements. The results of this preliminary study highlight that associations between demographic, dietary and lifestyle factors and use of different categories of food supplements differ according to products, and cannot be accounted for simply by dichotomizing individuals as users or non-users [12367].

Slovenia

Little is known about the prevalence of nutritional supplement use in European adolescents. We conducted the present study to analyse the prevalence of nutritional supplement use and factors associated with this use among Slovenian adolescents. The nutritional supplementation practices of 818 adolescents were studied using an anonymous questionnaire. Information was sought on the type of supplements used, frequency of use and sources of information. Some 19 percent of all the schoolchildren from twenty primary schools and twelve secondary schools reported using at least one nutritional supplement and the prevalence of use was significantly higher in adolescents who were members of sports clubs. Multivitamins were the most common nutritional supplement. Older adolescents were significantly more likely to be supplementing with iron, protein and minerals. Less than 16 percent of supplement users in the study sought information from health-care professionals. Nearly 62 percent obtained information from parents and coaches, and many adolescents appear to decide on nutritional supplementation themselves, without advice. Older adolescents were significantly more likely to combine supplements than younger adolescents. It was concluded that one-fifth of Slovenian adolescents use nutritional supplements. There are clear differences in supplement use between younger (age 12 years) and older (age 17 years) adolescents. Multiple use of supplements, coupled with self-managed supplementation in older adolescents, is concerning. Hence, there is an urgent need to provide accurate information regarding nutritional supplements, which will help adolescents, their parents and coaches to make informed choices about their use [12368].

Poland
The purpose of one study was to determine the prevalence of the use of dietary supplements among the young people exercising in fitness rooms in Kraków and environs. In the study, 81 percent of the population used some form of dietary supplement; nutritional supplements were used by 21 percent of women and 60 percent of men, and were used most frequently by young people 21-25 years of age. The most popular supplements were creatine (20 %) and HMB (beta-hydroxy-beta-methylbutyrate) (19 %). Only 14 percent of the respondents asked for the opinion of qualified personnel, such as doctors, pharmacists or dieticians.

Singapore

Nutritional supplements used by athletes can be classified as sports food, dietary supplements and ergogenic aids. The aim of one study was to examine the use of such supplements among university athletes in Singapore. Eighty-two athletes from 16 sport disciplines completed a questionnaire which sought information on demographical parameters, type of supplements, frequency of use, motivations, knowledge, expenditure and side effects. The prevalence of supplement use was 77 percent and 20 different products were used. Each individual consumed a mean and standard deviation of 3.2 ± 1.7 products over a 12-month period. The mean number of products consumed daily was 2.1 ± 1.2. Popular products included sports drinks, vitamin C, multivitamins and traditional/herbal preparations such as essence of chicken, bird’s nest and ginseng. Before using a product, 66 percent sought information, usually from the media, the Internet, coaches and fellow athletes. However, many did not know where to obtain reliable information and 86 percent were also unaware that supplementation can have adverse effects. Although there is a high prevalence of supplement use in the study population, many do not have accurate information about these products. Hence, there is an urgent need to provide athletes with education and access to scientific and unbiased information.

Canada

Dietary supplementation is a common practice in athletes with a desire to enhance performance, training, exercise recovery, and health. Supplementation habits of elite athletes in western Canada have been documented, but research is lacking on supplement use by athletes across Canada. The purpose of this descriptive study was to evaluate the dietary supplementation practices and perspectives of high-performance Canadian athletes affiliated with each of the country’s eight Canadian Sport Centres. Dietitians administered a validated survey to 440 athletes (63 % women, 37 % men; age 20 ± 5 years) representing 34 sports who predominantly trained ≥16 hr/week, most competing in “power” based sports. Within the previous 6 months, 87 percent declared having taken ≥3 dietary supplements, with sports drinks, multivitamin and mineral preparations, carbohydrate sports bars, protein powder, and meal-replacement products the most prevalent supplements reported. Primary sources of information on supplementation, supplementation justification, and preferred means of supplementation education were identified. Fifty-nine percent reported awareness of current World Anti-Doping Agency legislation, and 83 percent subjectively believed they were in compliance with such anti-doping regulations. It was concluded that supplementation rates are not declining in Canada, current advisors on supplementation for this athletic population are not credible, and sports medicine physicians and dietitians need to consider proactive strategies to improve their influence on supplementation practices in these elite athletes.

It is well documented that athletes report greater dietary supplement (DS) usage than nonathletes; however, limited data exist for Canadian athletes, especially relative to competitive performance levels. One descriptive and analytical, cross-sectional research
investigated DS practices and opinions, preferred means for DS education, and antidoping opinions among elite Canadian athletes competing at various performance levels. Subjects completed a validated questionnaire by recall. Combined, 582 high-performance athletes (314 M, 268 F) between the ages of 11 and 42 years and representing 27 sports activities participated. Respondents were categorized into five competitive performance levels: provincial (68), national (101), North America (61), international or professional (89), and varsity (263). Overall, most (88 %) reported taking one or more DS during the previous 6 months (mean 3 ± 2 DS per user). From a total of 1555 DS declared, sport drinks (22 %), sport bars (14 %), multivitamins and minerals (14 %), protein supplements (9 %), and vitamin C (6 %) were most frequently reported. Athletes at the highest performance level were significantly more likely to use protein supplements, to be advised by strength trainers regarding DS usage, to have a higher self-rating of their diet, to prefer individual interviews for DS educational purposes, to perceive greater awareness of antidoping legislation, and train more h/week. Furthermore, differences were observed for the types of DS reported and justifications for use. This dataset, the first of its kind in Canada, was generated with a validated and reliable questionnaire and has the potential to be extended nationally and internationally to provide greater insight into the patterns and opinions of elite athletes regarding supplementation and antidoping [06247].

To learn more about the prevalence of dietary supplement and medication use by Canadian athletes in the Olympic Games in Atlanta 1996 and Sydney 2000 data were collected from personal interviews with Canadian athletes who participated at the 1996 Atlanta and 2000 Sydney Olympic Games. The athletes were interviewed by Canadian physicians regarding the use of vitamins, minerals, nutritional supplements, and prescribed and over-the-counter medications. Of the 271 Canadian athletes who participated at the Atlanta Olympics, 257 athletes were interviewed; at the Sydney Olympics, 300 of 304 Canadian athletes were interviewed to get a quantitative and qualitative description of the use of dietary supplements by Canadian athletes at the Atlanta and Sydney Olympics. At the Atlanta Games, 69 percent of the athletes used some form of dietary supplements, whereas 74 percent of the athletes used dietary supplements at the Sydney Games. Vitamins were taken by 59 percent of men and 66 percent of women in Atlanta, and 65 percent of men and 58 percent women in Sydney. Mineral supplements were used by 16 percent of men and 45 percent of women in Atlanta, and 30 percent of men and 21 percent of women in Sydney. Nutritional supplements were used by 35 percent of men and 43 percent of women in Atlanta, and 43 percent of men and 51 percent of women in Sydney. The most popular vitamins were multivitamins in both Olympics. The most popular mineral supplements were iron supplements. The most commonly used nutritional supplement in Atlanta was creatine (14 %), but amino acids (15 %) were the most commonly used nutritional supplement in Sydney. In Atlanta, 61 percent of the athletes were using some form of medication, 54 percent of the athletes were using medications in Sydney. Nonsteroidal antiinflammatory drugs (NSAIDS) were the most commonly used medications at both Olympic Games. Among all sports, the highest prevalence of vitamin use occurred in boxing (91 %) in Atlanta and swimming (76 %) in Sydney. Rowers (56 %) and cyclists (73 %) demonstrated the highest use of mineral supplements. Nutritional supplement use occurred most often in swimming (56 %) and cycling (100 %). The use of NSAIDs was highest in softball (60 %) in Atlanta and gymnastics (100 %) in Sydney. The review demonstrates that dietary supplement use was common among Canadian athletes at both the Atlanta and Sydney Olympic Games. There was a slight increase in total dietary supplement use at the Sydney Games. Widespread use of supplements, combined with an absence of evidence of their efficacy and a concern for the possibility of “inadvertent” doping, underscore the need for appropriately focused educational initiatives in this area [06248].

Use of supplements administered by a doctor to the Canadian Olympic athletes was
measured by a questionnaire. The percentage of current users was 69 percent in the Atlanta Games and 74 percent in the Sydney Games. Use of supplements was also measured by a self-administered questionnaire distributed to the World Master Athletes in Germany. A total of 69 percent of the men used nutritional supplements. The use of supplements in the general US population of men was lower, around 47 percent in the National Health and Nutrition Examination Survey. This survey suggests that subjects reporting strenuous physical activity are more likely to be supplement consumers. The high use of supplements in professional athletes is not surprising, especially because they are recommended in order to reduce physical exercise-induced oxidative stress and tissue damage. Growing evidence indicates that reactive oxygen species are responsible for exercise-induced protein oxidation and contribute to muscle fatigue. In addition, high-level physical activity may lead to suppression of the immune system, which could be compensated for by supplementation with dietary micronutrients [07230].

To determine the nutritional intake of Canadian high-performance athletes a prospective survey study of 324 high-performance athletes (114 males and 201 females) from 8 Canadian sport centers participated in the study. Subjects prospectively completed a 3-day dietary records, reporting all food, fluid, and supplement consumption. Average daily energy intake was $2533 \pm 843$ Kcal/day with males consuming significantly more calories than females ($2918 \pm 927$ and $2304 \pm 713$ Kcal/day, respectively). Both genders consumed below recommended levels. Carbohydrate, protein, and fat accounted for 53, 19, and 28 percent of daily calorie intake, respectively. Average daily carbohydrate and protein intake was $5.1 \pm 1.8$ and $1.8 \pm 0.6$ g/kg body weight, respectively. Protein intake, but not carbohydrate intake, met recommendations. Supplementation significantly increased athletes' energy, total carbohydrate, protein, and fat intake. Of 17 micronutrients assessed, intake ranged between 120 and 366 percent of recommended daily intake with food alone and between 134 to 680 percent of recommended daily intake with supplements. It was concluded that Canadian high-performance athletes do not consume adequate energy or carbohydrates. However, their intake of micronutrients exceed current recommended daily intakes, even when supplements are not considered, indicating that athletes make high-quality food choices [09315].

**USA**

*College athletes*

The objective of one study was to assess dietary intakes and eating habits of female college athletes and compared them with the minimum sports nutrition standards. Data were obtained from 52 female college athletes from a National Collegiate Athletic Association (NCAA) Division I university between 2009 and 2010. Participants completed anthropometric measurements and dietary assessment using a 3-day food record, a 24-hour recall, and a nutrition questionnaire. Statistics indicated the energy and carbohydrate intakes were below the minimum recommended amount, with only 9 percent of the participants meeting their energy needs. Seventy-five percent of the participants failed to consume the minimum amount of carbohydrates that is required to support training. The majority of the participants reported no regular breakfast, 36 percent consumed <5 meals/day, and only 16 percent monitored their hydration status. Effective nutrition interventions are needed to improve dietary intakes and eating habits of female college athletes [13579].

While the use of performance enhancing substances by professional, collegiate, and Olympic athletes is well described, the rate of use in the general population is not well studied. It was explored the use of energy drinks, dietary supplements, and prescription medications for the enhancement of athletic performance among college students using an ongoing survey.
system. It was conducted a multi-round online questionnaire collecting data from self-identified students at two-year colleges, four-year colleges, online courses, or technical schools at least part-time during the specified sampling period. The sample is obtained through the use of a survey panel company in which respondents voluntarily register. Survey data were collected from December, 2010 through August, 2011. Subjects who reported participating in athletics were asked if they used any of the following substances to enhance athletic performance (1) energy drinks (2) dietary supplements (3) prescription medications within the last year. Data were analyzed from October, 2011 through January, 2012. There were 462 college students who responded to the survey reporting they participate in sports at various levels. Of these, 397 (86 %) responded that within the last year they used energy drinks, dietary supplements, or prescription medications to enhance athletic performance. Energy drinks had the highest prevalence (80 %), followed by dietary supplements (64 %) and prescription medications (53 %). Use was most prevalent amongst intercollegiate athletes (89 %) followed by club (89 %) and intermural (82 %) participants. The vast majority of survey respondents reported using energy drinks, dietary supplements, and prescription medications within the last year for athletic performance enhancement [13580].

**Supplement inquiries**

To characterize the types of drug and dietary supplement inquiries submitted to the National Center for Drug Free Sport through the Resource Exchange Center athletes and athletic personnel associated with the National Collegiate Athletic Association (NCAA) it was classification he drugs and dietary supplement of inquiries. Inquiries for prescription medications for albuterol inhalers, methylphenidate, amphetamines, and prednisone were the most common using a drug lookup function. The most common inquiries for over-the-counter medications included pseudoephedrine, loratadine, cetirizine, and caffeine. Among dietary supplements, inquiries for amino acids/metabolites, vitamins and minerals, and herbal products occurred most frequently. One dietary supplement, N.O.-Xplode (Bio-Engineered Supplements and Nutrition, Inc.), accounted for the majority of individual dietary supplement inquiries. Banned substances accounted for 30 percent of all inquiries submitted to the REC and 18 percent of medications searched in a drug lookup database. Almost 25,000 inquiries were submitted to the REC. Pharmacists can use this information to advise, counsel, and refer NCAA athletes regarding the use of banned and permitted substances. Education programs regarding stimulants, dietary supplements, and the risk of using substances such as animal byproducts are needed, and pharmacists can participate in these programs [13581].

**US position statement**

To help athletic trainers promote a “food-first” philosophy to support health and performance, understand federal and sport governing body rules and regulations regarding dietary supplements and banned substances, and become familiar with reliable resources for evaluating the safety, purity, and efficacy of dietary supplements a position statement was made. The dietary supplement industry is poorly regulated and takes in billions of dollars per year. Uneducated athletes need to gain a better understanding of the safety, eligibility, and efficacy concerns associated with choosing to take dietary supplements. The athletic trainer is a valuable athletic team member who can help in the educational process. In many cases, athletic trainers are asked to help evaluate the legality, safety, and efficacy of dietary supplements. Proper nutrition and changes in the athlete's habitual diet should be considered first when improved performance is the goal. Athletes need to understand the level of regulation (or lack thereof) governing the dietary supplement industry at the international, federal, state, and individual sport-participation levels. Athletes should not assume a product is safe simply because it is marketed over the counter. All products athletes are considering using should be evaluated for purity (i.e. truth in labeling), safety, and efficacy [13582].
US children

Dietary supplements may improve sport performance in adults. However, this has not been established in children. The aim of this study was to assess self-reported or parental-reported dietary supplement use to enhance sports performance among the child subset of the National Health Interview Survey (NHIS) dataset and determine national population estimates for that use. NHIS 2007 Child Alternative Medicine files containing records for children aged 18 years were used. Typical demographic variables were utilized as well as parental presence; parental education level; use of any herb, vitamin, and/or mineral use for sports performance by children; and age. Most (95%) who reported using supplements used multivitamin and/or mineral combinations followed by fish oil/omega-3 s, creatine, and fiber. Males were more likely users (OR = 2.1) and whites reported greater usage. Mean user age was 11. Most were US born and reported living with both parents. Parents and children report child use of a wide variety of herbal and vitamin/mineral supplements to improve sports performance. Usage could be predicted by age, gender, and level of education but less likely by parent-based demographics [12369].

Saudi Arabia

The objective of one study was to understand the usage patterns of dietary supplements among professional athletes in Saudi Arabia. The survey consisted of sixteen questions divided into four categories: use of supplements, reason for consumption of supplements, personal beliefs about supplements, and behavior. The questionnaires were given to the three teams residing in Riyadh: Al Hilal, Al Nasr, and Al-Shabab. Out of the 105 athletes surveyed, we found that only 98 are currently taking dietary supplements and the mean age was 26. The survey results showed a high percentage of athletes (93%; n=98) using different dietary supplements throughout the season, 44 percent (n=43) reported using supplements for performance, and 33 percent (n=32) believed in health benefits as a reason for using dietary supplements. The results showed that a total of 87 (89%), 81 (83%), and 51 (52%) athletes are consuming sports drinks, vitamin C, and multivitamins, respectively. Meanwhile, those supplements ranking among the least used included omega 6 (19%), creatine (16%), and Ginkgo biloba (10%). A majority of athletes indicated that their use of supplements was for the purpose of improving their health and performance [13583].

Oman

Adequate dietary intake is crucial for optimum training and performance of athletes. There is almost no available information related to dietary practices among Omani athletes, especially during the competition. One study aimed to assess the nutritional practices (nutritional knowledge, eating habits and daily nutrients intake) among Omani male handball athletes in Muscat city, Oman. It was a cross sectional study including 35 male handball athletes involved in serious training for no less than three years. Data collection was done through personal interviews using a study questionnaire which enlisted questions relating to socio-demographic information, anthropometric measurements and nutritional practices. All the study participants declared no intake of anabolic steroids. The mean age of the study participants was 27 ± 3 years. Their anthropometric assessment revealed that their mean height was 166 ± 12 cm, mean weight was 75 ± 10 kg, and body mass index was 27 ± 3. Nutritional knowledge analysis revealed that 80 percent had no nutritional supervision by a nutritionist/dietitian. Their knowledge of nutritional requirements was only 23 percent correct for total energy intake, 63 percent for protein intake, 46 percent for carbohydrate intake, 11 percent for fat intake and 83 percent for water intake. Eating habits indicated that 55 percent had <3 meals/day, 51 percent had lunch as the principal meal, 51 percent always added extra salt to their food, 28 percent took protein supplements on a daily basis, and 51% used pre-competition glycogen load diet. However, none consumed vitamins or mineral
supplements. The mean daily caloric intake was 3674 ± 265 kcal/day, which was roughly comprised of 596 ± 66 g carbohydrates, 147 ± 28 g of protein and 78 ± 20 g of total fat.

**Judo**

One research investigated the use of dietary supplement patterns and doping awareness among high-ranked Judoists from two countries. Korean (70 males and 31 females) and Japanese (37 males and 34 females) national Judo team members were divided into two groups (high and low competitive performance levels) according to their international and national rankings. Fifty-nine percent of Korean and 61 percent of Japanese Judoists consumed dietary supplements. Eighty-eight percent of high and 51% of low competitive performance level Korean Judoists consumed dietary supplements. Sixty-eight percent of high and 57 percent of low competitive performance level Japanese Judoists consumed dietary supplements. Oriental supplements (34 %), vitamins (23 %), and protein powder (12 %) were most commonly consumed dietary supplements in Korean Judoists. Otherwise, vitamins (45 %), protein powder (33 %), and minerals (15 %) were most commonly consumed dietary supplements in Japanese Judoists. Thirty-eight percent of Judoists from both countries had not received any proper education about anti-doping and 44 percent of Judoists from both countries had not received about knowledge of anti-doping legislation. There was a significant difference in education about anti-doping between high and low competitive performance levels of Korean Judoists. Korean Judoists received significantly less anti-doping education than Japanese Judoists. The findings showed Judoists' use of dietary supplement from both countries was increased followed by experiencing anti-doping education.

**Sailing**

Olympic sailing classes were first used in sailing (also known as yachting) during the 1896 Olympic Summer Games. Since then, 46 different classes have been used. As of this writing, 8 Olympic classes are currently used. Apart from tactical and strategic factors, performance
in Olympic sailing relates directly to the sailors’ ability to overcome the external forces imposed on the boat. Although dietary supplements (DSs) in sports are considered a natural need resulting from athletes’ increased physical demands, and although they are often consumed by athletes, data on DS usage in Olympic sailing are scarce. The aim of one study was to study the use of and attitudes towards DSs and doping problems in high-level competitive sailing. The sample consisted of 44 high-level sailing athletes (5 of whom were female; total mean age 24 years) and 34 coaches (1 of whom was female; total mean age 37). An extensive, self-administered questionnaire of substance use was used, and the subjects were asked about sociodemographic data, sport-related factors, DS-related factors (i.e., usage of and knowledge about DSs, sources of information), and doping-related factors. DS usage is relatively high. More than 77 percent of athletes consume DSs, and 38 percent do so on a regular basis (daily). The athletes place a high degree of trust in their coaches and/or physicians regarding DSs and doping. The most important reason for not consuming DSs is the opinion that DSs are useless and a lack of knowledge about DSs. The likelihood of doping is low, and one-third of the subjects believe that doping occurs in sailing (no significant differences between athletes and coaches). The logistic regression found crew number (i.e., single vs. double crew) to be the single significant predictor of DS usage, with a higher probability of DS consumption among single crews. It was concluded that because of the high consumption of DSs future investigations should focus on real nutritional needs in sailing sport. Also, since athletes reported that their coaches are the primary source of information about nutrition and DSs, further studies are necessary to determine the knowledge about nutrition, DSs and doping problems among athletes and their support teams (i.e. coaches, physicians, and strength and conditioning specialists) [12371].

Football

Physical training and competition in football markedly increase the need for macro- and micronutrient intake. This requirement can generally be met by dietary management without the need for dietary supplements. In fact, the efficacy of most supplements available on the market is unproven. In addition, players must be cautious of inadequate product labelling and supplement impurities that may cause a positive drug test. Nonetheless, a number of dietary supplements may beneficially affect football performance. A high endurance capacity is a prerequisite for optimal match performance, particularly if extra time is played. In this context, the potential of low-dose caffeine ingestion (2-5 mg/kg body mass) to enhance endurance performance is well established. However, in the case of football, care must be taken not to overdose because visual information processing might be impaired. Scoring and preventing goals as a rule requires production of high power output. Dietary creatine supplementation (loading dose: 10-20 g/day, 4-5 days; maintenance dose: 2-5 g/day) has been found to increase muscle power output, especially during intermittent sprint exercises. Furthermore, creatine intake can augment muscle adaptations to resistance training. Team success and performance also depend on player availability, and thus injury prevention and health maintenance. Glucosamine or chondroitin may be useful in the treatment of joint pain and osteoarthritis, but there is no evidence to support the view that the administration of these supplements will be preventative. Ephedra-containing weight-loss cocktails should certainly be avoided due to reported adverse health effects and positive doping outcomes. Finally, the efficacy of antioxidant or vitamin C intake in excess of the normal recommended dietary dose is equivocal. Responses to dietary supplements can vary substantially between individuals, and therefore the ingestion of any supplement must be assessed in training before being used in competition. It is recommended that dietary supplements are only used based on the advice of a qualified sports nutrition professional [06251].

There are surveys of the use of drugs in adolescents in school and college sport teams, but few data are available on professional players, mostly because access to large numbers of
elite athletes is restricted, and because elite athletes are generally reluctant to discuss their habits. One study conducted on English professional soccer players reported data on supplement and vitamin use in a selected sample of subjects, and another study reported information on permitted drug usage in Olympic athletes, not involving soccer. A cohort of 1041 professional soccer players from the two Italian major leagues was now assembled during the season 2003-4; 743 of the 785 (95 %) subjects available on the day of the interview answered an epidemiological questionnaire, which included questions on the type and frequency of use of several permitted drugs. The main aim of this study was to collect information about the health status of the soccer players; therefore the questionnaire did not ask about players' own use of drugs or about recreational drugs. In addition, a brief questionnaire was filled in by the team doctors, to gather details on the therapeutic schemes used for traumas and for other common pathologies, as well as the type and dosage of vitamins and supplements usually prescribed to the players. Information on recommended vaccinations and on general preventive activity performed by the doctor on the team was also collected. Of the 18 team doctors from the A league, 12 answered the questionnaire, and 17 of 18 doctors from the B league answered the questionnaire. Ninety-three percent of players reported having used oral anti-inflammatory products in the previous year, and most of them were current users (86 %). Thirty-six percent of the players, mostly current users, reported the use of analgesics. Eighty-three percent of the players reported current use of supplements, and 28 percent reported using vitamins. Some aspects of players' use of permitted drugs merit further comment. From the team doctors' questionnaires, it seems that the use of supplements and vitamins should be less than that reported by the players. This suggests that team doctors are not always consulted by players about their use of supplements and vitamins, and perhaps about the use of other drugs. This is of particular concern because creatine is often included among the supplements, and could consequently be used in excessively high and potentially dangerous doses. Another reason for concern is the reported possibility that supplements may be contaminated with banned substances; therefore unregulated use may create health concerns. It was concluded that the regular use of several permitted drugs is very high among professional soccer players. The description of players' behaviour is the first step towards regular monitoring of the players' need for, and use of, vitamins, supplements and other permitted drugs [07230].

**Track and field**

Many athletes use dietary supplements as part of their regular training or competition routine, including about 85 percent of elite track and field athletes. Supplements commonly used include vitamins, minerals, protein, creatine, and various "ergogenic" compounds. These supplements are often used without a full understanding or evaluation of the potential benefits and risks associated with their use, and without consultation with a sports nutrition professional. A few supplements may be helpful to athletes in specific circumstances, especially where food intake or food choice is restricted. Vitamin and mineral supplements should be used only when a food-based solution is not available. Sports drinks, energy bars, and protein-carbohydrate shakes may all be useful and convenient at specific times. There are well-documented roles for creatine, caffeine, and alkalinizing agents in enhancing performance in high-intensity exercise, although much of the evidence does not relate to specific athletic events. There are potential costs associated with all dietary supplements, including the risk of a positive doping result as a consequence of the presence of prohibited substances that are not declared on the label [07231].

**Rowing**
The aim of one study was to compare the effectiveness of different nutritional recovery strategies between weigh-in and racing on 2000-m rowing ergometer performance among oarsmen undertaking short-term weight loss before competition. Competitive rowers (n=12) completed four ergometer trials, each separated by 48 h. No weight restrictions were imposed for the first trial (TR1). Thereafter, athletes were required to reduce their body mass by 5.2 percent in the 24 h before trial 2 (TR2), again reaching this body mass before the final two trials (TR3 and TR4). Athletes were provided with one of three nutritional recovery strategies in the 2 h between weigh-in and racing in a counterbalanced fashion according to a Latin square design: fluid (2.8 kJ/kg, 0.0 g/kg carbohydrate, 0.6 mg/kg sodium, 28.5 mL/kg fluid; FLU), carbohydrate/sodium (45.3 kJ/kg, 2.2 g/kg carbohydrate, 32.9 mg/kg sodium, 7.2 mL/kg fluid; CHO), and a combination of water and carbohydrate/sodium (44.8 kJ/kg, 2.3 g/kg carbohydrate, 33 mg/kg sodium, 28.5 mL/kg fluid; COM). Performance was slower for CHO compared with both COM and FLU. However, FLU was not significantly slower than COM. The present investigation has shown that although carbohydrate and sodium intake may be important in the recovery period between weigh-in and 2000-m rowing ergometer performance, fluid intake has a greater influence on performance among lightweight male rowers who undertake short-term weight loss to achieve specified body-mass limits [07232].

**Males and females**

One study was designed to determine if differences exist between male and female collegiate athletes' supplement use and behaviors to modify body appearance. Collegiate athletes who participated in this study were 241 females and 210 males, aged 17 to 28 years. Participants completed a questionnaire about the average number of times each week they performed specific supplementing, exercise, or dietary behaviors. The authors found differences associated with gender for 9 of the 18 behaviors. Specifically, 2 of these 9 behaviors were dietary, 1 was supplementary, 3 were physique concerns, and 3 involved personal motivation for weightlifting. It was concluded that male athletes reported a higher drive for size, speed, and power, whereas female athletes were more concerned with body fat, more likely to restrict caloric intake, and more prone to consume weight loss supplements. No differences were found by gender regarding supplement use to increase body size [09319].

**Use of nutritional supplements among master athletes**

It was assessed the use of nutritional supplements among master athletes focusing on their source of information and source of supply of nutritional supplements. 1560 standardized, anonymous questionnaires were distributed among participants of the World Masters Athletics Championships Indoors 2004. These questions were related to biometric parameters, social indicators, training parameters, illicit drugs, and nutritional supplements. Chi2-tests were computed to reveal meaningful associations between basic information (age, gender, family status, children, education, country of origin, disciplines, training years, smoking, and the use of alcohol, illicit drugs, and doping) and the intake of nutritional supplements. Descriptive information on the history of their use of nutritional supplements was also provided. Sixty-one percent of all participants reported the actual use of nutritional supplements. It was found no significant differences between nutritional supplement users and non-users with regard to basic information. The substances predominantly used were vitamins (35 %) and minerals (30 %). In contrast to elite athletes who use nutritional supplements to increase their athletic performance, master athletes use these substances predominantly for health reasons and, thus, have a closer contact to the health care system. Physicians are their preferred source of information about nutritional supplements. More than half of the interviewed athletes obtain their nutritional supplements from pharmacies or
physicians. The results of this study indicate that nutritional supplement users in master athletics show no specific user profile. Since it is not rare for nutritional supplements to contain trace contaminations of anabolic androgenic steroids or pro-hormones, physicians should also inform master competitive athletes of the dangers of testing positive for doping substances due to their intake of nutritional supplements and advise them accordingly [06246].

Use of dietary supplements among adolescents

USA
The purpose of one study was to investigate the prevalence of dietary supplement use among adolescent athletes. The project was also directed at identifying the sources these student-athletes used for acquiring information about dietary supplements. One hundred thirty nine high school athletes (99 males; 34 females; mean age 16 years) volunteered to participate in this study. A 16-question anonymous survey instrument examined use of dietary supplements, reasons for use, type of sport participation, and sources of information regarding dietary supplements. Of the participants in this study, 22 percent (n=31) reported currently taking dietary supplements. There was no relationship found between dietary supplement use and age. There were a significantly higher number of males reporting current dietary supplement use. Of those who reported to be currently taking dietary supplements, sports performance (n=25) was the most reported reason for use. There were no significant differences found in reported dietary supplement use between any of the sports. Of the participants, 38 percent (n=53) listed their coach as their best source of information on dietary supplements. The results of this study offer the current literature some additional insight into trends in supplement use among high school student athletes. Practical implications suggest that it may be necessary to ensure coaches have sufficient knowledge about dietary supplements so that adolescent athletes are receiving accurate information [06249].

The role of the school nurse

An alarming trend in the United States is the use of performance-enhancing supplements by children and adolescents. These widely available over-the-counter products, often marketed as natural substances, are not regulated by the Food and Drug Administration and are thus widely available. High school and even middle school students are using these supplements because they are misled into thinking that supplements will enhance their athletic skills resulting in an improvement in their performance. Yet, the safety and long-term effects of these supplements have not been established in reputable or prevalent studies. School nurses have a unique opportunity and even an ethical responsibility to help in efforts to address this growing trend. Specific roles for the school nurse include serving as a student advocate for the health and safety of children and adolescents; identifying at-risk students; forming partnerships with teachers, parents, students, coaches, athletic trainers, and local health care providers; evaluating and refining health-oriented curricula; collecting and disseminating new knowledge; and staying abreast of new findings [06250].

Recovery after sports

To reduce the magnitude of fatigue and to accelerate the time to fully recover after completion, several recovery strategies are now used in professional soccer teams. During congested fixture schedules, recovery strategies are highly required to alleviate post-match fatigue, and then to regain performance faster and reduce the risk of injury. Fatigue following competition is multifactorial and mainly related to dehydration, glycogen depletion, muscle
damage and mental fatigue. Recovery strategies should consequently be targeted against the major causes of fatigue. Strategies reviewed of one article were nutritional intake, cold water immersion, sleeping, active recovery, stretching, compression garments, massage and electrical stimulation. Some strategies such as hydration, diet and sleep are effective in their ability to counteract the fatigue mechanisms. Providing milk drinks to players at the end of competition and a meal containing high-glycaemic index carbohydrate and protein within the hour following the match are effective in replenishing substrate stores and optimizing muscle-damage repair. Sleep is an essential part of recovery management. Sleep disturbance after a match is common and can negatively impact on the recovery process. Cold water immersion is effective during acute periods of match congestion in order to regain performance levels faster and repress the acute inflammatory process. Scientific evidence for other strategies reviewed in their ability to accelerate the return to the initial level of performance is still lacking. These include active recovery, stretching, compression garments, massage and electrical stimulation. While this does not mean that these strategies do not aid the recovery process, the protocols implemented up until now do not significantly accelerate the return to initial levels of performance in comparison with a control condition. In conclusion, scientific evidence to support the use of strategies commonly used during recovery is lacking. Additional research is required in this area in order to help practitioners establish an efficient recovery protocol immediately after matchplay, but also for the following days. Future studies could focus on the chronic effects of recovery strategies, on combinations of recovery protocols and on the effects of recovery strategies inducing an anti-inflammatory or a pro-inflammatory response [13586].

Individualized nutrition as doping

Food has societal, economic, medical and ethical implications, being fundamental for life. It plays an important role also in sports medicine, since a healthy diet is an important part of an athlete's training. Nutrigenomics and nutriproteomics are emerging as a result of a convergence of nutritional, genomics and proteomics knowledge strands in the postgenomics era. These fields of inquiry present an opportunity for the design of customized diets potentially able to counterbalance the extant obesity epidemic and remedy metabolic diseases, among others. They are noteworthy for sport medicine as well since they could provide athletes with crucial information for personalized training and nutrition, in order to achieve the best results possible and express one's own potential. But they could also be used as a form of personalized doping, thus constituting an advancement of "classical nutrition-based doping" (i.e. the use of nutraceuticals, stimulants and supplements). However, nutrigenomics (or nutriproteomics)-based nutritional doping is different from the first-generation doping because it is specifically tailored to the genomics and proteomics makeup of the athlete, although their effectiveness remain to be discerned in future systematic studies. Against this scientific background, ethical issues of nutrigenomics and nutriproteomics are discussed in the present paper with emphasis on the current limitations and the dizzying potentials of the omics data-intensive research for science and society. Additionally, I discuss the need to communicate uncertainty as a fundamental construct and intrinsic part of postgenomics personalized medicine, not to forget the gaps regarding the lack of adequate governance, and issues over providing a proper nutritional education to athletes as onus of the international sports organizations. "Let food be your medicine, and medicine be your food" Hippocrates [13587].

Side effects

Cholestatic jaundice
A 50-year-old Hispanic male presented to the hospital with a 1-week history of significant painless jaundice; total bilirubin on admission was 29.4 mg/dL. He reported use of both herbal (creatine and whey protein) and designer (Incredible Bulk and Spartan 45) supplements concurrently for approximately 2 months. Upon admission, all supplements were discontinued and multiple laboratory and diagnostic tests were ordered. On day 6 of his hospital admission, a liver biopsy was performed, the results of which indicated drug-induced hepatic toxicity. On day 9 he was discharged with prescriptions for ursodeoxycholic acid and hydroxyzine. Three months post hospital discharge, the patient continued to be supplement-free and bilirubin had decreased substantially. Anabolic-androgenic steroids are capable of causing hepatotoxicity, and multiple cases reported in the literature support this. A case report described hepatotoxicity secondary to both creatine and whey protein consumption, and several reports have described liver damage secondary to designer supplement use. This is the first case to describe hepatotoxicity as a result of combination herbal and designer supplement use. The Roussel Uclaf Causality Assessment Method score for drug-induced hepatotoxicity indicated a highly probable correlation between the use of combination supplements and cholestatic jaundice [13588].

Contaminated supplements

Based upon recent sales numbers, nutritional supplements play a key role in the lifestyle of a substantial proportion of the population. As well as products such as vitamins or minerals, several precursors of anabolic steroids are marketed as nutritional supplements. Another group of commercially available supplements are products for weight loss based upon herbal formulations originating from Ephedra species. Apart from supplements indicating the presence of these active compounds, numerous non-hormonal nutritional supplements were found that were contaminated with non-labelled anabolic steroids. Stimulating agents other than naturally occurring analogues of ephedrine were detected. A major group using dietary supplements are sportsmen, ranging from amateur level to elite athletes. Besides the possible health risks associated with the use of dietary supplements, athletes should take care not to violate the rules of the World Anti-Doping Agency because athletes remain responsible for substances detected in their biofluids, irrespective of their origin. Several analytical methods have been developed to determine the presence of doping agents as contaminants. One review attempted to address the issues concerning the use of nutritional supplements and the detection of doping agents as contaminants in dietary supplements [06243].

One of the key factors that elite athletes need to consider in negotiating the complex world of supplements and sports foods is whether the consumption of these products could lead to an inadvertent case of doping. Following the wave of nandrolone findings in the late 90s, several studies have sought to explore the extent of contamination. In 2000-2001, the International Olympic Committee (IOC) funded an extensive research project using the then IOC-accredited laboratory in Cologne to analyse independently 634 non-hormonal nutrition supplements purchased across 13 countries. This pivotal research confirmed the contamination issue, with 15 percent (94 products) being found to contain undeclared steroids banned by the World Anti-Doping Agency. Altogether, 289 samples (21 % positives) were from companies that were known to sell steroids/prohormones but, perhaps more worrying, 345 samples (10 % positives) came from companies that did not sell steroids/prohormones. In 2007, HFL Sport Science (a WADA-experienced laboratory, part of the Quotient Biosciences group) in the UK analysed 58 supplements purchased through standard retail outlets in the USA. They found that 25 percent were contaminated with
prohibited steroids and 11 percent were contaminated with prohibited stimulants. In 2008, HFL Sport Science followed this up with the analysis of 152 products purchased from standard retail outlets in the UK and found that over 10 percent were contaminated with steroids and/or stimulants. The amounts of steroids detected have been extremely variable, even within a single batch, but have generally been extremely small. However, very low levels of contamination (measured in parts per billion) can cause positive drug tests in an elite athlete at a level much lower than acceptable impurity levels (typically around 0.01 %) in good manufacturing practice regulations. It is important to note that, although this minimal amount of contamination could produce dire consequences for an athlete competing under the WADA code, in most cases, this amount is unlikely to cause detrimental health problems for the general consumer. There are daily food product withdrawals and recalls because of mislabelling and undeclared allergens. This, together with concerns about impurities and contamination from medicine residues, insects and small pieces of metal and plastic, shows that inadvertent contamination is not just a problem for sports nutrition products. The inadequate regulation of dietary supplements means there is no way for consumers to know what many supplements actually contain or how pure the product and its ingredients are. Manufacturers with good-quality controls and banned substance testing are better able to control the risk. The inception of the WADA code and the implications of strict liability means that an athlete is held responsible for whatever is in their body irrespective of how it got there. Therefore, athletes who compete under the WADA code should be extremely cautious about using supplements and always work with a qualified professional on risk minimisation of supplement use [09308].

Practitioners who work with elite athletes know that the pressure and considerable rewards involved with success provide a high level of motivation to look for any safe and legal strategy that might enhance performance, even by small margins. Dietary supplements operate in this space, whether they promise a large performance boost or just create the fear that an athlete cannot afford to miss out on what everyone else is using. It is often tempting to overlook the lack of evidence to support the claims made about a supplement on the basis that the stakes are higher for elite athletes; therefore the cost:benefit ratio favours experimentation in the absence of clear proof. Over the past decade, however, we have become aware that the cost of getting it wrong has also escalated for elite athletes. A new hazard related to supplement use has emerged: inadvertent ingestion of substances that are banned under the antidoping codes in place in elite sport, but present in supplement products. In some cases, the level of the presence, or contamination, of banned substances in supplements presents a health hazard for all consumers. In some cases, the concentration may be too small to achieve any health or performance effect but large enough to record an infringement for athletes who submit to doping tests. Newspapers, the internet and Courts of Arbitration in Sport now bear stories of dedicated athletes whose careers have been or are being jeopardized because of the ingestion of a banned substance via a dietary supplement [11425].

The increasing availability and use of sports supplements is of concern as highlighted by a number of studies reporting endocrine disruptor contamination in such products. The health food supplement market, including sport supplements, is growing across the Developed World. Therefore, the need to ensure the quality and safety of sport supplements for the consumer is essential. The development and validation of two reporter gene assays coupled with solid phase sample preparation enabling the detection of estrogenic and androgenic constituents in sport supplements is reported. Both assays were shown to be of high sensitivity with the estrogen and androgen reporter gene assays having an EC50 of 0.01 ng/mL and 0.16 ng/mL respectively. The developed assays were applied in a survey of 63 sport supplements samples obtained across the Island of Ireland with an additional seven reference samples previously investigated using LC-MS/MS. Androgen and estrogen bio-
activity was found in 71% of the investigated samples. Bio-activity profiling was further broken down into agonists, partial agonists and antagonists. Supplements (n=13) with the strongest estrogenic bio-activity were chosen for further investigation. LC-MS/MS analysis of these samples determined the presence of phytoestrogens in seven of them. Supplements (n=38) with androgen bio-activity were also selected for further investigation. Androgen agonist bio-activity was detected in 12 supplements, antagonistic bio-activity was detected in 16 and partial antagonistic bio-activity was detected in 10. A further group of supplements (n=7) did not present androgenic bio-activity when tested alone but enhanced the androgenic agonist bio-activity of dihydrotestosterone when combined. The developed assays offer advantages in detection of known, unknown and low-level mixtures of endocrine disruptors over existing analytical screening techniques. For the detection and identification of constituent hormonally active compounds the combination of biological and physio-chemical techniques is optimal [11371].

Since 1999 several groups have analyzed nutritional supplements with mass spectrometric methods (GC/MS, LC/MS/MS) for contaminations and adulterations with doping substances. These investigations showed that nutritional supplements contained prohibited stimulants as ephedrines, caffeine, methylenedioxy-metamphetamie and sibutramine, which were not declared on the labels. An international study performed in 2001 and 2002 on 634 nutritional supplements that were purchased in 13 different countries showed that about 15 percent of the nonhormonal nutritional supplements were contaminated with anabolic-androgenic steroids (mainly prohormones). Since 2002, also products intentionally faked with high amounts of "classic" anabolic steroids such as metandienone, stanozolol, boldenone, dehydrochloromethyl-testosterone, oxandrolone etc have been detected on the nutritional supplement market. These anabolic steroids were not declared on the labels either. The sources of these anabolic steroids are probably Chinese pharmaceutical companies, which sell bulk material of anabolic steroids. In 2005 vitamin C, multivitamin and magnesium tablets were confiscated, which contained cross-contaminations of stanozolol and metandienone. Since 2002 new "designer" steroids such as prostanozol, methasterone, androstatrienedione etc have been offered on the nutritional supplement market. In the near future also cross-contaminations with these steroids are expected. Recently a nutritional supplement for weight loss was found to contain the beta2-agonist clenbuterol. The application of such nutritional supplements is connected with a high risk of inadvertent doping cases and a health risk [08382].

The potential for contaminated dietary supplements to result in a failed doping test remains a concern for athletes, trainers, and sporting authorities despite improvements to regulatory guidelines. Previous surveys of readily available supplements confirm that many are contaminated with steroids and stimulants prohibited for use in elite sport. Suggested responses to this issue include the complete avoidance of all supplements. Many athletes, however, use nutritional supplements to achieve effective training and also to ensure that daily nutritional requirements are met (e.g. recommended levels of vitamins and minerals). This ensures that the use of supplements is and will remain the norm for a range of sports. As a result, an alternative approach of rigorous testing of materials destined for use by elite athletes has been introduced in several countries. While the testing of final product for banned substances may help mitigate the problem, it will not help to remove the underlying issue of contamination. In one article it was described an alternative approach that uses appropriate quality assurance procedures backed up by testing to remove sources of contamination. The decrease in the incidence of contamination amongst supplement companies adopting such a system is explained, and contrasted with the relatively high incidences of contamination found in products that are not part of a quality system. These findings are of key importance to both supplement manufacturers and those involved in advising athletes about supplement use [10310].
A simple and effective analytical method for the determination of anabolic steroids and related compounds in nutritional supplements was reported. Target compounds are extracted with ethyl acetate, crude extract is purified using dispersive solid-phase extraction (SPE) with primary secondary amine as sorbent, and finally they are identified and quantified as underivatized compounds using two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GCxGC-TOF MS). This method was validated for 25 steroids in two types of commercially available solid nutritional supplements: protein concentrate and creatine monohydrate. Repeatability expressed as the relative standard deviation of analyte concentration ranged from 4.1 to 20.5%. Recoveries between 70 and 123 percent were obtained for the target compounds except for oxymetholone in protein concentrate where the recovery was low as a result of strong interactions with primary secondary amine. Excellent linearity was obtained for six-point calibration with regression coefficients of 0.997-1.000 for all compounds. The limits of quantification ranged from 0.007 to 0.114 mg/kg. For a monitoring programme of 48 samples of nutritional supplements, three were positive. Nandrolone, testosterone, dehydroepiandrosterone (DHEA), 5alpha-androstan-3,17-dione, 19-norandrostendione and progesterone were found in positive samples at concentrations between 0.022 and 0.398 mg/kg.

In the 1990s the IOC issued a public warning when certain supplements appeared to contain unlabelled pseudo-ephedrine, at that time a prohibited substance. This issue was brought to a head by sprinter Linford Christie, who tested positive for pseudo-ephedrine during the Olympic Games of 1988, but (as a rare exception) was not sanctioned because the likely source was a cup of ginseng tea. Contamination of ginseng is often cited as a potential hazard. Christie also tested positive for nandrolone in 1999. In the UK, the number of nandrolone positives jumped from an average of 3.4 in the years 1994–1998 to 17 in 1999; an increase from 0.08 percent to 0.29 percent of all the samples analysed. A possible cause of this increase was the ingestion of contaminated nutritional supplements. A few years later, it was concluded that this statistical increase appeared to have been exceptional and was only present within the UK in 1999, but from 2000 onwards the subject of nutritional supplements and doping entered the limelight. In subsequent years, more and more studies were published that confirmed the hypothesis that supplements could indeed cause unintentional doping infractions. Other publications showed that unlabeled doping substances can be found in a variety of products and thoroughly discussed the risks for elite athletes. The information on this issue came mainly from the WADA-accredited laboratory in Cologne that conducted an IOC-sponsored study in 2004. This study showed that 15 percent of 634 freely available substances contained anabolic agents that were not declared on the label. These products were partly selected because the producers of these substances also sold pro-hormone containing products. The risk of buying a contaminated supplement is about twice as high in products from such companies. The amounts that were found varied from 10 ng/g or parts per billion (ppb) to 190 microg/g or parts per million (ppm). Later studies found even higher and profoundly dangerous amounts of anabolic agents, up to 17 mg of unlabeled metandienone per tablet.

The fact that nutritional supplements can lead to a positive urine test has been consistently found in various studies. The risk of producing a positive doping test can be present for hours to days after the ingestion of one single supplement, depending on the substance, dose, and individual variation in metabolism. The difficulty of finding possible contaminations of a nutritional supplement was shown in one of the very first studies addressing this problem. A group from an anti-doping laboratory in Los Angeles, California, USA proved the existence of tablet-to-tablet variation in contaminations. This variation was confirmed later that year and is still likely to exist. The experiences from the laboratories show that contaminations can be present in the raw materials that are used, both in the active ingredients and in the
substances used to make tablets or capsules. This type of contamination is often referred to as “cross-contamination”. A second source of contamination might result from a lack of sufficient hygiene in the machinery that is being used during the production process. Contamination problems in nutritional supplements can be found in any country. It concerns all types of nutritional supplements and all forms, including powders, pills, capsules, and liquids. Likewise, experience shows that contaminations can occur with a multitude of doping substances. Examples of nutritional supplements that have been contaminated with doping substances are branched-chain amino acids (BCAAs), creatine, glutamine, guarana, minerals, ornithine-alpha-ketoglutarate (OKG), proteins, pyruvate, ribose, saw palmetto, Tribulus terrestris, vitamins, zinc, 4-androsten-3,17-diol, 4-androsten-3,17-dion, 5-androsten-3,17-diol, 19-nor-4-androsten-3,17-diol, 19-nor-4-androsten-3,17-dion, 19-nor-5-androsten-3,17-diol, 19-nortestosterone (nandrolone), benzylpiperazine, caffeine (off the WADA doping list since 1 January 2004), dehydroepiandrosterone (DHEA), ephedrine, methandienone, methylenedioxymethylamphetamine (MDMA or XTC), nor-pseudo-ephedrine, sibutramine, stanozolol, and testosterone. Even though these lists might not be complete, the variety of substances found indicates the magnitude of the problem. It also shows that contaminations in nutritional supplements are most likely to occur with substances that are part of the groups of anabolic agents or stimulants. The problems surrounding nutritional supplements that contain unlabeled doping substances have often been attributed to the Dietary Supplement Health Education Act (DSHEA) that was passed in the USA in 1994. The DSHEA has often been accused of introducing a system where quality control is lacking. This Act has undoubtedly played a large role in creating the problem of contaminated substances, because of the strong influence of the USA on the global market and the consequent spread of (traces of) anabolic agents and strong stimulants. But this problem does not only originate from the USA. Any facility that is part of the production or storage process of nutritional supplements, or that handles doping substances in addition to doping-free products, could be a source of the eventual downfall of an ignorant athlete. Globally there is a great difference in the quality procedures surrounding pharmacological medications and nutritional supplements, but the difference between these two groups is not always as clear as it should be. Based on the published facts that a precursor of an anabolic steroid in an amount between 1 and 10 microg can cause a positive doping test, and based on the fact that athletes easily use 50 g of supplements per day or more, a reporting threshold value of 10 ng/g or 10 ppb for all anabolic steroids is used in all tests. This value also allows for individual variations in metabolism. Excretion studies for stimulants are rare, but similar considerations led to the conclusion that for stimulants, a reporting threshold value of 100 ppb is opportune. However, the only way athletes are able to enjoy a 100 percent guarantee is when they decide not to take any supplements at all. But there are certainly some circumstances when dietary supplements provide an added benefit to diet and, in the world of elite sport where the ultimate goal is to reach one's best, it is not fair to deny athletes the use of legal substances that could improve their health, such as anti-oxidants and multivitamins. Although there are some studies that suggest that there is a relationship between (legal) supplement use and (illegal) doping use, these are only based on epidemiological data and causality has never been established [07025].

Even athletes who are careful can test positive for doping because of a contaminated nutritional supplement. This is a disconcerting thought, but an unavoidable consequence of the current situation in the nutritional supplement industry. However, years after the sudden rise in nandrolone positives in the UK (around 1998-1999) and the wealth of experience with this issue in the meantime, ignorance is no excuse for today's elite athletes. Two types of contamination can be identified. The first type is from malpracticing producers who do not care about consumer health or even deliberately spike products with known effective substances such as anabolic steroids or their precursors. Generally, such companies change
identity quickly and most often sell their products over the Internet. Occasionally, the products might emerge in regular shops, but a country’s health directorate is highly likely to pick up such products and take them off the market. Most of the time this type of company uses advertisements with unrealistic claims, and athletes and their support personnel should be able to avoid this type of supplement easily. The second type of contamination is more subtle and more difficult to detect, and thus more of a concern to athletes with good intentions. The nature of the origin of such contaminations (mostly cross-contamination from other products, frequently not in the facility where the end-product is made and packed and therefore always unexpected) makes it very difficult to pick up such contaminations, even for well-intentioned producers who follow strict quality procedures. Experience shows that such supplements can contain doping substances despite these extra efforts. Even though the level of such contaminations might be low, even very low amounts of doping substances can suffice to cause a positive urine sample for a window of several hours after the consumption of such a product [07025].

The Cologne study, performed in 2001, showed that the Netherlands faced one of the biggest contamination issues in Europe. In November 2001, the athletes nominated to go to the Winter Olympics in Salt Lake City 2002 were given an opportunity to have their supplements tested for doping substances. They were asked to buy a supply of the nutritional supplements they were going to use during their preparation for the Olympics from a controlled sample of one batch. From this supply, a random selection of supplements was tested for several anabolic steroids, their precursors, and several stimulants. The results of this preliminary study gave a clear insight of the seriousness, size, and scope of the problem. Of the 69 supplements that were submitted (mainly vitamins, minerals and creatine), 13 (19%) contained unlabeled doping substances. Most products showed traces of caffeine and/or ephedrine, one product contained a small amount of 3,4-methylenedioxymethamphetamine (better known as MDMA or XTC), and five products contained anabolic steroids. By pure chance, two different batches of one single product were tested as well, yielding one positive and one negative finding [07025].

Despite ongoing improvements to regulatory and manufacturing guidelines, the potential for contaminated nutritional supplements to cause a failed doping test for an athlete remains a concern. Several surveys of supplements available through the internet and at retail have confirmed that many are contaminated with steroids and stimulants that are prohibited for use in elite sport. Suggested responses to this issue include the complete avoidance of all supplements. However, this approach seems to be unrealistic as many athletes use nutritional supplements for very different reasons. In addition, the number of publications describing trials that demonstrate the benefit of certain nutritional products has also increased over the last decade or so. This ensures that for many sports the use of supplements will remain a common practice. In response to the issue of contamination in nutritional supplements, many reputable manufacturers have their products rigorously tested by sports anti-doping laboratories to help ensure as far as possible that the risks to an athlete remain minimal. In this chapter we review the issue of supplements and contamination, and look at how this might be addressed through effective quality control procedures at the manufacturing facility and through the highly sensitive testing of finished products using appropriately accredited tests [12372].

Previously developed estrogen and androgen mammalian reporter gene assays (RGAs) were assessed for their potential use as a quantitative screening method in the detection of estrogenic and androgenic endocrine disruptors (EDs) in sport supplements. The validation of both RGAs coupled with dispersive solid phase extraction (dSPE) was performed in accordance with European Commission Decision EC/2002/6579 for biological screening methods. Decision limits (CC-alpha) and detection capabilities (CC-beta) were established
for both the estrogen and androgen RGAs. All samples were compliant with CC-alpha and CC-beta in both bioassays. Recovery rates were 96 percent for 17beta-estradiol and 115 percent for dihydrotestosterone as obtained in their corresponding RGA. Both estrogens and androgens were stable in samples for more than 3 weeks, when stored at -20 °C. Specificity, good repeatability (coefficients of variation (CV), 12-25 %), reproducibility and robustness of both bioassays were also observed. Four different ED modes of action were determined for estrogens and androgens in 53 sport supplements, using the validated RGAs. This study revealed that 89 percent of the investigated sport supplements contained estrogenic EDs and 51 percent contained androgenic compounds. In conclusion, both bioassays are suitable for sport supplement screening of estrogenic and androgenic EDs [12373].

**Anabolic steroids**

Nineteen different dietary supplements, ordered through the internet and intercepted by the Belgian pharmaceutical inspection at the post office, were analyzed by means of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the presence of anabolic steroids. After a methanolic extraction the samples were screened for the presence of 49 compounds. This resulted in almost 60 percent of the samples being suspected of containing one of these 49 anabolic compounds and being subjected to a confirmatory product ion scan. In all of these suspected samples we were able to confirm at least one anabolic steroid with concentrations between 0.01 and 2.5 mg/unit (unit: one capsule or tablet or for liquids: the prescribed dose). The anabolic steroids that was mostly encountered was testosterone (50 %) followed by beta-boldenone (25 %). These results once more confirm the dubious reputation of over-the-counter dietary supplements [07245].

Several studies have highlighted that nutritional supplements may contain undeclared substances that are banned by the International Olympic Committee (IOC)/World Anti-Doping Agency (WADA). This paper describes a qualitative liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) method to detect anabolic androgenic steroids (4-androsten-3,17-dione, 4-oestrin-3,17-dione, 5alpha-androsten-17beta-ol-3-one, boldenone, nandrolone, nandrolone decanoate, testosterone, and testosterone decanoate) and ephedrine in food supplements. The products are dissolved in methanol and analysed by gas chromatography-mass spectrometry (GC-MS). The methanolic solution was added to testosterone-d(3), evaporated to dryness, mixed with NaOH and extracted with n-pentane:diethyl ether (9:1). LC-MS/MS analyses were performed in selected reaction monitoring (SRM) on an ion-trap equipped with an atmospheric pressure chemical ionization (APCI) probe operating in positive-ion mode. The method was applied to 64 nutritional supplements. A total of 13 percent of the nutritional supplements analysed contained banned substances not declared on the label (anabolic steroids and ephedrine). Detection limits were in the range 1-25 ng/g [07246].

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**Androgens on the nutritional supplement market**

During recent years, the nutritional supplement market has expanded and in 2006, the worldwide market was estimated to be worth more than US dollar 60 billion. The use of supplements is popular in the general population; however, athletes corner the market with 44-100 percent prevalence, dependent on age, gender, level of competition and the type of sport. Whilst vitamins, minerals, proteins and creatine, are not prohibited by WADA, the problem arises when supplements contain additives that are not on their label. This can occur inadvertently through cross-contamination from a production line and/or transport in unclean containers or it can occur through direct addition. This level of contamination can be sufficient to create a positive doping test. For example, 634 nutritional supplements purchased from 13 different countries were analyzed with mass spectrometric methods (GC/MS, LC/MS/MS) for undeclared doping substances and 15 percent of these minerals, vitamins, creatine or protein supplements contained androgens (mostly prohormones) that were not declared on the label. In another example, bioassays were used to screen a range of nutritional supplements for hormonal activity and found that for 63 supplements, only 13 were negative for estrogenic activity and that only 18 were negative for androgenic activity. All of the other supplements showed agonistic, partial agonistic and/or antagonistic hormone activities, indicating that these health food products contain hormonally active constituents. Since 2002, nutritional supplements have been found to be intentionally spiked with androgens at concentrations higher than 1 mg/g. The androgens found include metandienone, stanozolol, boldenone, oxandrolone and dehydrochloromethyltestosterone. These androgens were either not declared, or were declared but with non-approved names on the labels. The nutritional supplements can be purchased without restriction by telephone order or internet purchase and are delivered by ordinary mail. This allows easy access by the general population for non-medical reasons. Not only do sport nutritional supplements face the problem of androgen spiking, either deliberate or accidental, but “natural” products have also been found to contain hormonally active substances. For example, a herbal product used for the treatment of high level prostate-specific antigen (PSA) was found to contain an estrogenic substance, diethylstilbestrol (DES) using a yeast estrogen bioassay, together with nuclear magnetic resonance (NMR) and liquid chromatography/ time-of-flight MS (LC/TOFMS). This was identifiable clinically because of the development of gynecomastia. Therefore, it is evident that the intentional or unintentional addition of hormonally active substances is an ongoing problem. It is important then that methods are available for the exquisite detection of these hormonally active substances, especially those that are androgenic [13084].

**Methylstenbolone**

The use of "nutritional supplements" containing unapproved substances has become a regular practice in amateur and professional athletes. This represents a dangerous habit for their health once no data about toxicological or pharmacological effects of these supplements are available. Most of them are freely commercialized online and any person can buy them without medical surveillance. Usually, the steroids intentionally added to the "nutritional supplements" are testosterone analogues with some structural modifications. In this study, the analyzed product was bought online and a new anabolic steroid known as methylstenbolone (2,17alpha-dimethyl-17beta-hydroxy-5alpha-androst-1-en-3-one) was detected, as described on label. Generally, anabolic steroids are extensively metabolized, thus in-depth knowledge of their metabolism is mandatory for doping control purposes. For this reason, a human excretion study was carried out with four volunteers after a single oral dose to determine the urinary metabolites of the steroid. Urine samples were submitted to enzymatic hydrolysis of glucuron conjugated metabolites followed by liquid-liquid extraction and analysis of the trimethylsilyl derivatives by gas chromatography coupled to tandem mass spectrometry. Mass spectrometric data allowed the proposal of two plausible metabolites:
2,17α-dimethyl-16ε,17β-dihydroxy-5α-androst-1-en-3-one (S1), 2,17α-dimethyl-3α,16ε,17β-trihydroxy-5α-androst-1-ene (S2). Their electron impact mass spectra are compatible with 16-hydroxylated steroids O-TMS derivatives presenting diagnostic ions such as m/z 231 and m/z 218. These metabolites were detectable after one week post administration while unchanged methylstenbolone was only detectable in a brief period of 45 h [13605].

**Anabolic steroids and stimulants**

The purpose of one study was to analyze the composition of 103 dietary supplements bought on the internet. The supplements were dispatched in four different categories according to their announced contents [creatine, prohormones, "mental enhancers" and branched chain amino acids, BCAA]. All the supplements were screened for the presence of stimulants and main anabolic steroids parent compounds. At the same time, the research was focused on the precursors and metabolites of testosterone and nandrolone. The study pointed out three products containing an anabolic steroid, metandienone, in a very high amount. The ingestion of such products induced a high quantity of metandienone metabolites in urines that would be considered as a positive antidoping test. The results have also shown that one creatine product and three "mental enhancers" contained traces of hormones or prohormones not claimed on the labels and 14 prohormone products contained substances other than those indicated by the manufacturer. The oral intake of the creatine product revealed the presence of the two main nandrolone metabolites (19-norandrosterone and 19-noretiocholanolone) in urine [06245].

**Ephedrine**

Several studies have highlighted that nutritional supplements may contain undeclared substances that are banned by the International Olympic Committee (IOC)/World Anti-Doping Agency (WADA). One paper described a qualitative liquid chromatography coupled with tandem mass spectrometry detection (LC-MS/MS) method to detect anabolic androgenic steroids (4-androst-3,17-dion, 4-oestren-3,17-dion, 5α-androst-17β-ol-3-one, boldenone, nandrolone, nandrolone decanoate, testosterone, and testosterone decanoate) and ephedrine in food supplements. The products are dissolved in methanol and analysed by gas chromatography-mass spectrometry (GC-MS). The methanolic solution was added to testosterone-d(3), evaporated to dryness, mixed with NaOH and extracted with n-pentane:diethyl ether (9:1). LC-MS/MS analyses were performed in selected reaction monitoring (SRM) on an ion-trap equipped with an atmospheric pressure chemical ionization (APCI) probe operating in positive-ion mode. The method was applied to 64 nutritional supplements. A total of 13 percent of the nutritional supplements analysed contained banned substances not declared on the label (anabolic steroids and ephedrine). Detection limits were in the range 1-25 ng/g [07247].

**Hepatotoxicity**

The aim of one article was to re-emphasize the hepatotoxicity associated with the use of anabolic androgenic steroids and to highlight the marketing and sale of anabolic androgenic steroids as dietary supplements. It was a case series of two patients who developed a cholestatic liver panel after consumption of anabolic androgenic steroids. It was presented two young men who developed significant cholestatic liver injury after consumption of anabolic androgenic steroids. This was associated with considerable morbidity, although both patients recovered without the need for a liver transplant. Both of these anabolic androgenic steroids were being marketed as dietary supplements. It was concluded that despite being classified as class III controlled substances, anabolic androgenic steroids are
still a cause for serious hepatotoxicity. Some of these anabolic androgenic steroids are being marketed as dietary supplements. Increased vigilance is required from the medical profession [07248].

*How to deal with supplements in the field of practice*

Many athletes tend to take supplements for a variety of reasons. The first rule in any educational effort regarding supplements is that athletes should be cautioned against their indiscriminate use. Supplements can play a role in an athlete's diet, but confirmation of their added benefit should be sought with an appropriate expert before using them. The second step is to try and identify those supplements that have the slimmest chance of being contaminated with doping substances. Companies that sell products containing doping substances should definitely be avoided, and it is prudent to disregard companies with unrealistic claims in their advertisements. This includes advertisements that mention "IOC approved" or "WADA tested" on their label, as no such approvals exist. The basic anti-doping rule remains that at all times athletes are responsible for the substances that are within their bodies, and a simple appeal based on an advertisement does not lift this rule of strict liability. Athletes and their support personnel should be aware that no system is able to provide a 100% guarantee of doping free supplements. Contaminations can occur in many ways, which leads to possible package-to-package or even tablet-to-tablet variation. No sampling protocol is able to cater for all these possibilities. However, there are protective systems that can be used to bring down the chances of ingesting contaminated supplements to very close to 0 percent. Such athlete-friendly systems should address the problems surrounding possible contaminations mentioned in this article, and well intentioned producers should acknowledge that contaminations can occur outside their control. Generally speaking, any system that ensures that the particular product is produced in a "doping-free environment", meaning that all parts of the production process are free of any substances prohibited by WADA, will provide an athlete with a trustworthy product. But as the NZVT experience has shown, even quality systems that are foolproof on paper cannot prevent contaminations with doping substances. Therefore, the best available option for athletes is to only use supplements that have been analysed in a knowledgeable laboratory on a batch-by-batch basis [07025].

*Recovery time after sports*

To study the effects of a single soccer game on indices of performance, muscle damage, and inflammation during a 6-day recovery period an investigation was done. In 24 players a single soccer game induces short-term muscle damage and marked but transient inflammatory responses. Anaerobic performance seems to deteriorate for as long as 72-hour post-game. The acute phase inflammatory response in soccer appears to follow the same pattern as in other forms of exercise [08384].

*Self-reported recovery*

The aims of one study were to assess the dietary intake and monitor self-reported recovery quality and clinical symptomology of a male ultra-endurance runner who completed a multi-day ultra-endurance running challenge covering 4254 km from North Scotland to the Moroccan Sahara desert over 78 consecutive days. Food and fluid intakes were recorded and analysed through dietary analysis software. Body mass (BM) was determined before and after running each day, and before sleep. Clinical symptomology and perceived recovery quality were recorded each day. Whole blood haemoglobin and serum ferritin were
determined before and after the challenge. Total daily energy (mean ± SD: 23.2 ±3.2 MJ/day) and macronutrient intake (182 ± 31 g/day; protein, 842 ± 115g /day carbohydrate, 159 ± 55 g/day fat) met consensus nutritional guidelines for endurance performance. Total daily water intake through foods and fluids was 4.8 ± 2.0 L/day. Water and carbohydrate intake rates during running were 239 ± 143 ml/h and 56 ± 19 g/h, respectively. Immediately after running, carbohydrate and protein intakes were 1.3 ± 1.0 g/kg BM and 0.4 ± 0.2 g/kg BM, respectively. Daily micronutrient intakes ranged from 109-662 % of UK RNIs. Pre-running BM was generally maintained throughout. Overall exercise-induced BM loss averaged 0.8 ± 1.0 PERCENT, although BM losses of ≥2 PERCENT occurred in the latter stages, a reflection of the warmer climate. Varying degrees of self-reported perceived recovery quality and clinical symptomology occurred throughout the challenge. This case study highlights oscillations in dietary habits along 78 consecutive days of ultra-endurance running, dependent on changes in ambient conditions and course topography. Nevertheless, nutrition and hydration status were maintained throughout the challenge. Despite dietary iron intake above RNI and iron supplementation, this alone did not prevent deficiency symptoms [13602].

**Practical nutritional recommendations**

The aim of training is to achieve optimum performance on the day of competition via three processes or paradigms; training hard to create the required training stimulus, training smart to maximize adaptations to the training stimulus, and training specifically to fine-turn the behaviors or physiology needed for competition strategies. Dietary strategies for competition must target the factors that would otherwise cause fatigue during the event, promoting an enhancement of performance by reducing or delaying the onset of these factors. In some cases, the nutritional strategies needed to achieve these various paradigms are different, and even opposite to each other, so athletes need to periodize their nutrition, just as they periodize their training program. The evolution of new knowledge from sports nutrition research, such as presented in this book, usually starts with a stark concept that must be further refined; to move from individual nutrients to food, from "one size fits all" to the individual needs and practices of different athletes, and from single issues to an integrated picture of sports nutrition. The translation from science to practice usually requires a large body of follow-up studies as well as experimentation in the field [12366].

**Adolescents’ nutrition**

Athletics is a popular sport among young people. To maintain health and optimize growth and athletic performance, young athletes need to consume an appropriate diet. Unfortunately, the dietary intake of many young athletes follows population trends rather than public health or sports nutrition recommendations. To optimize performance in some disciplines, young athletes may strive to achieve a lower body weight or body fat content and this may increase their risk for delayed growth and maturation, amenorrhoea, reduced bone density, and eating disorders. Although many of the sports nutrition principles identified for adults are similar to those for young athletes, there are some important differences. These include a higher metabolic cost of locomotion and preferential fat oxidation in young athletes during exercise. Young athletes, particularly children, are at a thermoregulatory disadvantage due to a higher surface area to weight ratio, a slower acclimatization, and lower sweating rate. An appropriate dietary intake rather than use of supplements (except when clinically indicated) is recommended to ensure young athletes participate fully and safely in athletics [07236].
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Self-regulation concepts

To test self-regulation concepts in relation to dietary intake and physical activity patterns in adolescence, which we predicted to be influenced by components of a self-control model a survey was conducted with a multiethnic sample of 9th grade public school students in a metropolitan area (n=539). Confirmatory analysis tested the measurement structure of self-control. Structural equation modeling tested the association of self-control constructs with measures of fruit and vegetable intake, saturated fat intake, physical activity, and sedentary behavior. Confirmatory analysis of 14 indicators of self-control showed best fit for a two-factor structure, with latent constructs of good self-control (planfulness) and poor self-control (impulsiveness). Good self-control was related to more fruit and vegetable intake, more participation in sports, and less sedentary behavior. Poor self-control was related to more saturated fat intake and less vigorous exercise. These effects were independent of gender, ethnicity, and parental education, which themselves had relations to diet and exercise measures. Multiple-group modeling indicated that effects of self-control were comparable across gender and ethnicity subgroups. Self-control concepts are relevant for patterns of dietary intake and physical activity among adolescents. Attention to self-control processes may be warranted for prevention programs to improve health behaviors in childhood and adolescence [07238].

Adolescents aged 11-14 years (n=326), belonging to organized sports federations in the Federal District, Brazil were interviewed. Subjects (n=107) provided four non-consecutive days of food consumption and 219 subjects provided two non-consecutive days of intake. The objective was to assess their nutrient and water intake according to dietary reference intake values and their energy and macronutrient intake by sex and sports groups they were engaged in: endurance, strength-skill or mixed, according to the guidelines established by the American College of Sport Medicine (ACSM). Dietary data were corrected for intra-individual variation. Total energy expenditure was higher among endurance athletes following their higher training time when compared to adolescents engaged in strength-skill or mixed sports. Total energy intake was only significantly higher among endurance-engaged females. Protein intake of males was above the guidelines established by the ACSM for all sports groups. All male sport groups fulfilled the intake levels of carbohydrate per kg body weight but only females engaged in endurance sports fulfilled carbohydrate guidelines. Intakes of micronutrients with low prevalence of adequate intake were: vitamins B1, E and folate, magnesium and phosphorus. Few adolescents presented adequate intake for calcium, fibre, drinking water and beverages. For micronutrients, prevalence of adequacies were
lower for females than males, except for liquids and water. Nutrition guidance is needed to help adolescents fulfil specific guidelines of macronutrient intake for their sports and to improve their intake of micronutrients and water. Special attention should be given to female adolescent athletes [07239].

**Food preferences**

To assess the influence of preferences on food and nutritional intake in a group of adolescent high-level athletes, 22 male soccer players (14-16 years) were recruited. Individuals were asked to fill in a specific questionnaire including 15 food groups that had to be ranked according to their preferences. Three categories were established: “Like” (ranked 1-5), “Indifferent” (6-10), and “Dislike” (11-15). Dietary intake was assessed using the weighed food method (for nutrient intake) and a quantitative open-ended food frequency questionnaire (for the number of standard portions of each food group ingested daily). The main preferences were meat, poultry and derivates (ranked 1-5 in 83 % of individuals) and pasta (58 %), while vegetables (ranked 11-15 in 82 %) and fish (64 %) were the main dislikes. The most frequently consumed food groups were fruits and fruit juices (3.9 portions/day), Bread (3.0), and biscuits, confecionery and sweets (3.0). No statistical differences were found in food consumption between preference groups, and no relation was found between preferences and nutritional intake, except for those individuals who especially like bread, which had statistically higher energy and carbohydrate intake. Food preferences and food and nutritional intake of adolescent high-level soccer players were, effectively, unrelated [07240].

**Excess of protein and fat**

The purpose of one study was to determine whether 34 young Spanish males belonging to a cyclist team, follows the optimal macronutrients intake based on the recommended dietary guidelines. The deficits in nutrition jeopardise the sportive performances, but what about the diets with excessive intake of macronutrients? Furthermore, is there an association between their sports achievements and the psychological profile? Surely, but the problem is to determine which psychological variables are involved. Nutritional evaluation based on a questionnaire of 7 consecutive days. Cyclists consume an excessive quantity of proteins and lipids in their diets. The average consumption of proteins is 16 percent of their caloric intake (the recommended quantity is less than 10 %). The average consumption of fats is 39 percent (the recommended is less than 30 %). The same tendency is found in the homologous Spanish young people of the enKID study, where the percentage of energy from fat and saturated fat is much higher than the recommended one. The cyclists consume insufficient quantities of carbohydrates (average is 45 % of their caloric intake, the recommended is more than 60 %), therefore the reload of their glycogen stores may not be complete on each competition stage. No association has been found between the excessive intake of referred macronutrients and the achieved sports performances. This work contributes to the knowledge of the diets of very active young cyclists. Excessive intake of proteins and fats do not jeopardise their sportive performances. The commonly studied psychological variables in sport, are not determinant of sports achievements of young cyclists; additional work is needed to determine the psychological profile playing a determinant role in success of young cyclists [07241].

**Female needs in nutrition and hydration**

In general, differences in nutritional needs between males and females are smaller than differences between individuals, so that principles developed for male players also apply to
women. There is a need to address energy balance and body composition: prolonged energy deficits cannot be sustained without harm to health and performance. Published reports show mean carbohydrate intakes for female players of about 5 g/kg/day, and this seems to be too low to sustain consistent intensive training. The timing of protein intake may be as important as the amounts consumed, provided that the total intake is adequate. Dehydration adversely affects skill and stamina in women as it does in men, so an individualised hydration strategy should be developed. The prevalence of iron deficiency in women generally is high, but it seems to be alarmingly high in female players. All players should adopt dietary habits that ensure adequate iron intake. Football training seems to increase bone mass in the weight-bearing limbs, with positive implications for bone health in later life, but some players may be at risk from inadequate calcium dietary intake. An extensive series of reviews on all aspects of nutrition for football has been published. It is immediately evident from these reviews that there are abundant experimental data relating to the nutrition and hydration practices of male football players, but information on female players is less easy to find. There are many reasons for this, but the growing popularity of the women's game means that the nutritional needs of the female player need to be urgently considered. Owing to the shortage of gender-specific information, nutrition and hydration guidelines developed for male players are often applied to the female player. This is sometimes appropriate, but for many reasons this may not always be so. There are gender-related differences in the physical demands of training and match play, and also some differences in the nutrition needs of men and women generally, and of male and female athletes in particular. It is important to recognise at the outset, though, that the differences between the nutritional needs of men and women are generally small in comparison with the wide range of nutritional needs of the population as a whole. In addition to the physiological and metabolic demands that shape nutritional needs and eating habits, players are subject to many of the societal issues that affect the general population. Female football players are not immune to these external pressures. The women's game is generally less well developed – at least in most countries – than the men's game. This may mean that less emphasis is placed on training, nutrition and other means of enhancing performance. It may also mean that there is more scope for the committed player to gain an advantage by exploiting these opportunities. Most of the world's football players take part for enjoyment: only a few are professionals. The nutritional strategies of the elite player, who trains most days of the week and perhaps competes more than once most weeks of the year, will be different from those of the player who trains no more than once a week. The general nutritional principles are the same, however, and are aimed at promoting health, fitness and match performance [07242].

A triad

The female athlete triad (Triad) refers to the interrelationships among energy availability, menstrual function, and bone mineral density, which may have clinical manifestations including eating disorders, functional hypothalamic amenorrhea, and osteoporosis. With proper nutrition, these same relationships promote robust health. Athletes are distributed along a spectrum between health and disease, and those at the pathological end may not exhibit all these clinical conditions simultaneously. Energy availability is defined as dietary energy intake minus exercise energy expenditure. Low energy availability appears to be the factor that impairs reproductive and skeletal health in the Triad, and it may be inadvertent, intentional, or psychopathological. Most effects appear to occur below an energy availability of 30 kcal/kg of fat-free mass per day. Restrictive eating behaviors practiced by girls and women in sports or physical activities that emphasize leanness are of special concern. For prevention and early intervention, education of athletes, parents, coaches, trainers, judges, and administrators is a priority. Athletes should be assessed for the Triad at the preparticipation physical and/or annual health screening exam, and whenever an athlete
presents with any of the Triad's clinical conditions. Sport administrators should also consider rule changes to discourage unhealthy weight loss practices. A multidisciplinary treatment team should include a physician or other health-care professional, a registered dietitian, and, for athletes with eating disorders, a mental health practitioner. Additional valuable team members may include a certified athletic trainer, an exercise physiologist, and the athlete's coach, parents and other family members. The first aim of treatment for any Triad component is to increase energy availability by increasing energy intake and/or reducing exercise energy expenditure. Nutrition counseling and monitoring are sufficient interventions for many athletes, but eating disorders warrant psychotherapy. Athletes with eating disorders should be required to meet established criteria to continue exercising, and their training and competition may need to be modified. No pharmacological agent adequately restores bone loss or corrects metabolic abnormalities that impair health and performance in athletes with functional hypothalamic amenorrhea [07243].

Energy need

From a review of energy intake of male and female athletes in different sports it was concluded that the energy intake of female athletes, expressed relative to body mass, is about 70 percent of that of their male counterparts. This can be explained by the lower intensity, frequency and duration of the training programmes of most female athletes. Many studies, however, report that some athletes seem to be in negative energy balance, and such observations seem to apply more often to female athletes than to their male counterparts: these observations and the potential explanations have been reviewed in detail. It does seem that some female athletes are in precarious energy balance, and maintain a low body mass and low body fat content by prolonged energy restriction, including some periods of negative energy balance. This is not unique to female athletes, in many societies women are under greater pressure to maintain a low body fat content. At a time when the prevalence of obesity is increasing rapidly, some parts of the population are moving in the opposite direction. There seem to be rather few data on the energy intakes of female players, and most of those are based on short-term measurements (typically three days) using household measures to estimate portion sizes that were then recorded in a food diary. In one study it was used a seven-day food record: indirect calorimetry was used to assess resting metabolic rate and a physical activity diary was used to estimate energy expenditure. The mean estimated energy expenditure (9.42 MJ/day) was not different from the mean estimated energy intake (8.97 MJ/day). It is interesting to note that the estimated energy intake was also equal to energy expenditure in a control group in this study, but a group of gymnasts recorded energy expenditure that was more than 3 MJ/day higher than the estimated energy intake. Any restriction of energy intake with the aim of reducing body fat content requires careful attention if injury and illness are to be avoided. This is because of the potential for adverse effects of prolonged energy restriction, including delayed recovery from exercise, impairment of adaptation to the training stimulus, suppression of immune function, and disruption of reproductive function [07242].

Women football

There is little information on the nutritional habits of female football players at any level of the game. There is also a shortage of information on the nutrition and hydration strategies that players should adopt. In general, differences in nutritional needs between males and females are smaller than differences between individuals, so that principles developed for male players also apply to women. There is a need to address energy balance and body composition: prolonged energy deficits cannot be sustained without harm to health and performance. Published reports show mean carbohydrate intakes for female players of about 5 g/kg/day, and this seems to be too low to sustain consistent intensive training. The timing
of protein intake may be as important as the amounts consumed, provided that the total intake is adequate. Dehydration adversely affects skill and stamina in women as it does in men, so an individualised hydration strategy should be developed. The prevalence of iron deficiency in women generally is high, but it seems to be alarmingly high in female players. All players should adopt dietary habits that ensure adequate iron intake. Football training seems to increase bone mass in the weight-bearing limbs, with positive implications for bone health in later life, but some players may be at risk from inadequate calcium dietary intake [07244].

**Women endurance running**

The aim of one case study was to describe the race nutrition practices of a female runner who completed her first 100-km off-road ultraendurance running event in 12 hr 48 min 55 s. Food and fluid intake during the race provided 10,890 kJ (736 kJ/hr) and 6,150 ml (415 ml/hr) of fluid. Hourly reported carbohydrate intake was 44 g, with 34 percent provided by sports drink. Hourly carbohydrate intake increased in the second half (53 g/hr) compared with the first half (34 g/hr) of the race, as the athlete did not have access to individualized food and fluid choices at the early checkpoints and felt satiated in the early stages of the race after consuming a prerace breakfast. Mean sodium intake was 500 mg/hr (52 mmol/L), with a homemade savory broth and sports drink (Gatorade Endurance®) being the major contributors. The athlete consumed a variety of foods of varying textures and tastes with no complaints of gastrointestinal discomfort. Despite thinking she would consume sweet foods exclusively, as she had done in training, the athlete preferred savory foods and fluids at checkpoints during the latter stages of the race. This case study highlights the importance of the sports nutrition team in educating athletes about race-day nutrition strategies and devising a simple yet effective system to allow them to manipulate their race-day food and fluid intake to meet their nutritional goals [11380].

**Carbohydrate need**

Carbohydrate, in the form of glycogen stores in liver and skeletal muscle, is an essential fuel during training and match play, and carbohydrate availability is a limiting factor during prolonged fatiguing exercise. In male players, the glycogen content of the active muscles substantially decreases during training and match play, but the muscle glycogen content at the end of a game remains much higher than the values found in typical laboratory studies in which subjects run to fatigue. However, studies (on male players) have shown that an inadequate intake of dietary carbohydrate in the days before a match will impair running performance, reducing both the distance covered and the speed of running, especially during the second half of a game. This last finding suggests that, even though there is no good understanding of why players slow down and show signs of fatigue late in a game, it seems prudent to ensure that the muscle glycogen stores have been replenished since the last training session or game. This can be a problem for elite players when the fixture list becomes congested. However, it is less of a problem for those who play only once a week and can adjust training to ensure sufficient recovery prior to games. It is no longer appropriate to think of carbohydrate needs as a fraction of total energy intake: rather they should be expressed as an amount of carbohydrate relative to body mass. It was considered primarily male players, recommended that daily carbohydrate intake during periods of moderate training, periods of energy restriction to achieve fat loss, and for less mobile players, should be about 5-7 g/kg body mass. For more mobile players and during periods of twice-daily training or in preparation for match play, they recommended increasing this to 7-12 g/kg. This is a wide range, and it has important implications for overall energy balance and also raises practical issues relating to the amount of food that must be consumed. From the limited data available, it seems that female players typically consume rather less than
these recommendations. Although most of the players may be able to cope at this level of intake, those at the lower end might well benefit from an increased intake while remaining within their total energy budget. There are, however, consistent with data suggesting that early reports of women being less able than men to achieve supercompensation of muscle glycogen stores by manipulation of diet and exercise were the result of inadequate carbohydrate intake of the female subjects [07242].

Protein need

If the diet does not supply sufficient protein, and more particularly enough essential amino acids, the functional capacity of muscles and of all other tissues will eventually decline. An adequate intake of protein is essential for muscle growth and repair, and also for a healthy immune system and a whole range of other physiological functions. However, what constitutes an adequate protein intake has been widely debated. More recent investigations suggest that besides the amount of protein, the timing of intake and the presence or absence of other nutrients can affect the ability of an athlete to adapt to a training stimulus. The aim of training is to promote changes in tissue structure and function that lead to improved performance. This requires a constant turnover of protein molecules with a net breakdown of those proteins that are not required and a net synthesis of proteins that are required. These changes require the application of an appropriate exercise stimulus; the nature of the adaptation is specific to the type of training stimulus, and the extent of adaptation is a function of the training load. It is now known that the nutritional and hormonal environment will modulate these changes, making training either more or less effective. Whereas it was formerly thought that the main role of nutrition was to enhance recovery, thus allowing athletes to train harder, it is now recognised that a good dietary strategy can perhaps allow the same training adaptations with a reduced training load. This is important in reducing the risk of chronic fatigue and injury, and may allow more time and effort to be devoted to technical training rather than to strength and endurance training. There is some increase in the protein requirement during periods of stress, including those imposed by exercise training and competition. The protein requirement, assuming that the diet provides mixed proteins containing all of the essential amino acids, is about 0.6 g/kg/day. However, a safety margin is added to this to account for variations in the composition of the protein ingested as well as individual variation in the requirement, so that the recommended intake is usually set at about 0.8 g/kg/day for both men and women. This is easily achieved with normal levels of intake. Even a player eating a diet that provides only 2200 kcal/day (8.8 MJ/day) with 10-15 percent of total energy from protein will have an intake of 55-82 g/day. Assuming a body mass of 60 kg, this will give a daily protein intake of about 0.9-1.4 g/kg/day. Recommendations for football players have been set at various levels, but are commonly in the range of 1.4-1.7 g/kg/day. It is difficult not to reach this value with a typical Western diet if energy demand is met, but some players with restrictive eating practices and limited food choices may not. Millward's Adaptive Metabolic Demands model for protein says that a protein balance can be sustained on a wide range of protein intakes, but that sudden changes in intake are accommodated only slowly. The athlete on a high protein intake (3-4 g/kg/day) who suddenly reduces this will experience a loss of muscle during the process of accommodation but will eventually adapt to the new lower intake [07242].

Hydration during sports

Sweat rates in women are lower than in men across most activities for which data are available. In female football players sweat rates (mean 0.8 L/h in training, 0.8 L/h in a match) and drinking rates (0.4 L/h in training, 0.4 L/h in a match) tended to be lower than those observed in elite male players in training. This reflects in part the smaller mass of female
participants but is largely a consequence of the lower rates of metabolic heat production. Losses in sweat electrolytes in both England U21 female players (n=14) during training and England senior international players (n=25) during a friendly match were less than those of their male counterparts, but this was solely due to smaller volume losses: the sodium concentrations were similar (mean 44 mmol/L). Where sweat losses are small, there is little need for fluid replacement during training or match play. Performance, however, is impaired when the sweat-induced loss of body mass reaches about 1-2 percent of the pre-exercise body mass, and sufficient fluid should be consumed to prevent net loss exceeding this amount. Drinking fluids containing small amounts of carbohydrate and electrolytes may be better than drinking plain water. Water is adequate when sweat losses are small or when activity levels are not high. Where large sweat losses are anticipated as in games or hard training in the heat, players should be careful to ensure they are well hydrated beforehand. Self-monitoring by attention to the frequency, volume and colour of urine may be helpful when fluid balance is stressed – for example, in tournament situations in warm climates [07242].

**Calcium**

Most of the body's store of calcium is found in the bones, and the amount of calcium deposited there as calcium phosphate reaches a peak in young adulthood before beginning to decline thereafter. After the age of 40, bone mass is lost typically at a rate of about 0.5 to 1.0 percent a year. Anyone who reaches adult life with a low peak bone mass, or who experiences a high rate of loss, is at risk of increased bone fragility: a third of women who reach the age of 90 will have fractured a hip, about twice the rate in men. Estrogen has a major role in calcium homoeostasis in women, so women with late menarche and early menopause are more likely to have osteoporosis and associated weakening of the bone structure. An adequate intake of calcium is essential for bone health, but so too is vitamin D, either ingested as the preformed vitamin or synthesised in the skin on exposure to sunlight. Imposed stress, in the form of weight-bearing exercise, stimulates bone formation, while bedrest promotes bone loss. Female football players have higher bone mineral density than sedentary women. Lean players who experience amenorrhoea should be especially conscious of the risk of problems in later life. Dairy produce is the best source of dietary calcium, but it is often avoided by weight-conscious athletes because of the fat content. All players should be encouraged to consume adequate low fat dairy produce [07242].

**Iron**

Iron is a key component of hemoglobin, the protein responsible for transport of oxygen from the lungs to the active tissues, and low levels of haemoglobin are associated with reduced exercise performance. Iron deficiency affects both men and women: the consequences are probably the same for both genders, but it seems that more women are at risk. The prevalence of iron deficiency in the general population is higher in women than it is in men, so it is not surprising that female athletes also show a higher prevalence than their male counterparts. It is difficult to compare studies as many different criteria have been used, and generalisations are also fraught with difficulties. Data from Australia suggest that the prevalence of deficiency, defined as abnormal serum ferritin and/or transferrin saturation with or without anemia, is about 8 percent in women and 2 percent in men: in the vegetarian population, however, this rises to 27 percent of females and 5 percent of males. Data from the USA show an increased prevalence in women from minority populations and from lower socioeconomic groups. This high prevalence in disadvantaged populations is a particular concern, but in the USA the prevalence of iron deficiency has been estimated to be up to 20 percent in young women. This figure may increase to 25-35 percent or even more in women.
competing in a variety of sports. Of 28 players in the Swedish national squad, 59 percent were found to have iron deficiency and 29 percent iron deficiency anaemia six months prior to the Fédération Internationale de Football Association (FIFA) Women's World Cup. These values are alarmingly high, and although iron deficiency without anaemia has little effect on exercise performance, it may be the prelude to a fall in circulating haemoglobin concentration and lead to a fall in aerobic capacity. All individuals diagnosed as having iron deficiency anaemia should take iron supplements in the form of ferrous sulphate in an appropriate dose. The treatment will probably take months rather than weeks to be effective, and dietary changes should be started concurrently. Strategies should include:

- an increased intake of iron-rich foods, such as red meat that contain haem iron
- use of fortified foods, such as breakfast cereals
- use of nutrients that promote iron intake, such as vitamin C and meat with the iron-rich meals
- a reduction in the intake of inhibitors of iron absorption, such as fibre and tannin.

Note that supplementation should not be started on a “just in case” basis, but should follow a full investigation that includes blood parameters. The routine use of iron supplementation is common, but is probably not wise as it may do more harm than good [07242].

*Creatine*

Creatine has attracted enormous attention over recent years, and there is a solid body of evidence showing that some individuals may benefit by an increased ability to perform repeated sprints and an increased lean body mass. Studies that have used performance tasks that simulate football have not been entirely convincing. Equally, there is no evidence of harm, so players may feel inclined to see whether they experience a benefit. Any player adopting this approach must be aware that a large fraction of the dietary supplements on sale to athletes may contain agents that will cause a positive doping test, even though these are not declared on the label [07242].

*Nutrition in the aged athletes*

To evaluate the evidence for dietary recommendations in older adult athletes a review of the literature was performed. Regarding resistance training, a protein intake of slightly more than 0.8 g/kg/d is required to optimize gains in muscle strength. The early provision of protein and carbohydrate following a weight training session can enhance resultant strength and fat-free mass gains. Supplementation with creatine monohydrate (approximately 5 g/d) can potentiate some of the gains in strength and fat free mass attained through resistance exercise training. Regarding endurance exercise training, there are no studies evaluating carbohydrate loading, during-event, or postexercise carbohydrate/nutritional replacement in older adults. It was concluded that the amount and timing of dietary protein is important to maximize strength and gains in fat-free mass during resistance exercise training. Creatine monohydrate supplementation can potentiate some of these gains during the first 4 to 6 months of training. Older adults should consume adequate carbohydrates during endurance training (6-8 g/kg/d) and may benefit from the provision of carbohydrate and protein in the early recovery phase following endurance exercise to maximize glycogen re-synthesis for a subsequent exercise bout. There is no scientific reason to assume that older athletes will respond differently to the pre- and during-race fluid and carbohydrate replacement strategies suggested for younger athletes. The consensus guidelines outlined by the American College of Sports Medicine should therefore be followed for all athletes, regardless of their age.
Dietary recommendation

Impact of dietary recommendations

The aim of one study was to evaluate the nutritional trends in young elite male soccer players, attending national soccer league at RFC Bruges over the last two decades. At the start of each season, players and parents are instructed about normal healthy nutrition and fluid intake by dieticians. Since 1983, dieticians perform dietary habit surveys in the adolescent player groups. They instruct players and parents how to record all food and fluid intake during 3 days, a training-day, a match-day and an off -day. It is asked to do the recordings when players and parents are together and parents are asked to supervise the recording. Intakes are calculated using the Becel institute nutrition A significant decrease of energy intake/m² is observed over the last 20 years. Body composition, measured as age-matched body mass index remained at median levels for the population during this period. An important modification of dietary content towards the recommended daily intakes is observed. Fat, saturated fat and cholesterol intake decreased dramatically. Carbohydrate intake increased. It was concluded that a positive evolution towards the recommended dietary composition is observed over the years. However, the decrease in caloric intake without influence on the body mass index could suggest that these elite male footballers have a decreased physical activity as compared to 20 years ago [11378].

Energy intake

Previous intervention studies suggest that leptin, insulin, insulin-like growth factor 1 (IGF-1), and triiodothyronine (T3) are sensitive markers of inadequate energy intake in relation to exercise expenditures. Because of limitations in metabolic hormone measurements, self-reported energy availability (EA) based on food and activity records may present an alternative for characterizing energy status in young athletes. The purpose of one study was to assess whether self-reported EA is related to leptin, insulin, IGF-1, and T3 in 352 young athletes. Sex, body composition, sport participation, and acute weight changes were considered as confounding variables. Multiple linear regression revealed that EA was negatively associated with leptin but not with insulin, IGF-1, or T3. Female athletes with low EA (<30 kcal/kg fat-free mass (FFM)) had higher leptin concentrations (5.0 ± 4.7 ng/mL) and more body fat (18.3 % ± 5.1 %) than did females with normal EA (leptin, 3.1 ± 2.4 ng/mL; body fat, 15.8 % ± 4.2 %; both). Athletes reporting acute weight loss (>1 kg/week) had a lower EA (18.9 ± 7.4 kcal/kg FFM) than did weight-stable athletes (30.0 ± 11.2 kcal/kg FFM) or athletes reporting weight gain (>1 kg; 49.7 ± 13.1 kcal/kg FFM). IGF-1 and T3 were also significantly reduced in athletes who lost weight. This cross-sectional study reveals a lack of association between self-reported EA and metabolic hormones indicative of energy status in young athletes. Further studies are needed to investigate whether self-reported EA and metabolic hormones are in better agreement when measured repeatedly [13589].

Chewing gum

Although chewing gum has been considered a potential method for reducing energy intake, little empirical data exist to support this idea. The purpose of one study was to test the hypothesis that chewing gum before eating reduces motivation to eat, hunger, and energy intake. In order to test this hypothesis, we conducted two experiments in which participants chewed gum prior to completing a food reinforcement task or before all eating occasions for two of three weeks. In Experiment 1, we found that chewing gum had no influence on the
reinforcing value of food, but chewing mint gum reduced liking of and energy intake from fruit. In addition, chewing gum reduced self-reported hunger immediately after gum chewing and after eating compared with the no gum condition. In Experiment 2, gum chewing had no significant effect on total energy intake, but participants consumed fewer meals, consumed more energy per meal, and had a lower nutrient adequacy ratio during the gum chewing weeks. These studies provide no evidence that acute or chronic gum chewing reduces hunger or energy intake. In fact, chewing mint-flavored gum may deter consumption of fruit and reduce diet quality [13590].

For team sports

Abstract Implementation of a nutrition programme for team sports involves application of scientific research together with the social skills necessary to work with a sports medicine and coaching staff. Both field and court team sports are characterized by intermittent activity requiring a heavy reliance on dietary carbohydrate sources to maintain and replenish glycogen. Energy and substrate demands are high during pre-season training and matches, and moderate during training in the competitive season. Dietary planning must include enough carbohydrate on a moderate energy budget, while also meeting protein needs. Strength and power team sports require muscle-building programmes that must be accompanied by adequate nutrition, and simple anthropometric measurements can help the nutrition practitioner monitor and assess body composition periodically. Use of a body mass scale and a urine specific gravity refractometer can help identify athletes prone to dehydration. Sports beverages and caffeine are the most common supplements, while opinion on the practical effectiveness of creatine is divided. Late-maturing adolescent athletes become concerned about gaining size and muscle, and assessment of maturity status can be carried out with anthropometric procedures. An overriding consideration is that an individual approach is needed to meet each athlete's nutritional needs [11374].

For power sports

Contemporary training for power sports involves diverse routines that place a wide array of physiological demands on the athlete. This requires a multi-faceted nutritional strategy to support both general training needs – tailored to specific training phases – as well as the acute demands of competition. Elite power sport athletes have high training intensities and volumes for most of the training season, so energy intake must be sufficient to support recovery and adaptation. Low pre-exercise muscle glycogen reduces high-intensity performance, so daily carbohydrate intake must be emphasized throughout training and competition phases. There is strong evidence to suggest that the timing, type, and amount of protein intake influence post-exercise recovery and adaptation. Most power sports feature demanding competition schedules, which require aggressive nutritional recovery strategies to optimize muscle glycogen resynthesis. Various power sports have different optimum body compositions and body weight requirements, but increasing the power-to-weight ratio during the championship season can lead to significant performance benefits for most athletes. Both intra- and extracellular buffering agents may enhance performance, but more research is needed to examine the potential long-term impact of buffering agents on training adaptation. Interactions between training, desired physiological adaptations, competition, and nutrition require an individual approach and should be continuously adjusted and adapted [11375].

For endurance sports

Endurance sports are increasing in popularity and athletes at all levels are looking for ways to optimize their performance by training and nutrition. For endurance exercise lasting
30 min or more, the most likely contributors to fatigue are dehydration and carbohydrate depletion, whereas gastrointestinal problems, hyperthermia, and hyponatraemia can reduce endurance exercise performance and are potentially health threatening, especially in longer events (>4 h). Although high muscle glycogen concentrations at the start may be beneficial for endurance exercise, this does not necessarily have to be achieved by the traditional supercompensation protocol. An individualized nutritional strategy can be developed that aims to deliver carbohydrate to the working muscle at a rate that is dependent on the absolute exercise intensity as well as the duration of the event. Endurance athletes should attempt to minimize dehydration and limit body mass losses through sweating to 2-3 percent of body mass. Gastrointestinal problems occur frequently, especially in long-distance races. Problems seem to be highly individual and perhaps genetically determined but may also be related to the intake of highly concentrated carbohydrate solutions, hyperosmotic drinks, as well as the intake of fibre, fat, and protein. Hyponatraemia has occasionally been reported, especially among slower competitors with very high intakes of water or other low sodium drinks [11376].

For young soccer players

Limited data exist concerning the dietary practices of young professional soccer players that compete within the United Kingdom. Therefore, the purpose of this study was to investigate the nutritional and activity habits of professional male soccer players (n=10; age: 17 ± 1 years, height: 172 ± 5 m, mass: 68 ± 2 kg, estimated maximal aerobic capacity: 58 ± 1 mL/kg/min) who played for the youth team of a UK-based Championship club. All players recorded their 7-day dietary intake and activity habits during a competitive week that included a match day, 4 training days, and 2 rest days in the first half of the 2009/2010 playing season. The intake of carbohydrates (5.9 ± 0.4 g/kg/d), proteins (1.7 ± 0.1 g/kg/d), and fats (1.5 ± 0.1 g/kg/d) represented 56 ± 1, 16 ± 1, and 31 ± 1 percent of the mean daily energy intake respectively. The intake of fiber was found to be significantly lower than Recommended Nutrient Intake (RNI) values (67 % of RNI), whereas all other analyzed micronutrients met or exceeded recommended values. A mean daily energy deficit of 788 ± 174 kcal existed because daily energy expenditures exceeded that of intake (3,618 ± 61 vs. 2831 ± 164 kcal). The mean daily fluid intake was 3.2 ± 0.3 L. Consequently, the nutritional practices of the sampled group of professional youth soccer players were inadequate to sustain optimized performance throughout training and match play. Youth soccer players should therefore seek to ensure that their diets contain adequate energy through increased total caloric intake, while also optimizing the proportion of energy derived from carbohydrates and ensuring that enough fiber-rich foods are consumed [11377].

Response to long distance running (energy needed)

Marathoners are at an increased risk of developing upper respiratory tract infections following races and periods of hard training, which are associated with temporary changes in the immune system. The majority of the reported changes are decreases in function or concentration of certain immune cells. During this period of immune suppression, by some referred to as an “open window” in immune function, it has been hypothesized that viruses and bacteria might gain a foothold, which would increase the risk of infections. In light of this, nutritional interventions that can enhance immune function and reduce the risk of upper respiratory tract infections have been sought. One paper focused on the effect of glutamine, vitamin C, bovine colostrum and glucose. Although, some of these supplements can affect the physiological and immune changes associated with marathon racing, none of the supplements discussed have consistently been shown to reduce the risk of upper respiratory
tract infections and therefore cannot be recommended for use as enhancers of immune function in marathon runners [08426].

Many components of the immune system exhibit adverse change after marathon-type exertion. These immune changes occur in several compartments of the immune system and body (e.g. the skin, upper respiratory tract mucosal tissue, lung, peritoneal cavity, blood and muscle). Of all immune cells, natural killer (NK) cells, neutrophils and macrophages (of the innate immune system) exhibit the greatest changes in response to marathon competition, both in terms of numbers and function. Many mechanisms appear to be involved, including exercise-induced changes in stress hormone and cytokine concentrations, body temperature changes, increases in blood flow and dehydration. During this “open window” of immune dysfunction (which may last between 3 and 72 hours, depending on the immune measure), viruses and bacteria may gain a foothold, increasing the risk of subclinical and clinical infection. Of the various nutritional and pharmacological countermeasures to marathon-induced immune perturbations that have been evaluated thus far, ingestion of carbohydrate beverages during intense and prolonged exercise has emerged as the most effective. However, carbohydrate ingestion during a marathon attenuates increases in plasma cytokines and stress hormones, but is largely ineffective against changes in other immune components including suppression of NK and T-cell function, and salivary IgA output. Other countermeasures, such as glutamine, antioxidant supplements and ibuprofen, have had disappointing results and thus the search for companion agents to carbohydrate continues [08427].

It was investigated the nutritional habits of ultra-endurance runners before, during and after the Deutschlandlauf 2006 in Germany, from the north (Kap Arkona-Rügen) to the south (Lörrach), over 1,200 km and 17 stages. Twenty male ultra-runners completed a questionnaire about their nutrition before, during and after the race. In the 4 weeks, and the day before the race, 70 percent of the runners followed no special diet. In the morning before the start of a stage, the main nutrients were buns with jam, butter and cheese and the preferred drink was coffee. During the stages, the athletes preferred to consume bread, bananas and chocolate and preferably drank pure water, Apfelschorle and Coca Cola. In the evening, the athletes preferred to consume meat, noodles, pure water and beer. During the run, 40 percent of the athletes had a special desire for salty and fatty food and 10 percent a particular reluctance for sweet and carbohydrate-rich products. After the race, the runners preferred apples, vegetables, rice, bread, pure water, Apfelschorle and beer. Multi-vitamin products, multi-mineral products as well as magnesium were the preferred supplements before, during and after the race. It was conclude that 70 percent of the ultra-endurance runners in the Deutschlandlauf 2006 followed no special diet before the race. Multi-vitamins, multi-minerals and magnesium were preferably consumed as ergogenic supplements. Before the start of a stage they ate a normal breakfast; during a stage they preferred carbohydrate-rich products and water; and in the evening after a stage they preferred to consume meat with a carbohydrate-rich nutrition and drank water as well as beer [08428].

The objective of the study was to describe the food intake of adventure racers during a competition simulated in the laboratory. Ten male athletes with international experience in adventure races took part in the study. The experiment lasted 67 hr (total distance covered 477 km), but 3 athletes did not finish the race. Food intake was recorded throughout the simulation. Athletes’ total energy expenditure was greater than their total energy intake (24,516 vs. 14,738 kcal), and the athletes obtained significantly more energy from food than from supplements. Carbohydrate intake was below the recommendation of 0.5-1.0 g per kg and hour. These results indicate that guidelines for multiday adventure races are needed [08429].
Food provision during Olympic Games

The history of food provision at the summer Olympic Games over the past century (1896-2008) provides insight into the evolution of sports nutrition research and the dietary strategies of athletes. Early research favoring protein as the main fuel for exercise was reflected in Olympic Games menus from 1932 to 1968. Despite conclusive research from the 1960s demonstrating the clear benefit of carbohydrate on exercise performance, a specific emphasis on carbohydrate-rich foods was not noted until the 1970s. Athlete food preferences and catering complexity evolved rapidly between 1970 and 2000, driven predominantly by a dramatic expansion of the Olympic Games and the emergence of systematic sports nutrition research. Nutritional advice by experts and sponsorship by food companies became increasingly important beginning with the 1984 Los Angeles Olympic Games. More recent developments include nutritional labeling of menu items and provision of a nutrition information desk (Barcelona 1992), demand for a "high-starch, low-fat menu" (Atlanta 1996), the addition of a dedicated menu website and the systematic gathering of information on athletes' apparent consumption (Sydney 2000), and appointment of the first international dietetic review committee (Beijing 2008). The history of catering at the Olympic Games tracks the evolution of sports nutrition practice from anecdotes and myth towards an established specialty in nutrition and dietetics grounded in evidence-based science [11243].

General recommendations

It is the position of the American Dietetic Association, Dietitians of Canada, and the American College of Sports Medicine that physical activity, athletic performance, and recovery from exercise are enhanced by optimal nutrition. These organizations recommend appropriate selection of foods and fluids, timing of intake, and supplement choices for optimal health and exercise performance. This updated position paper couples a rigorous, systematic, evidence-based analysis of nutrition and performance-specific literature with current scientific data related to energy needs, assessment of body composition, strategies for weight change, nutrient and fluid needs, special nutrient needs during training and competition, the use of supplements and ergogenic aids, nutrition recommendations for vegetarian athletes, and the roles and responsibilities of sports dietitians. Energy and macronutrient needs, especially carbohydrate and protein, must be met during times of high physical activity to maintain body weight, replenish glycogen stores, and provide adequate protein to build and repair tissue. Fat intake should be sufficient to provide the essential fatty acids and fat-soluble vitamins, as well as contribute energy for weight maintenance. Although exercise performance can be affected by body weight and composition, these physical measures should not be a criterion for sports performance and daily weigh-ins are discouraged. Adequate food and fluid should be consumed before, during, and after exercise to help maintain blood glucose concentration during exercise, maximize exercise performance, and improve recovery time. Athletes should be well hydrated before exercise and drink enough fluid during and after exercise to balance fluid losses. Sports beverages containing carbohydrates and electrolytes may be consumed before, during, and after exercise to help maintain blood glucose concentration, provide fuel for muscles, and decrease risk of dehydration and hyponatremia. Vitamin and mineral supplements are not needed if adequate energy to maintain body weight is consumed from a variety of foods. However, athletes who restrict energy intake, use severe weight-loss practices, eliminate one or more food groups from their diet, or consume unbalanced diets with low micronutrient density, may require supplements. Because regulations specific to nutritional ergogenic aids are poorly enforced, they should be used with caution, and only after careful product evaluation for safety, efficacy, potency, and legality [09311, 09312].
Mineral nutrients, vitamins, and trace elements are essential for the growth and development of a multicellular organism. Today, an adequate supply of nutrients is often unattainable solely through a well-balanced diet, so a targeted, individually designed dietary supplement regime is necessary. Nutrient deficiency, which is impossible to detect through plasma levels alone, is reliably detected through the intracellular measurement of the nutrient levels in the blood. Two case studies presented here indicate the need for supplementation as improvement in nutritional behavior could not replenish already exhausted nutrient reservoirs. Only supplementation was able to significantly boost nutrient levels and confer beneficial effects on general welfare, physical performance, and resistance to infections. Therefore, it appears that nutritional supplements are advisable for everyone, but more research is needed, especially on an intracellular level, to corroborate these findings [07220].

Vitamins and minerals are necessary for many metabolic processes in the body and are important in supporting growth and development. Vitamins and minerals also are required in numerous reactions involved with exercise and physical activity, including energy, carbohydrate, fat and protein metabolism, oxygen transfer and delivery, and tissue repair. The vitamin and mineral needs of athletes have always been a topic of discussion. Some researchers state that athletes require more vitamins and minerals than their sedentary counterparts, whereas other researchers do not report greater micronutrient requirements. The intensity, duration, and frequency of the sport/workout and the overall energy and nutrient intakes of the individual all have an impact on whether or not micronutrients are required in greater amounts [07221].

**Nutrition for sprinters**

The primary roles for nutrition in sprints are for recovery from training and competition and influencing training adaptations. Sprint success is determined largely by the power-to-mass ratio, so sprinters aim to increase muscle mass and power. However, extra mass that does not increase power may be detrimental. Energy and protein intake are important for increasing muscle mass. If energy balance is maintained, increased mass and strength are possible on a wide range of protein intakes, so energy intake is crucial. Most sprinters likely consume ample protein. The quantity of energy and protein intake necessary for optimal training adaptations depends on the individual athlete and training demands; specific recommendations for all sprinters are, at best, useless, and are potentially harmful. However, if carbohydrate and fat intake are sufficient to maintain energy levels, then increased protein intake is unlikely to be detrimental. The type and timing of protein intake and nutrients ingested concurrently must be considered when designing optimal nutritional strategies for increasing muscle mass and power. On race day, athletes should avoid foods that result in gastrointestinal discomfort, dehydration or sluggishness. Several supplements potentially influence sprint training or performance. Beta-alanine and bicarbonate may be useful as buffering agents in longer sprints. Creatine may be efficacious for increasing muscle mass and strength and perhaps increasing intensity of repeat sprint performance during training [07222].

**Nutrition for runners**

The high prevalence of obesity in Western societies has been attributed in part to high-fat low-CHO food consumption. However, people have also become less active, and inactivity may have increased the risk for weight gain from poor dietary choices. Analyses were performed to test whether diet-weight relationships were attenuated by vigorous exercise. Age- and education-adjusted cross-sectional regression analyses of 62,042 men and 44,695
women recruited for the National Runners’ Health Study were conducted. Reported meat and fruit intakes were analyzed separately and as indicators of high-risk diets. The runners were generally lean as measured by body mass index (BMI), and middle-aged, who ran 5.30 ± 3.23 km/day if male and 4.79 ± 3.00 km/day if female. Running significantly attenuated BMI’s relationship to reported meat and fruit intakes in men and women. Specifically, compared with running <2 km/day running >8 km/day reduced the apparent BMI increase per serving of meat by 43 percent in men and 55 percent in women and reduced the apparent BMI reduction per serving of fruit by 75 percent in men and 94 percent in women. Running also significantly attenuated the concordant relationship between reported meat intake and waist and chest circumferences in men and women and the concordant relationship between meat intake and hip circumference in women. It was concluded that vigorous exercise may mitigate diet-induced weight gain, albeit not guaranteeing protection from poor dietary choices [11515].

Nutrition for triathlon and marathon

Triathlon is a sport consisting of sequential swimming, cycling and running. The main diversity within the sport of triathlon resides in the varying event distances, which creates specific technical, physiological and nutritional considerations for athlete and practitioner alike. The purpose of this article is to review physiological as well as nutritional aspects of triathlon and to make recommendations on ways to enhance performance. Aside from progressive conditioning and training, areas that have shown potential to improve triathlon performance include drafting when possible during both the swim and cycle phase, wearing a wetsuit, and selecting a lower cadence (60-80 rpm) in the final stages of the cycle phase. Adoption of a more even racing pace during cycling may optimise cycling performance and induce a “metabolic reserve” necessary for elevated running performance in longer distance triathlon events. In contrast, drafting in swimming and cycling may result a better tactical approach to increase overall performance in elite Olympic distance triathlons. Daily energy intake should be modified to reflect daily training demands to assist triathletes in achieving body weight and body composition targets. Carbohydrate loading strategies and within exercise carbohydrate intake should reflect the specific requirements of the triathlon event contested. Development of an individualised fluid plan based on previous fluid balance observations may assist to avoid both dehydration and hyponatremia during prolonged triathlon racing [07223].

Muscle glycogen provides a key fuel for training and racing a marathon. Carbohydrate “loading” can enhance marathon performance by allowing the competitor to run at their optimal pace for a longer period before fatiguing. For the well trained runner, this may be achieved by tapering exercise over the final days before the marathon and ensuring carbohydrate intakes of 10-12 g/kg/day over the 36-48 hours prior to the race. Sports nutrition guidelines recommend that the runner consumes sufficient carbohydrate to promote restoration of muscle glycogen between training sessions. This strategy should allow the runner to ‘train harder’ and recover optimally between workouts. A recent hypothesis suggests that runners might ‘train smarter’ by training with low glycogen stores, since this might promote greater stimulation of the training response. However, there is no evidence that a low carbohydrate diet enhances the outcomes of training or provides benefits as a depletion phase prior to carbohydrate loading. In fact, a low carbohydrate diet may even impair performance if carried out for extended periods. If there are benefits to manipulating glycogen stores for some workouts, this is likely to happen as the natural outcome of the periodisation of the high-volume programme of an elite runner [07224].
The energy required to run a marathon is mainly provided through oxidative phosphorylation in the mitochondria of the active muscles. Small amounts of energy from substrate phosphorylation are also required during transitions and short periods when running speed is increased. The three inputs for adenosine triphosphate production in the mitochondria include oxygen, free adenosine diphosphate and inorganic phosphate, and reducing equivalents. The reducing equivalents are derived from the metabolism of fat and carbohydrate (CHO), which are mobilised from intramuscular stores and also delivered from adipose tissue and liver, respectively. The metabolism of fat and CHO is tightly controlled at several regulatory sites during marathon running. Slower, recreational runners run at 60-65 percent maximal oxygen uptake ($\text{VO}_2\text{max}$) for approximately 3:45:00 and faster athletes run at 70-75 percent for approximately 2:45:00. Both groups rely heavily on fat and CHO fuels. However, elite athletes run marathons at speeds requiring between 80 and 90 percent $\text{VO}_2\text{max}$, and finish in times between 2:05:00 and 2:20:00. They are highly adapted to oxidise fat and must do so during training. However, they compete at such high running speeds, that CHO oxidation (also highly adapted) may be the exclusive source of energy while racing. Further work with elite athletes is needed to examine this possibility [07225].

**Nutrition for football players**

The physical demands in soccer have been studied intensively, and the aim of the present review is to provide an overview of metabolic changes during a game and their relation to the development of fatigue. Heart-rate and body-temperature measurements suggest that for elite soccer players the average oxygen uptake during a match is around 70 percent of maximum oxygen uptake ($\text{VO}_2\text{max}$). A top-class player has 150 to 250 brief intense actions during a game, indicating that the rates of creatine-phosphate (CP) utilization and glycolysis are frequently high during a game, which is supported by findings of reduced muscle CP levels and severalfold increases in blood and muscle lactate concentrations. Likewise, muscle pH is lowered and muscle inosine monophosphate (IMP) elevated during a soccer game. Fatigue appears to occur temporarily during a game, but it is not likely to be caused by elevated muscle lactate, lowered muscle pH, or change in muscle-energy status. It is unclear what causes the transient reduced ability of players to perform maximally. Muscle glycogen is reduced by 40 to 90 PERCENT during a game and is probably the most important substrate for energy production, and fatigue toward the end of a game might be related to depletion of glycogen in some muscle fibers. Blood glucose and catecholamines are elevated and insulin lowered during a game. The blood free-fatty-acid levels increase progressively during a game, probably reflecting an increasing fat oxidation compensating for the lowering of muscle glycogen. Thus, elite soccer players have high aerobic requirements throughout a game and extensive anaerobic demands during periods of a match leading to major metabolic changes, which might contribute to the observed development of fatigue during and toward the end of a game [07226].

**General recommendatons regarding the use of dietary supplements**

Many athletes continue to take supplements to try and improve recovery from training or to gain a performance edge in competition or are advised that supplements are necessary for health maintenance. Dietary supplement use by high-level athletes is estimated at 65-95 percentage. Supplement commercialization is a multibillion dollar industry where many claims are made with little scientific evidence; regulation for purity or side effects is still lacking in many countries. Members of the athletes' entourage often push substances without sufficiently understanding physiology or nutrition. There is a very limited number of dietary supplements which are permitted and considered ergogenic. Athletes need to focus
on proper training, optimal recovery practices and wholesome nutrition regimens before they even consider supplements. Some education programmes appear to be resulting in decreased supplement use among Olympic athletes. If an athlete truly believes he/she should take a dietary supplement, everything should be done to minimise the risk [13015]:

1. Do not rely on advice from friends, fellow athletes or coaches but undergo a proper evaluation by a qualified physician and/or sports nutrition professional familiar with sport and anti-doping rules. It is quite likely that dietary supplements are not necessary and nutrient deficiencies may be corrected from food sources.
2. Avoid any product making claims of performance enhancement or any exaggerated claims or uses the words: “stimulant, energy or muscle booster, enhancer, legal or alternate steroid, extreme, blast, weight lost”. Even if no prohibited substance is listed on the label, the product may be spiked with one.
3. Herbal stimulants and prohormones are especially high risk. Use of the terms herbal or natural does not in any way mean that the product does not contain a prohibited substance.
4. Some companies offer guarantees of purity or are certified by other companies that do quality control. Verify the third party testing system reputation and remember there are no absolute guarantees.
5. Avoid any company that states their products are WADA approved. WADA or its accredited laboratories never test supplements or any products when not part of a doping control process. WADA cannot recommend any company or quality control system. In order to guarantee purity, each product batch would have to be tested for all prohibited substances.
6. Avoid products containing multiple ingredients as there is a higher risk of contamination. Vitamins and minerals (often classified as supplements) should be from reputable pharmaceutical companies and should not be mixed with other products.
7. Seek guidance from your anti-doping organisation about recent information on contaminated or dangerous products in your part of the world.

Although athletes must exhibit utmost caution when using supplements due to risk of contamination, governmental authorities also have a duty to endeavour to ensure proper regulation and quality control within the supplement industry. Some initiatives are trying to improve this situation. According to Article 10, UNESCO International Convention against Doping in Sport, governments must encourage producers and distributors of dietary or nutritional supplements to establish marketing best practices, including accurate labelling, quality assurance and avoidance of false marketing. In a survey (UNESCO Conference of Parties 2011), only 43 percent of governments responded that they implemented extensive or substantial measures to address these issues [13015].

Furthermore, a more systematic approach is needed for analyzing risks and benefits of dietary supplements. The real risks to health of ingesting potentially dangerous substances contained in poorly regulated supplements are occasionally lost in the discussion of inadvertent doping. Anti-doping regulations were developed over many years to promote fairness in sport and to protect the health of the athlete. Athletes are keen to improve their performance and nutrition may play an integral part in their overall plan. However, when athletes embark upon using performance-enhancing supplements, the risks often outweigh the benefits. Improved regulation of the dietary supplement industry would go a long way towards reducing the risks, but the onus remains on the athlete to make the right choices [13015].
Recommendation of the International Society for Sports Nutrition

Sports nutrition is a constantly evolving field with hundreds of research papers published annually. For this reason, keeping up to date with the literature is often difficult. One paper was a five year update of the sports nutrition review article published as the lead paper to launch the ISSN in 2004 and presents a well-referenced overview of the current state of the science related to how to optimize training and athletic performance through nutrition. More specifically, the paper provides an overview of:

- the definitional category of ergogenic aids and dietary supplements
- how dietary supplements are legally regulated
- how to evaluate the scientific merit of nutritional supplements
- general nutritional strategies to optimize performance and enhance recovery
- an overview of our current understanding of the ergogenic value of nutrition and dietary supplementation in regards to weight gain, weight loss, and performance enhancement

The association also categorized nutritional supplements into “apparently effective”, “possibly effective”, “too early to tell”, and “apparently ineffective” as well a description of the general approach into educating athletes about sports nutrition. Over the last five years there have been many changes to the original categorization of supplements. In addition, a number of new supplements have been introduced to the market. Their classifications are based on current available scientific evidence and have been well received within the broader scientific community [10218].

It was first stated that an ergogenic aid is any training technique, mechanical device, nutritional practice, pharmacological method, or psychological technique that can improve exercise performance capacity and/or enhance training adaptations. This includes aids that may help prepare an individual to exercise, improve the efficiency of exercise, and/or enhance recovery from exercise. Ergogenic aids may also allow an individual to tolerate heavy training to a greater degree by helping them recover faster or help them stay injury-free and/or healthy during intense training. Although this definition seems rather straightforward, there is considerable debate regarding the ergogenic value of various nutritional supplements. Some sports nutrition specialists only consider a supplement ergogenic if studies show that the supplement significantly enhances exercise performance (e.g. helps you run faster, lift more weight, and/or perform more work during a given exercise task). On the other hand, some feel that if a supplement helps prepare an athlete to perform or enhances recovery from exercise, it has the potential to improve training adaptations and therefore should be considered ergogenic. In the view of the ISSN, one should take a broader view about the ergogenic value of supplements. According to the Food and Drug Administration (FDA), dietary supplements were regulated in the same manner as food prior to 1994 [4]. Consequently, the FDA monitored the manufacturing processes, quality, and labeling of dietary supplements. Products sold as dietary supplements must be clearly labeled as a dietary supplement, and dietary supplements are not drugs [10218].

One of the most common questions raised by athletes, parents, and professionals regarding dietary supplements relates to how they are manufactured and consumer awareness of supplement quality. In a number of cases, reputable companies who develop dietary supplements have research teams who scour the medical and scientific literature looking for potentially effective nutrients. These research teams often attend scientific meetings and review the latest patents, research abstracts presented at scientific meetings, and research
publications. They may also consult with leading researchers to discuss ideas about dietary supplements that can be commercialized. Leading companies invest in basic research on nutrients before developing their supplement formulations. Others wait until research has been presented in patents, research abstracts, or publications before developing nutritional formulations featuring the nutrient. Once a new nutrient or formulation has been identified, the next step is to contact raw ingredient suppliers to see if the nutrient can be obtained in a highly pure source and/or if it's affordable. Sometimes, companies develop and patent new processing and purification processes because the nutrient has not yet been extracted in a pure form or is not available in large quantities. Reputable raw material manufacturers conduct extensive tests to examine purity of their raw ingredients. If the company is working on a new ingredient, they often conduct toxicity studies on the new nutrient once a purified source has been identified [10218].

**Energy need**

Dietary supplements may contain carbohydrate, protein, fat, minerals, vitamins, herbs, enzymes, metabolic intermediates (like amino acids), and/or various plant/food extracts. Supplements can generally be classified as convenience supplements (e.g. energy bars, meal replacement powders, ready to drink supplements) designed to provide a convenient means of meeting caloric needs and/or managing caloric intake, weight gain, weight loss, and/or performance enhancement. The first component to optimize training and performance through nutrition is to ensure the athlete is consuming enough calories to offset energy expenditure. People who participate in a general fitness program (e.g. exercising 30-40 minutes per day, 3 times per week) can typically meet nutritional needs following a normal diet (e.g. 1,800-2,400 kcals/day or about 25-35 kcals/kg/day for a 50-80 kg individual) because their caloric demands from exercise are not too great (e.g. 200-400 kcals/session). However, athletes involved in moderate levels of intense training (e.g. 2-3 hours per day of intense exercise performed 5-6 times per week) or high volume intense training (e.g. 3-6 hours per day of intense training in 1-2 workouts for 5-6 days per week) may expend 600-1,200 kcals or more per hour during exercise. For this reason, their caloric needs may approach 50-80 kcals/kg/day (2,500-8,000 kcals/day for a 50-100 kg athlete). For elite athletes, energy expenditure during heavy training or competition may be enormous. For example, energy expenditure for cyclists to compete in the Tour de France has been estimated as high as 12,000 kcals/day (150-200 kcals/kg/d for a 60-80 kg athlete). Additionally, caloric needs for large athletes (i.e. 100-150 kg) may range between 6,000-12,000 kcals/day depending on the volume and intensity of different training phases [10218].

Although some argue that athletes can meet caloric needs simply by consuming a well-balanced diet, it is often very difficult for larger athletes and/or athletes engaged in high volume/intense training to be able to eat enough food in order to meet caloric needs. Maintaining an energy deficient diet during training often leads to significant weight loss (including muscle mass), illness, onset of physical and psychological symptoms of overtraining, and reductions in performance. Nutritional analyses of athletes' diets have revealed that many are susceptible to maintaining negative energy intakes during training. Susceptible populations include runners, cyclists, swimmers, triathletes, gymnasts, skaters, dancers, wrestlers, boxers, and athletes attempting to lose weight too quickly. Additionally, female athletes have been reported to have a high incidence of eating disorders. Consequently, it is important for the sports nutrition specialist working with athletes to ensure that athletes are well-fed and consume enough calories to offset the increased energy demands of training, and maintain body weight. Although this sounds relatively simple, intense training often suppresses appetite and/or alters hunger patterns so that many athletes do not feel like eating. Some athletes do not like to exercise within several hours after eating because of sensations of fullness and/or a predisposition to cause
gastrointestinal distress. Further, travel and training schedules may limit food availability and/or the types of food athletes are accustomed to eating. This means that care should be taken to plan meal times in concert with training, as well as to make sure athletes have sufficient availability of nutrient dense foods throughout the day for snacking between meals (e.g. drinks, fruit, carbohydrate/protein bars, etc). For this reason, sports nutritionists’ often recommend that athletes consume 4-6 meals per day and snacks in between meals in order to meet energy needs. Use of nutrient dense energy bars and high calorie carbohydrate/protein supplements provides a convenient way for athletes to supplement their diet in order to maintain energy intake during training [10218].

Carbohydrate

The second component to optimizing training and performance through nutrition is to ensure that athletes consume the proper amounts of carbohydrate (CHO), protein (PRO) and fat in their diet. Individuals engaged in a general fitness program can typically meet macronutrient needs by consuming a normal diet (i.e. 45-55 % CHO, 3-5 grams/kg/day) 10-15 % PRO, 0.8-1.0 gram/kg/day, and 25-35 % fat, 0.5-1.5 grams/kg/day). However, athletes involved in moderate and high volume training need greater amounts of carbohydrate and protein in their diet to meet macronutrient needs. For example, in terms of carbohydrate needs, athletes involved in moderate amounts of intense training (e.g. 2-3 hours per day of intense exercise performed 5-6 times per week) typically need to consume a diet consisting of 55-65 % carbohydrate (i.e. 5-8 grams/kg/day or 250-1,200 grams/day for 50-150 kg athletes) in order to maintain liver and muscle glycogen stores. Research has also shown that athletes involved in high volume intense training (e.g. 3-6 hours per day of intense training in 1-2 workouts for 5-6 days per week) may need to consume 8-10 grams/day of carbohydrate (i.e. 400-1,500 grams/day for 50-150 kg athletes) in order to maintain muscle glycogen levels. This would be equivalent to consuming 0.5-2.0 kg of spaghetti. Preferably, the majority of dietary carbohydrate should come from complex carbohydrates with a low to moderate glycemic index (e.g. whole grains, vegetables, fruit, etc). However, since it is physically difficult to consume that much carbohydrate per day when an athlete is involved in intense training, many nutritionists and the sports nutrition specialist recommend that athletes consume concentrated carbohydrate juices/drinks and/or consume high carbohydrate supplements to meet carbohydrate needs [10218].

It should also be noted that exogenous carbohydrate oxidation rates have been shown to differ based on the type of carbohydrate consumed because they are taken up by different transporters. For example, oxidation rates of disaccharides and polysaccharides like sucrose, maltose, and maltodextrins are high while fructose, galactose, trehalose, and isomaltulose are lower. Ingesting combinations of glucose and sucrose or maltodextrin and fructose have been reported to promote greater exogenous carbohydrate oxidation than other forms of carbohydrate. These studies generally indicate a ratio of 1-1.2 for maltodextrin to 0.8-1.0 fructose. For this reason, it was recommended that care should be taken to consider the type of carbohydrate to ingest prior to, during, and following intense exercise in order to optimize carbohydrate availability [10218].

Protein

There has been considerable debate regarding protein needs of athletes. For people involved in a general fitness program, protein needs can generally be met by ingesting 0.8-1.0 grams/kg/day of protein. Older individuals may also benefit from a higher protein intake (e.g. 1.0-1.2 grams/kg/day of protein) in order to help prevent sarcopenia. It is recommended that athletes involved in moderate amounts of intense training consume 1-1.5 grams/kg/day of protein (50-225 grams/day for a 50-150 kg athlete) while athletes involved in high volume
intense training consume 1.5-2.0 grams/kg/day of protein (75-300 grams/day for a 50-150 kg athlete). This protein need would be equivalent to ingesting 3-11 servings of chicken or fish per day for a 50-150 kg athlete. Although smaller athletes typically can ingest this amount of protein in their normal diet, larger athletes often have difficulty consuming this much dietary protein. Additionally, a number of athletic populations have been reported to be susceptible to protein malnutrition (e.g. runners, cyclists, swimmers, triathletes, gymnasts, dancers, skaters, wrestlers, boxers, etc). Therefore, care should be taken to ensure that athletes consume a sufficient amount of quality protein in their diet in order to maintain nitrogen balance (e.g. 1.5-2 grams/kg/day) [10218].

However, it should be noted that not all protein is the same. Proteins differ based on the source that the protein was obtained, the amino acid profile of the protein, and the methods of processing or isolating the protein. These differences influence availability of amino acids and peptides that have been reported to possess biological activity (e.g. alpha-lactalumin, beta-lactoglobulin, glycomacropeptide, immunoglobulins, lactoperoxidases, lactoferrin, etc). Additionally, the rate of digestion and/or absorption and metabolic activity of the protein also are important considerations. For example, different types of proteins (e.g. casein and whey) are digested at different rates, which directly affect whole body catabolism and anabolism. Therefore, care should be taken not only to make sure the athlete consumes enough protein in their diet but also that the protein is high quality. The best dietary sources of low fat, high quality protein are light skinless chicken, fish, egg white and skim milk (casein and whey). The best sources of high quality protein found in nutritional supplements are whey, colostrum, casein, milk proteins and egg protein. Although some athletes may not need to supplement their diet with protein and some sports nutrition specialists may not think that protein supplements are necessary, it is common for a sports nutrition specialist to recommend that some athletes supplement their diet with protein in order to meet dietary protein needs and/or provide essential amino acids following exercise in order to optimize protein synthesis. The timing of protein intake in the time period encompassing the exercise session has several benefits including improved recovery and greater gains in fat free mass. Protein residues such as branched chain amino acids have been shown to be beneficial for the exercising individual, including increasing the rates of protein synthesis, decreasing the rate of protein degradation, and possibly aiding in recovery from exercise [10218].

**Fat**

The dietary recommendations of fat intake for athletes are similar to or slightly greater than those recommended for non-athletes in order to promote health. Maintenance of energy balance, replenishment of intramuscular triacylglycerol stores and adequate consumption of essential fatty acids are of greater importance among athletes and allow for somewhat increased intake. This depends on the athlete’s training state and goals. For example, higher-fat diets appear to maintain circulating testosterone concentrations better than low-fat diets. This has relevance to the documented testosterone suppression which can occur during volume-type overtraining. Generally, it is recommended that athletes consume a moderate amount of fat (approximately 30 % of their daily caloric intake), while increases up to 50 percent of kcal can be safely ingested by athletes during regular high-volume training. For athletes attempting to decrease body fat, however, it has been recommended that they consume 0.5 to 1 g/kg/d of fat. The reason for this is that some weight loss studies indicate that people who are most successful in losing weight and maintaining the weight loss are those who ingest less than 40 g/d of fat in their diet although this is not always the case. Certainly, the type of dietary fat (e.g. n-6 versus n-3; saturation state) is a factor in such research and could play an important role in any discrepancies [10218].

**Strategic eating and refueling**
In addition to the general nutritional guidelines described above, research has also demonstrated that timing and composition of meals consumed may play a role in optimizing performance, training adaptations, and preventing overtraining. In this regard, it takes about 4 hours for carbohydrate to be digested and begin being stored as muscle and liver glycogen. Consequently, pre-exercise meals should be consumed about 4 to 6 h before exercise. This means that if an athlete trains in the afternoon, breakfast is the most important meal to top off muscle and liver glycogen levels. Research has also indicated that ingesting a light carbohydrate and protein snack 30 to 60 min prior to exercise (e.g. 50 g of carbohydrate and 5 to 10 g of protein) serves to increase carbohydrate availability toward the end of an intense exercise bout. This also serves to increase availability of amino acids and decrease exercise-induced catabolism of protein [10218].

When exercise lasts more than one hour, athletes should ingest glucose/electrolyte solution drinks in order to maintain blood glucose levels, help prevent dehydration, and reduce the immunosuppressive effects of intense exercise. Following intense exercise, athletes should consume carbohydrate and protein (e.g. 1 g/kg of carbohydrate and 0.5 g/kg of protein) within 30 min after exercise as well as consume a high carbohydrate meal within two hours following exercise. This nutritional strategy has been found to accelerate glycogen resynthesis as well as promote a more anabolic hormonal profile that may hasten recovery. Finally, for 2 to 3 days prior to competition, athletes should taper training by 30 to 50 percent and consume 200 to 300 g/d of extra carbohydrate in their diet. This carbohydrate loading technique has been shown to supersaturate carbohydrate stores prior to competition and improve endurance exercise capacity. During intense exercise, regular consumption (10-15 fl oz.) of a carbohydrate/electrolyte solution delivering 6-8 percent CHO (6-8 g CHO/100 ml fluid) should be consumed every 15-20 min to sustain blood glucose levels. Glucose, fructose, sucrose and other high-glycemic CHO sources are easily digested, but fructose consumption should be minimized as it is absorbed at a slower rate and increases the likelihood of gastrointestinal problems. The addition of protein (0.15-0.25 g PRO/kg/day) to CHO at all time points, especially post-exercise, is well tolerated and may promote greater restoration of muscle glycogen when carbohydrate intakes are suboptimal. Ingestion of 6-20 grams of essential amino acids (EAA) and 30 - 40 grams of high-glycemic CHO within three hours after an exercise bout and immediately before exercise has been shown to significantly stimulate muscle PRO synthesis. Milk PRO sources (e.g. whey and casein) exhibit different kinetic digestion patterns and may subsequently differ in their support of training adaptations. Addition of creatine monohydrate to a CHO + PRO supplement in conjunction with regular resistance training facilitates greater improvements in strength and body composition as compared with when no creatine is consumed. Irrespective of timing, regular ingestion of snacks or meals providing both CHO and PRO (3:1 CHO: PRO ratio) helps to promote recovery and replenishment of muscle glycogen when lesser amounts of carbohydrate are consumed [10218].

**Vitamins**

Vitamins are essential organic compounds that serve to regulate metabolic processes, energy synthesis, neurological processes, and prevent destruction of cells. There are two primary classifications of vitamins: fat and water soluble. The fat soluble vitamins include vitamins A, D, E, and K. The body stores fat soluble vitamins and therefore excessive intake may result in toxicity. Water soluble vitamins are B vitamins and vitamin C. Since these vitamins are water soluble, excessive intake of these vitamins are eliminated in urine, with few exceptions (e.g. vitamin B6, which can cause peripheral nerve damage when consumed in excessive amounts). Although research has demonstrated that specific vitamins may possess some health benefit (e.g. Vitamin E, niacin, folic acid, vitamin C, etc), few have been
reported to directly provide ergogenic value for athletes. However, some vitamins may help athletes tolerate training to a greater degree by reducing oxidative damage (Vitamin E, C) and/or help to maintain a healthy immune system during heavy training (Vitamin C). Theoretically, this may help athletes tolerate heavy training leading to improved performance. The remaining vitamins reviewed appear to have little ergogenic value for athletes who consume a normal, nutrient dense diet. Since dietary analyses of athletes have found deficiencies in caloric and vitamin intake, many sports nutritionists' recommend that athletes consume a low-dose daily multivitamin and/or a vitamin enriched post-workout carbohydrate/protein supplement during periods of heavy training. Suggestions that there is no benefit of vitamin supplementation for athletes and/or it is unethical for an sports nutrition specialist to recommend that their clients take a one-a-day multi-vitamin and/or suggest taking other vitamins that may raise HDL cholesterol levels and decrease risk of heart disease (niacin), serve as antioxidants (Vitamin E), preserve musculoskeletal function and skeletal mass (vitamin D), or may help maintain a health immune system (Vitamin C) is not consistent with current available literature [10218].

Minerals

Minerals are essential inorganic elements necessary for a host of metabolic processes. Minerals serve as structure for tissue, important components of enzymes and hormones, and regulators of metabolic and neural control. Some minerals have been found to be deficient in athletes or become deficient in response to training and/or prolonged exercise. When mineral status is inadequate, exercise capacity may be reduced. Dietary supplementation of minerals in deficient athletes has generally been found to improve exercise capacity. Additionally, supplementation of specific minerals in non-deficient athletes has also been reported to affect exercise capacity. Of the minerals reviewed, several appear to possess health and/or ergogenic value for athletes under certain conditions. For example, calcium supplementation in athletes susceptible to premature osteoporosis may help maintain bone mass. There is also recent evidence that dietary calcium may help manage body composition. Iron supplementation in athletes prone to iron deficiencies and/or anaemia has been reported to improve exercise capacity. Sodium phosphate loading has been reported to increase maximal oxygen uptake, anaerobic threshold, and improve endurance exercise capacity by 8 to 10 percent. Increasing dietary availability of salt (sodium chloride) during the initial days of exercise training in the heat has been reported to help maintain fluid balance and prevent dehydration. ACSM recommendations for sodium levels (340 mg) represent the amount of sodium in less than 1/8 teaspoon of salt and meet recommended guidelines for sodium ingestion during exercise (300-600 mg per hour or 1.7-2.9 grams of salt during a prolonged exercise bout). Finally, zinc supplementation during training has been reported to decrease exercise-induced changes in immune function. Consequently, somewhat in contrast to vitamins, there appear to be several minerals that may enhance exercise capacity and/or training adaptations for athletes under certain conditions. However, although ergogenic value has been purported for remaining minerals, there is little evidence that boron, chromium, magnesium, or vanadium affect exercise capacity or training adaptations in healthy individuals eating a normal diet [10218].

Water

The most important nutritional ergogenic aid for athletes is water. Exercise performance can be significantly impaired when 2 percent or more of body weight is lost through sweat. For example, when a 70-kg athlete loses more than 1.4 kg of body weight during exercise (2 %), performance capacity is often significantly decreased. Further, weight loss of more than 4 percent of body weight during exercise may lead to heat illness, heat exhaustion, heat stroke, and possibly death. For this reason, it is critical that athletes consume a sufficient
amount of water and/or carbohydrates and electrolyte sports drinks during exercise in order to maintain hydration status. The normal sweat rate of athletes ranges from 0.5 to 2.0 L/h depending on temperature, humidity, exercise intensity, and their sweat response to exercise. This means that in order to maintain fluid balance and prevent dehydration, athletes need to ingest 0.5 to 2 L/h of fluid in order to offset weight loss. This requires frequent ingestion of 6-8 oz of cold water or sports drink every 5 to 15-min during exercise. Athletes should not depend on thirst to prompt them to drink because people do not typically get thirsty until they have lost a significant amount of fluid through sweat. Additionally, athletes should weigh themselves prior to and following exercise training to ensure that they maintain proper hydration. The athlete should consume 3 cups of water for every pound lost during exercise in order adequately rehydrate themselves. Athletes should train themselves to tolerate drinking greater amounts of water during training and make sure that they consume more fluid in hotter/humid environments. Preventing dehydration during exercise is one of the most effective ways to maintain exercise capacity. Finally, inappropriate and excessive weight loss techniques (e.g. cutting weight in saunas, wearing rubber suits, severe dieting, vomiting, using diuretics, etc) are extremely dangerous and should be prohibited [10218].

Convenience supplements

Convenience supplements are meal replacement powders (MRP’s), ready to drink supplements (RTD’s), energy bars, and energy gels. They currently represent the largest segment of the dietary supplement industry representing 50-75 percent of most company’s sales. They are typically fortified with vitamins and minerals and differ on the amount of carbohydrate, protein, and/or fat they contain. They may also vary based whether they are fortified with various nutrients purported to promote weight gain, enhance weight loss, and/or improve performance. Most people view these supplements as a nutrient dense snack and/or use them to help control caloric intake when trying to gain and/or lose weight. In our view, MRP’s, RTD’s, and energy bars/gels can provide a convenient way for people to meet specific dietary needs and/or serve as good alternatives to fast food other foods of lower nutritional value. Use of these types of products can be particularly helpful in providing carbohydrate, protein, and other nutrients prior to and/or following exercise in an attempt to optimize nutrient intake when an athlete doesn't have time to sit down for a good meal or wants to minimize food volume. However, they should be used to improve dietary availability of macronutrients, not as a replacement for a good diet. Care should also be taken to make sure they do not contain any banned or prohibited nutrients [10218].

Muscle building supplements

One of the most common means athletes have employed to increase muscle mass is to add extra calories to the diet. Most athletes "bulk up" in this manner by consuming extra food and/or weight gain powders. In order to increase skeletal muscle mass, there must be adequate energy intake (anabolic reactions are endergonic and therefore require adequate energy intake). Studies have consistently shown that simply adding an extra 500-1,000 calories per day to your diet in conjunction with resistance training will promote weight gain. However, only about 30-50 percent of the weight gained on high calorie diets is muscle while the remaining amount of weight gained is fat. Consequently, increasing muscle mass by ingesting a high calorie diet can help build muscle but the accompanying increase in body fat may not be desirable for everyone. Therefore, it is not recommend this type of weight gain approach [10218].

Creatine
The most effective nutritional supplement available to athletes to increase high intensity exercise capacity and muscle mass during training is creatine monohydrate. Numerous studies have indicated that creatine supplementation increases body mass and/or muscle mass during training. Gains are typically 2-5 pounds greater than controls during 4-12 weeks of training. The gains in muscle mass appear to be a result of an improved ability to perform high intensity exercise enabling an athlete to train harder and thereby promote greater training adaptations and muscle hypertrophy. The only clinically significant side effect occasionally reported from creatine monohydrate supplementation has been the potential for weight gain. Although concerns have been raised about the safety and possible side effects of creatine supplementation, recent long-term safety studies have reported no apparent side effects and/or that creatine monohydrate may lessen the incidence of injury during training [10218].

It was concluded that creatine monohydrate is the most effective ergogenic nutritional supplement currently available to athletes in terms of increasing high-intensity exercise capacity and lean body mass during training. There is no compelling scientific evidence that the short- or long-term use of creatine monohydrate has any detrimental effects on otherwise healthy individuals. If proper precautions and supervision are provided, supplementation in young athletes is acceptable and may provide a nutritional alternative to potentially dangerous anabolic drugs. The addition of carbohydrate or carbohydrate and protein to a creatine supplement appears to increase muscular retention of creatine, although the effect on performance measures may not be greater than using creatine monohydrate alone. The quickest method of increasing muscle creatine stores appears to be to consume ~0.3 grams/kg/day of creatine monohydrate for at least 3 days followed by 3-5 g/d thereafter to maintain elevated stores. Ingesting smaller amounts of creatine monohydrate (e.g. 2-3 g/d) will increase muscle creatine stores over a 3-4 week period, however, the performance effects of this method of supplementation are less supported [10218].

**Essential amino acids (EAA)**

Recent studies have indicated that ingesting 3 to 6 g of EAA prior to and/or following exercise stimulates protein synthesis. Theoretically, this may enhance gains in muscle mass during training. Because EAA's include branch chain amino acids (BCAA) it is probable that positive effects on protein synthesis from EAA ingestion are likely due to the BCAA content. It was thus concluded that BCAAs have been shown to acutely stimulate protein synthesis, aid in glycogen resynthesis, delaying the onset of fatigue, and help maintain mental function in aerobic-based exercise. It was concluded that consuming BCAAs (in addition to carbohydrates) before, during, and following an exercise bout would be recommended safe and effective [10218].

**Beta-hydroxy beta-methylbutyrate (HMB)**

HMB is a metabolite of the amino acid leucine. Leucine and metabolites of leucine have been reported to inhibit protein degradation. Supplementing the diet with 1.5 to 3 g/d of calcium HMB during training has been typically reported to increase muscle mass and strength particularly among untrained subjects initiating training and the elderly. Gains in muscle mass are typically 0.5 to 1 kg greater than controls during 3-6 weeks of training. There is also evidence that HMB may lessen the catabolic effects of prolonged exercise and that there may be additive effects of co-ingesting HMB with creatine. However, the effects of HMB supplementation in athletes are less clear. Most studies conducted on trained subjects have reported non-significant gains in muscle mass possibly due to a greater variability in response of HMB supplementation among athletes [10218].
Amino acids such as leucine and its metabolite alpha-ketoisocaproate (KIC), are returning to be the focus of studies, mainly because of their anti-catabolic properties, through inhibition of muscle proteolysis and enhancement of protein synthesis. It is clear that these effects may counteract catabolic conditions, as well as enhance skeletal muscle mass and strength in athletes. Moreover, beta-hydroxy-beta-methylbutyrate (HMB) has been shown to produce an important effect in reducing muscle damage induced by mechanical stimuli of skeletal muscle. One review aimed to describe the general scientific evidence of KIC and HMB supplementation clinical relevance, as well as their effects (e.g. increases in skeletal muscle mass and/or strength), associated with resistance training or other sports. Moreover, the possible mechanisms of cell signaling regulation leading to increases and/or sparing (during catabolic conditions) of skeletal muscle mass were discussed in detail [10415].

Beta-hydroxy-beta-methylbutyrate (HMBeta) is a metabolite of leucine widely used for improving sports performance. Although HMB is recognized to promote anabolic or anti-catabolic effects on protein metabolism, the impact of its long-term use on skeletal muscle and/or genes that control the skeletal protein balance is not fully known. This study aimed to investigate whether chronic HMBeta treatment affects the activity of GH/IGF-I axis and skeletal muscle IGF-I and myostatin mRNA expression. Rats were treated with HMBeta (320mg/kg BW) or vehicle, by gavage, for 4 weeks, and killed by decapitation. Blood was collected for evaluation of serum insulin, glucose and IGF-I concentrations. Samples of pituitary, liver, extensor digitorum longus (EDL) and soleus muscles were collected for total RNA or protein extraction to evaluate the expression of pituitary growth hormone (GH) gene (mRNA and protein), hepatic insulin-like growth factor I (IGF-I) mRNA, skeletal muscle IGF-I and myostatin mRNA by Northern blotting/real time-PCR, or Western blotting. Chronic HMBeta treatment increased the content of pituitary GH mRNA and GH, hepatic IGF-I mRNA and serum IGF-I concentration. No changes were detected on skeletal muscle IGF-I and myostatin mRNA expression. However, the HMBeta-treated rats although normoglycemic, exhibited hyperinsulinemia. The data presented herein extend the body of evidence on the potential role of HMBeta-treatment in stimulating GH/IGF-I axis activity. In spite of this effect, HMBeta supplementation also induces an apparent insulin resistance state which might limit the beneficial aspects of the former results, at least in rats under normal nutritional status and health conditions [11236].

The use of ergogenic nutritional supplements is becoming inseparable from competitive sports. beta-Hydroxy-beta-methylbutyric acid (HMB) has recently been suggested to promote fat-free mass (FFM) and strength gains during resistance training in adults. In this prospective randomized, double-blind, placebo-controlled study, it was studied the effect of HMB (3 g/day) supplementation on body composition, muscle strength, anaerobic and aerobic capacity, anabolic/catabolic hormones and inflammatory mediators in elite, national team level adolescent volleyball players (14-18 years, 14 males, 14 females, Tanner stage 4-5) during the first 7 weeks of the training season. HMB led to a significant greater increase in FFM by skinfold thickness. HMB led to a significant greater increase in both dominant and non-dominant knee flexion isokinetic force/FFM, measured at fast (180°/sec) and slow (60°/sec) angle speeds, but had no significant effect on knee extension and elbow flexion and extension. HMB led to a significant greater increase in peak and mean anaerobic power determined by the Wingate anaerobic test (peak power and mean power), with no effect on fatigue index. HMB had no significant effect on aerobic fitness or on anabolic (growth hormone, IGF-I, testosterone), catabolic (cortisol) and inflammatory mediators (IL-6 and IL-1 receptor antagonist). HMB supplementation was associated with greater increases in muscle mass, muscle strength and anaerobic properties with no effect on aerobic capacity suggesting some advantage for its use in elite adolescent volleyball players during the initial phases of the training season. These effects were not accompanied by hormonal and
beta-Hydroxy-beta-methylbutyrate (HMBeta) supplementation is used to treat cancer, sepsis and exercise-induced muscle damage. However, its effects on animal and human health and the consequences of this treatment in other tissues (e.g. fat and liver) have not been examined. The purpose of one study was to evaluate the effects of HMBeta supplementation on skeletal muscle hypertrophy and the expression of proteins involved in insulin signalling. Rats were treated with HMBeta (320 mg/kg body weight) or saline for one month. The skeletal muscle hypertrophy and insulin signalling were evaluated by western blotting, and hormonal concentrations were evaluated using ELISAs. HMBeta supplementation induced muscle hypertrophy in the extensor digitorum longus (EDL) and soleus muscles and increased serum insulin levels, the expression of the mammalian target of rapamycin (mTOR) and phosphorylation of p70S6K in the EDL muscle. Expression of the insulin receptor was increased only in liver. Thus, the results suggest that HMBeta supplementation can be used to increase muscle mass without adverse health effects [11238].

beta-Hydroxy-beta-methylbutyrate (HMBeta) is a metabolite of leucine widely used for improving sports performance. Although HMBeta is recognized to promote anabolic or anti-catabolic effects on protein metabolism, the impact of its long-term use on skeletal muscle and/or genes that control the skeletal protein balance is not fully known. One study aimed to investigate whether chronic HMBeta treatment affects the activity of GH/IGF-I axis and skeletal muscle IGF-I and myostatin mRNA expression. Rats were treated with HMBeta (320 mg/kg BW) or vehicle, by gavage, for 4 weeks, and killed by decapitation. Blood was collected for evaluation of serum insulin, glucose and IGF-I concentrations. Samples of pituitary, liver, extensor digitorum longus (EDL) and soleus muscles were collected for total RNA or protein extraction to evaluate the expression of pituitary growth hormone (GH) gene (mRNA and protein), hepatic insulin-like growth factor I (IGF-I) mRNA, skeletal muscle IGF-I and myostatin mRNA by Northern blotting/real time-PCR, or Western blotting. Chronic HMBeta treatment increased the content of pituitary GH mRNA and GH, hepatic IGF-I mRNA and serum IGF-I concentration. No changes were detected on skeletal muscle IGF-I and myostatin mRNA expression. However, the HMBeta-treated rats although normoglycemic, exhibited hyperinsulinemia. The data presented extend the body of evidence on the potential role of HMB-treatment in stimulating GH/IGF-I axis activity. In spite of this effect, HMBeta supplementation also induces an apparent insulin resistance state which might limit the beneficial aspects of the former results, at least in rats under normal nutritional status and health conditions [11239].

Amino acids such as leucine and its metabolite alpha-ketoisocaprate (KIC), are returning to be the focus of studies, mainly because of their anti-catabolic properties, through inhibition of muscle proteolysis and enhancement of protein synthesis. It is clear that these effects may counteract catabolic conditions, as well as enhance skeletal muscle mass and strength in athletes. Moreover, beta-hydroxy-beta-methylbutyrate (HMB) has been shown to produce an important effect in reducing muscle damage induced by mechanical stimuli of skeletal muscle. One review aimed to describe the general scientific evidence of KIC and HMB supplementation clinical relevance, as well as their effects (e.g. increases in skeletal muscle mass and/or strength), associated with resistance training or other sports. Moreover, the possible mechanisms of cell signaling regulation leading to increases and/or sparing (during catabolic conditions) of skeletal muscle mass are discussed in detail based on the recent literature [11240].

HMB is a metabolite of the essential branched chain amino acid leucine, via alpha-ketoisocaprate, the transamination product of leucine. Approximately 2-10 percent of leucine oxidation proceeds to HMB. Much initial research on HMB focused on animals,
assessing effects on immune function, morbidity and mortality, colostral milk fat content, growth rates, safety and toxicity. Despite unconvincing results in animal research, HMB supplementation was used in humans in the mid-1990s on the presumption that it might enhance gains in muscle size and strength while reducing muscle damage and soreness associated with resistance training, and possibly also enhance aerobic capacity. Supplementation with either 1.5 or 3.0 g/day of HMB had a favourable impact on indirect indices of muscle protein breakdown and muscle damage, with concomitant trends for enhanced strength and skeletal muscle hypertrophy. Consequently, the popularity of HMB supplementation increased dramatically, becoming one of the top supplements used by athletes in the late 1990s. More recently, interest in HMB supplementation among athletes has probably declined. Despite this, HMB research reports within the sports environment continue to emerge. There are several recent HMB reviews, with results of resistance training research from 2001 to 2007 consolidated into two meta-analyses. It was concluded that HMB supplementation augmented lean mass and strength gains associated with resistance training, although the magnitude of effect was trivial (0.28% increase in lean mass gain per week). It was also identified similar small benefits from HMB supplementation in untrained males but effects were trivial for strength and non-existent for body composition in trained lifters. This disparity in responsiveness to HMB supplementation relative to resistance training status might be expected, given the suppression in skeletal muscle protein breakdown as a consequence of resistance training adaptations. Furthermore, if HMB does enhance net protein balance following resistance training as a consequence of reducing protein degradation, any effect on adaptations is likely to be blunted compared with interventions that enhance protein synthesis as the protein synthetic response is many times more sensitive to nutrition interventions than degradation. An exception may be in clinical conditions such as cancer in which skeletal muscle atrophy results from an elevation in skeletal muscle protein breakdown. Short-term HMB supplementation appears to be safe, with daily doses equivalent to approximately 6 g/day (76 mg/kg) having no impact on indices of hepatic, renal or immune function. Based on current available reports, the potential for HMB supplementation to enhance strength training adaptations appears to be small in previously untrained individuals and negligible in resistance-trained athletes. Given that the protein synthetic response is much more sensitive to nutrition interventions than protein breakdown, the resistance-trained athlete is advised to focus on proven strategies such as post-exercise ingestion of high biological value proteins rich in leucine to maximise adaptation to the resistance training stimulus [11241].

There is a huge market for ergogenic supplements for athletes. However, only a few products have been proven to have ergogenic effects and to be effective at improving muscle strength and body composition. One such supplement is beta-hydroxy beta-methylbutyrate (HMB). Derived from the amino acid leucine and its keto acid alpha-ketoisocaproate (KIC), HMB has been well documented as an oral ergogenic supplement commonly used by athletes. Several studies have shown that combining exercise training with HMB supplementation leads to increased muscle mass and strength, and there is some anecdotal evidence of aerobic improvement. However, HMB supplementation has been found to be effective mainly for untrained individuals. While previous reviews have emphasized three main pathways for HMB's mode of action, i.e. enhancement of sarcolemmal integrity via cytosolic cholesterol, inhibition of protein degradation via proteasomes, and increased protein synthesis via the mTOR pathway, more recent studies have suggested additional possible mechanisms for its physiological effects. These include decreased cell apoptosis and enhanced cell survival, increased proliferation, differentiation and fusion via the MAPK/ERK and PI3K/Akt pathways, and enhanced IGF-I transcription [10416].
Branched chain amino acids (BCAA)

BCAA supplementation has been reported to decrease exercise-induced protein degradation and/or muscle enzyme release (an indicator of muscle damage) possibly by promoting an anti-catabolic hormonal profile. Theoretically, BCAA supplementation during intense training may help minimize protein degradation and thereby lead to greater gains in fat-free mass. There is some evidence to support this hypothesis. Although more research is necessary, findings suggest that BCAA supplementation may have some impact on body composition [10218].

alpha-Ketoglutarate (alpha-KG)

alpha-KG is an intermediate in the Krebs cycle that is involved in aerobic energy metabolism. There is some clinical evidence that alpha-KG may serve as an anticitabolotic nutrient after surgery. However, it is unclear whether alpha-KG supplementation during training may affect training adaptations [10218].

alpha-Ketoisocaproate (KIC)

KIC is a branched-chain keto acid that is a metabolite of leucine metabolism. In a similar manner as HMB, leucine and metabolites of leucine are believed to possess anticitabolotic properties. There is some clinical evidence that KIC may spare protein degradation in clinical populations [10218].

Ecdysterones

Ecdysterones (also known as ectysterone, 20 Beta-Hydroxyecdysterone, turkesterone, ponasterone, ec dysone, or ec dys tene) are naturally derived phytoecdysteroids (i.e. insect hormones). They are typically extracted from the herbs Leuza raptonticum sp., Rhaponticum carthamoides, or Cyanotis vaga. They can also be found in high concentrations in the herb Suma (also known as Brazilian Ginseng or Pfaffia). Research from Russia and Czechoslovakia conducted over the last 30 years indicates that ecdysterones may possess some potentially beneficial physiological effects in insects and animals. It is too early to tell whether phytoecdysteroids serve as a safe and effective nutritional supplement for athletes [10218].

Growth hormone releasing peptides (GHRP) and secretagogues

Research has indicated that growth hormone releasing peptides (GHRP) and other non-peptide compounds (secretagogues) appear to help regulate growth hormone (GH) release. These observations have served as the basis for development of nutritionally-based GH stimulators (e.g. amino acids, pituitary peptides, "pituitary substances", Macuna pruriens, broad bean, alpha-GPC, etc). Although there is clinical evidence that pharmaceutical grade GHRP's and some non-peptide secretagogues can increase GH and IGF-1 levels at rest and in response to exercise, it has not been demonstrated that such increases lead to an increase in skeletal muscle mass [10218].

Ornithine-alpha-ketoglutarate (OKG)

OKG (via enteral feeding) has been shown to significantly shorten wound healing time and improve nitrogen balance in severe burn patients. Because of its ability to improve nitrogen
balance, OKG may provide some value for athletes engaged in intense training. However, additional research is needed before conclusions can be drawn [10218].

**Zinc/Magnesium aspartate (ZMA)**

The main ingredients in ZMA formulations are zinc monomethionine aspartate, magnesium aspartate, and vitamin B-6. The rationale of ZMA supplementation is based on studies suggesting that zinc and magnesium deficiency may reduce the production of testosterone and insulin-like growth factor (IGF-1). ZMA supplementation has been theorized to increase testosterone and IGF-1 leading to greater recovery, anabolism, and strength during training. More research is needed to further evaluate the role of ZMA on body composition and strength during training before definitive conclusions can be drawn [10218].

**Glutamine**

Glutamine is the most plentiful non-essential amino acid in the body and plays a number of important physiological roles. Glutamine has been reported to increase cell volume and stimulate protein and glycogen synthesis. Despite its important role in physiological roles, there is no compelling evidence to support glutamine supplementation in terms of increasing lean body mass [10218].

**Isoflavones**

Isoflavones are naturally occurring non-steroidal phytoestrogens that have a similar chemical structure as ipriflavone (a synthetic flavonoid drug used in the treatment of osteoporosis). For this reason, soy protein (which is an excellent source of isoflavones) and isoflavone extracts have been investigated in the possible treatment of osteoporosis. Results of these studies have shown promise in preventing declines in bone mass in post-menopausal women as well as reducing risks to side effects associated with estrogen replacement therapy. However, there are currently no peer-reviewed data indicating that isoflavone supplementation affects exercise, body composition, or training adaptations in physically active individuals [10218].

**Sulfo-polysaccharides (myostatin inhibitors)**

Myostatin or growth differentiation factor 8 (GDF-8) is a transforming growth factor that has been shown to serve as a genetic determinant of the upper limit of muscle size and growth. Recent research has indicated that eliminating and/or inhibiting myostatin gene expression in mice and cattle promotes marked increases in muscle mass during early growth and development. The result is that these animals experience what has been termed as a "double-muscle" phenomenon apparently by allowing muscle to grow beyond its normal genetic limit. In agriculture research, eliminating and/or inhibiting myostatin may serve as an effective way to optimize animal growth leading to larger, leaner, and a more profitable livestock yield. In humans, inhibiting myostatin gene expression has been theorized as a way to prevent or slow down muscle wasting in various diseases, speed up recovery of injured muscles, and/or promote increases in muscle mass and strength in athletes. While these theoretical possibilities may have great promise, research on the role of myostatin inhibition on muscle growth and repair is in the very early stages, particularly in humans. Though the research is limited, there is currently no published data supporting the use of sulfo-polysaccharides as a muscle building supplement [10218].

**Boron**
Boron is a trace mineral proposed to increase testosterone levels and promote anabolism. Several studies have evaluated the effects of boron supplementation during training on strength and body composition alterations. These studies (conducted on male bodybuilders) indicate that boron supplementation (2.5 mg/d) appears to have no impact on muscle mass or strength [10218].

**Chromium**

Chromium is a trace mineral that is involved in carbohydrate and fat metabolism. Clinical studies have suggested that chromium may enhance the effects of insulin particularly in diabetic populations. Since insulin is an anti-catabolic hormone and has been reported to affect protein synthesis, chromium supplementation has been theorized to serve as an anabolic nutrient. Theoretically, this may increase anabolic responses to exercise. Although some initial studies reported that chromium supplementation increased gains in muscle mass and strength during training particularly in women, most well-controlled studies that have been conducted since then have reported no benefit in healthy individuals taking chromium (200-800 mcg/d) for 4 to 16-weeks during training [10218].

**Conjugated linoleic acids (CLA)**

Animal studies indicate that adding CLA to dietary feed decreases body fat, increases muscle and bone mass, has anti-cancer properties, enhances immunity, and inhibits progression of heart disease. Consequently, CLA supplementation in humans has been suggested to help manage body composition, delay loss of bone, and provide health benefit. Although animal studies are impressive and some studies suggests benefit over time at some but not all dosages, there is little current evidence that CLA supplementation during training can affect lean tissue accretion [10218].

**Gamma oryzanol (Ferulic acid)**

Gamma oryzanol is a plant sterol theorized to increase anabolic hormonal responses during training. Although data are limited, one study reported no effect of 0.5 g/d of gamma oryzanol supplementation on strength, muscle mass, or anabolic hormonal profiles during 9-weeks of training [10218].

**Prohormones**

Testosterone and growth hormone are two primary hormones in the body that serve to promote gains in muscle mass and strength while decreasing muscle breakdown and fat mass. Prohormones (androstenedione, 4-androstenediol, 19-nor-4-androstenedione, 19-nor-4-androstenediol, 7-keto DHEA, and DHEA, etc) are naturally derived precursors to testosterone or other anabolic steroids. Prohormones have become popular among body builders because they believe they are natural boosters of anabolic hormones. Consequently, a number of over-the-counter supplements contain prohormones. While there is some data indicating that prohormones increase testosterone levels, there is virtually no evidence that these compounds affect training adaptations in younger men with normal hormone levels. In fact, most studies indicate that they do not affect testosterone and that some may actually increase estrogen levels and reduce HDL-cholesterol. Consequently, although there may be some potential applications for older individuals to replace diminishing androgen levels, it appears that prohormones have no training value. Since prohormones are "steroid-like compounds", most athletic organizations have banned their use. Use of nutritional supplements containing prohormones will result in a positive drug test for anabolic
steroids. Use of supplements knowingly or unknowingly containing prohormones have been believed to have contributed to a number of recent positive drug tests among athletes. Consequently, care should be taken to make sure that any supplement an athlete considers taking does not contain prohormone precursors particularly if their sport bans and tests for use of such compounds. Rather than provide the body with a precursor to testosterone, a more recent technique to enhance endogenous testosterone has been to inhibit aromatase activity. Two studies have investigated the effects of aromatase inhibitors (androst-4-ene-3,6,17-trione) and (hydroxyandrost-4-ene-6,17-dioxo-3-THP ether and 3,17-diketo-androst-1,4,6-triene]). In both of these investigations, it was reported that free testosterone and dihydrotestosterone levels were significantly increased. Muscle mass/fat free mass was not measured in one investigation and no changes were observed in fat free mass in the other investigation [10218].

Tribulus terrestris

Tribulus terrestris (also known as puncture weed/vine or caltrops) is a plant extract that has been suggested to stimulate leutinizing hormone (LH) which stimulates the natural production of testosterone. Consequently, Tribulus has been marketed as a supplement that can increase testosterone and promote greater gains in strength and muscle mass during training. Several recent studies have indicated that Tribulus supplementation appears to have no effects on body composition or strength during training [10218].

Vanadyl sulfate (Vanadium)

In a similar manner as chromium, vanadyl sulfate is a trace mineral that has been found to affect insulin-sensitivity and may affect protein and glucose metabolism. For this reason, vanadyl sulfate has been purported to increase muscle mass and strength during training. Although there may be some clinical benefits for diabetics (with a therapeutic dose of at least 50 mg vanadyl sulfate twice daily, vanadyl sulfate supplementation does not appear to have any effect on strength or muscle mass during training in non-diabetic, weight training individuals [10218].

Green tea extract

Green tea is now one of the most common herbal supplements that is being added to thermogenic products because it has been suggested to affect weight loss and is now the fourth most commonly used dietary supplement in the US. Green tea contains high amounts of caffeine and catechin polyphenols. The primary catechin that is associated to the potential effects on weight loss through diet induced thermogenesis is the catechin epigallocatechin gallate, also known as EGCG. Research suggests that catechin polyphenols possess antioxidant properties and the intake of tea catechins is associated with a reduced risk of cardiovascular disease. However, it must be noted that both human and animal studies have not supported positive findings and have reported that supplementation of these extracts does not affect weight loss [10218].

Phosphatidyl choline (Lecithin)

Choline is considered an essential nutrient that is needed for cell membrane integrity and to facilitate the movement of fats in and out of cells. It is also a component of the neurotransmitter acetylcholine and is needed for normal brain functioning, particularly in infants. For this reason, phosphatidyl choline has been purported as a potentially effective supplement to promote fat loss as well as improve neuromuscular function. However, despite
these alleged benefits of lecithin supplementation, there are no clinical trials in humans to support a potential role of lecithin supplementation affecting weight loss [10218]

*Dehydroepiandrosterone (DHEA) and 7-Keto DHEA*

Dehydroepiandrosterone (DHEA) and its sulfated conjugate DHEAS represent the most abundant adrenal steroids in circulation. Although, DHEA is considered a weak androgen, it can be converted to the more potent androgens testosterone and dihydrotestosterone in tissues. In addition, DHEAS can be converted into androstenedione and testosterone. DHEA levels have been reported to decline with age in humans. The decline in DHEA levels with aging has been associated with increased fat accumulation and risk to heart disease. Since DHEA is a naturally occurring compound, it has been suggested that dietary supplementation of DHEA may help maintain DHEA availability, maintain and/or increase testosterone levels, reduce body fat accumulation, and/or reduce risk to heart disease as one ages. Although animal studies have generally supported this theory, the effects of DHEA supplementation on body composition in human trials have been mixed [10218].
NUTRITIONAL SUPPLEMENTS: WATER, CARBOHYDRATE, PROTEIN, AND FAT

Studies failing to show a negative effect of rapid weight loss (RWL) on performance have been conducted in athletes who have been cycling weight for years. It has been suggested that chronic weight cycling could lead combat athletes to become resistant to the stresses associated with weight loss. To investigate the effects of RWL up to 5 percent of body mass on high-intensity intermittent performance in weight cyclers (WC) and non-weight cyclers (non-WC). Eighteen male combat athletes (WC: n=10; non-WC: n=8) reduced up to 5 percent of their body mass in 5 days. Body composition, high-intensity performance and plasma lactate were assessed preweight loss and postweight loss. Athletes had 4 h to refeed and rehydrate following the weigh-in. Food intake was recorded during the weight loss and the recovery periods. Athletes significantly decreased body mass, lean body mass (most likely due to fluid loss) and fat mass following weight loss. No significant changes in performance were found from preweight loss to postweight loss in both groups. Plasma lactate was significantly elevated after exercise in both groups, but no differences were found between groups and in response to RWL. For all these variables no differences were observed between groups. Athletes from both groups ingested high amounts of energy and carbohydrates during the recovery period after the weigh-in. Thus, chronic weight cycling does not protect athletes from the negative impact of RWL on performance. The time to recover after weigh-in and the patterns of food and fluid ingestion during this period is likely to play the major role in restoring performance to baseline levels [13591].

The purpose of one study was to compare fluid balance between a resistance and aerobic training session, in elite rugby players. It is hypothesised that resistance exercise will result in a higher prevalence of over-drinking whereas during the aerobic session under-drinking will be more prevalent. As with previous fluid balance studies, this was an observational study. Twenty-six players completed the resistance training session and twenty players completed the aerobic training session. All players were members of an elite rugby union squad competing in the southern hemisphere’s premier competition. For both sessions players provided a pre-exercise urine sample to determine hydration status, pre- and post-exercise measures of body mass and blood sodium concentration were taken and the weight of drinks bottles were recorded to calculate sweat rates and fluid intake rates. Sweat patches were positioned on the shoulder of the players and these remained in place throughout each training session, and were later analysed for sodium concentration. The percentage of sweat loss replaced was higher in the resistance (196 ± 130 %) than the aerobic training session (56 ± 17 %). Despite this, no cases of hyponatremia were detected. The results also indicated that over 80% of players started training in a hypohydrated state. Fluid intake appears to differ depending on the nature of the exercise session. In this group of athletes, players did not match their fluid intakes with their sweat loss, resulting in over-drinking during resistance training and under-drinking in aerobic training. Therefore, hydration strategies and education need to be tailored to the exercise session. Furthermore, given the large number of players arriving at training hypohydrated, improved hydration strategies away from the training venue are required [13592].

High temperature environment causes detrimental effects on health. In the present study, the effects of intake of several kinds of beverage on blood components during exercise under the high temperature environment were evaluated. The 10 subjects were student of the H University. Exercise intensity was 50-60 percent $O_{2max}$ and treadmill exercise was continued for 1 h. The kinds of beverage were water, ion beverage, cucumber drink. Blood sampling was performed before the exercise, immediately finishing exercise, and 30 min after rest. In
the present results, glucose concentration was increased by intake of water, ion beverage, and cucumber drink immediately after exercise. In the water intake group, glucose concentration was decreased 30 min after rest. Free fatty acid concentration was increased by intake of water, ion beverage, and cucumber drink 60 min after exercise. In the ion beverage and cucumber drink intake groups, free fatty acid concentration was decreased 30 min after rest. Insulin concentration was increased by intake of water, ion beverage, and cucumber drink 60 min after exercise. In the water intake group, insulin concentration was decrease 30 min after rest. There was no difference in the efficacy among water, ion beverage, and cucumber drink, but ion beverage and cucumber drink showed more potent effect on metabolic parameters [13593].

Hydration

It has now been established that exercise performed under various environmental conditions may affect acute energy intake and appetite-related hormones. Long-term maintenance of a healthy body weight (BW) is becoming increasingly important as rates of obesity and obesity-related comorbidities continue to rise. While the "energy balance equation" is understood to underpin long-term weight maintenance, several studies have shown that, without an accompanying conscious reduction in energy intake, weight loss is not maintained during the longer-term through exercise alone. The exact mechanism linking acute energy intake and exercise remains unknown, although indirect evidence suggests a possible role for hydration status. External factors such as the ambient temperature during exercise and the exercise intensity may also affect short-term energy intake. Of particular interest, an acute bout of exercise performed at 36°C resulted in lower relative energy intake (REI) in the subsequent meal as well as an increased circulating concentration of peptide YY (PYY; appetite-suppressing peptide) when compared to a resting control trial, whereas an equivalent bout of exercise in a temperate environment (25°C) did not. The reduced REI after exercise in the heat raises the possibility that dehydration may play a role in modulating energy intake after exercise. That hydration levels may reduce appetite and feeding behavior in animals has long been acknowledged, and indeed, the term dehydration-induced anorexia is commonly used in animal-based research. Therefore, the purpose of one study was to investigate the interaction of exercise and hydration status on subsequent energy intake and appetite-related hormones. In a randomized, counterbalanced design, 10 physically active males completed three experimental trials in a fasted state: exercise when hydrated (0 % to 1 % of body mass), exercise when dehydrated (-1 % to -2 % of body mass), and a hydrated resting control. Exercise consisted of treadmill running for 45 min at 70 percent V·O$_2$peak. Participants were then given access to a buffet-style breakfast from which they could consume ad libitum. Blood was sampled regularly during trials for appetite-related hormones. There were no significant differences in total energy intake between trials, however, relative energy intake was significantly higher in the control (4839 ± 415 kJ) compared to hydrated (1749 ± 403 kJ) and dehydrated exercise (1656 ± 413 kJ) conditions. Exercise performed in a dehydrated state resulted in significantly lower concentrations of ghrelin compared with control and hydrated exercise conditions. Exercise significantly decreased relative energy intake compared with resting control; however, energy intake (relative and total) was no different between the exercise conditions (dehydrated vs hydrated). Despite similar energy intake between trials, exercise in a dehydrated state resulted in a significantly lower concentration of ghrelin, a hormone responsible for stimulating appetite [11379].

People perform physical activity throughout a range of environmental conditions (humidity, temperature, sun, wind exposure). Depending upon the metabolic rate, environmental conditions and clothing worn, exercise can induce significant elevations in body (core and
skin) temperatures. Body temperature elevations elicit heat loss responses of increased skin blood flow and increased sweat secretion. Sweat evaporation provides the primary avenue of heat loss during vigorous exercise in warm hot weather; therefore sweat losses can be substantial. Besides containing water, sweat contains electrolytes that are lost. If not appropriately replaced, water and electrolytes imbalances (dehydration and hyponatremia) can develop and adversely impact on the individuals exercise performance and perhaps health. One “Position Stand” provides guidance on fluid replacement to sustain appropriate hydration of individuals performing physical activity. Throughout the position stand, the term “euhydration” refers to “normal” body water content, while the terms “hypohydration” and “hyperhydration” refer to body water content deficits and excesses beyond the normal fluctuation in body water content, respectively. The term “dehydration” refers to the loss of body water. The hypohydration that occurs during exercise is usually characterized as hyperosmotic hypovolemia (because sweat is hypotonic to plasma), although iso-osmotic hypovolemia can occur when taking some medications (e.g., diuretics) or exposure to cold and hypoxia. For simplicity, the term dehydration will be used to describe both the process of body water loss and hypohydration in this position statement, unless stated otherwise. The goal of prehydrating is to start the activity euhydrated and with normal plasma electrolyte levels. Prehydrating with beverages, in addition to normal meals and fluid intake, should be initiated when needed at least several hours before the activity to enable fluid absorption and allow urine output to return to normal levels. The goal of drinking during exercise is to prevent excessive (>2 % body weight loss from water deficit) dehydration and excessive changes in electrolyte balance to avert compromised performance. Because there is considerable variability in sweating rates and sweat electrolyte content between individuals, customized fluid replacement programs are recommended. Individual sweat rates can be estimated by measuring body weight before and after exercise. During exercise, consuming beverages containing electrolytes and carbohydrates can provide benefits over water alone under certain circumstances. After exercise, the goal is to replace any fluid electrolyte deficit. The speed with which rehydration is needed and the magnitude of fluid electrolyte deficits will determine if an aggressive replacement program is merited [07249].

Water is the largest single component of the human body. It is obtained from food and beverage consumption and by metabolic production in the body. One study investigated water consumption in adults living in the UK. Forty males and 40 females (mean ± SD age 34 ± 13 years, body mass 72 ± 19 kg, height 169 ± 10 cm, body mass index 25 ± 6 kg/m², mean arterial blood pressure 97 ± 13 mm Hg) completed a three consecutive day dietary record for 1 weekend and 2 week days. Every item consumed (food and drinks), the eating occasion (meal or snack), time, food preparation method, brand of product, location of consumption and details of any people with them were recorded. Females tended to consume more water than males (2402 ± 827 ml/day, 2056 ± 911 mL/day respectively. More water was consumed via nonwater beverages than food and plain water, equivalent to 27 ± 11 percent of total daily water intake from food, 45 ± 20 percent from nonwater beverages and 28 ± 21 percent from plain water. There was no difference in total water intake on weekend days compared to week days for females 2488 ± 1041 ml, 2446 ± 831 ml and 2272 ± 9 21 mL or for males 2132 ±1 353 ml, 2101 ± 1295 ml and 1934 ± 876 mL. There was no difference in total water intake among females and males relative to weekend days and week day 1 and week day 2 [10506].

Research into exercise and hydration is not new. Twenty-five years ago, White and Ford1 reported on the voluntary dehydration (hypohydration) during a competitive cycling road race; subjects' mean body mass losses were greater than 3 percent, and there were low rates of fluid ingestion despite fluid being freely available. It is notable that there was no relationship between fluid intake and finishing position. The British Journal of Sports Medicine published one of the first consensus statements on fluid replacement2 during and after exercise. It has
now been challenged the common belief that (full) fluid replacement is necessary to maintain/improve high-intensity exercise performance in moderate and warm conditions. Data show neuromuscular adjustments according to hydration status allowing the attainment of similar peak and terminal core body temperatures and heart rates, and performance times. Therefore, subjects performed equally well during 60 min of cycling at 20°C when fully hydrated, as at 33°C without any hydration (with body mass losses of 2.1 %). Interestingly, this is not the first study to demonstrate this occurrence. In another study it was utilised a similar 60 min cycling protocol performed at 20°C and reported no benefit of replacing fluid losses during exercise when compared to complete fluid restriction (2.2 % body mass loss); in fact, when receiving fluid, subjects actually performed worse! So, why does the sports medicine and exercise science community advocate that “during prolonged exercise the ingestion of appropriate fluids will improve performance” and “dehydration >2% body weight degrades aerobic exercise”, when fluid restriction or ad libitum ingestion has been shown to exert no deleterious performance effect? Substantial literature demonstrates that prolonged exercise can cause considerable body water deficit (dehydration), which adversely affects blood (plasma) volume and therefore cardiac stroke volume and output, osmolality, mean arterial pressure, body temperature(s), perception of effort and exercise duration; fluid replacement can attenuate these effects. Heat stress usually accelerates this exercise-induced dehydration and therefore exacerbates the forementioned consequences. Most, if not all, of these observations were made employing fixed-intensity exercise protocols – an experimental model that controls numerous confounding factors. Ideally then, the investigators can confidently conclude that independent variable x (e.g. fluid intake) exerts an effect on dependent variable y (e.g. exercise performance). While “as fast as possible” not “as long as possible” is reasonable, and one on which much of our understanding of physiological responses to exercise has been built, there is one area where this model is falling out of favour – exercise “performance”. Traditionally, exercising to exhaustion, that is, volitional fatigue, has been the performance measure of choice, yet the ecological validity of such a test is low as most “real-life” sporting situations require us to complete a set distance as quickly as possible rather than for as long as possible. Importantly, exercise is usually self-paced – the athlete can adjust “pace” or workload as desired. Thus, while fixed-intensity exercise to exhaustion only allows an all-or-none response (exercise continuance vs termination), self-paced exercise of a set load (distance/work/time) allows continual behavioural adjustments that may affect performance. This explains the need to re-examine the literature on hydration and exercise performance, such as the improved exercise duration with adequate hydration observed by the same group who 1 year later observed no such performance improvement. One study is an innovative foray into studies of self-paced exercise performance and its determinants, such as hydration. Importantly, this new study suggests that previous conclusions, based on fixed-intensity exercise models, cannot be assumed to apply to the more realistic setting of self-paced competition [10507].

Twelve adolescent athletes underwent, in a crossover-design study, 3 separate 90-min training sessions in the following conditions: no fluid ingestion allowed (NF), ad libitum ingestion of water (W), and ad libitum ingestion of a commercial 8 percent carbohydrate-electrolyte sports beverage (CSB). After each session athletes performed a set of basketball drills (2-point, 3-point, and free-throw shootout, suicide sprints, and defensive zigzags). Body weight (before and after sessions), rating of perceived exertion (RPE), urine color, and beverage acceptability were determined in each session. Athletes also completed a survey about their knowledge and behaviors regarding hydration and fluid replacement. The percentage of weight loss was significantly higher in NF than in the other 2 conditions but also higher in W than CSB. RPE was higher in NF than in the W and CSB trials. Athletes' fluid intake was positively correlated with proper self-reported behaviors and knowledge about fluid and hydration. In conclusion, fluid restriction during exercise was associated with a greater level of dehydration and increased perceived exertion but had no impact on
basketball performance compared with ad libitum drinking of water or a CSB. Athletes with more knowledge about hydration and better self-reported hydration behaviors ingested more fluids during training sessions [11247].

The field of research examining the link between dehydration and endurance performance is at the dawn of a new era. This article reviews the latest findings describing the relationship between exercise-induced dehydration and endurance performance and provides the knowledge necessary for competitive, endurance-trained athletes to develop a winning hydration strategy. Acute, pre-exercise body weight loss at or above 3 percent may decrease subsequent endurance performance. Therefore, endurance athletes should strive to start exercise well hydrated, which can be achieved by keeping thirst sensation low and urine color pale and drinking approximately 5-10 mL/kg body weight of water 2 h before exercise. During exercise lasting 1 h or less, dehydration does not decrease endurance performance, but athletes are encouraged to mouth-rinse with sports drinks. During exercise lasting longer than 1 h, in which fluid is readily available, drinking according to the dictates of thirst maximizes endurance performance. In athletes whose thirst sensation is untrustworthy or when external factors such as psychological stress or repeated food intake may blunt thirst sensation, it is recommended to program fluid intake to maintain exercise-induced body weight loss around 2 to 3 percent [12376].

Another review also presented recommendations for fluid needs and hydration assessment for recreational activity. Fluid needs are based on sweat losses, dependent on intensity and duration of the activity, and will vary among individuals. Prolonged aerobic activity is adversely influenced by dehydration, and heat exposure will magnify this effect. Fluid needs predicted for running 5-42 km at recreational paces show that fluid losses are <2 percent body mass; thus, aggressive fluid replacement may not be necessary. Competitive paces result in greater fluid losses and greater fluid needs. Fluid needs for recreational activity may be low; however, carbohydrate consumption (sport drinks, gels, bars) can benefit high-intensity (≤ 1 h) and less-intense, long-duration activity (≥ 1 h). Spot measures of urine color or urine-specific gravity to assess hydration status have limitations. First morning urine concentration and body mass with gross thirst perception can be simple ways to assess hydration status [12377].

Loss of body water, if sufficiently severe, impairs most physiological functions, but the body water content fluctuates over the course of a normal day with no implications for physical or mental performance. The point at which an effect of dehydration becomes apparent has been the subject of much debate, in part, at least, because of the different tests that have been applied, differences in the methodologies used to induce dehydration and also because of differences in the fitness and other physiological characteristics of the subjects studied. The act of drinking itself and the conscious denial of access to water will also have implications for subjective responses to the exercise task. In many published studies, it is difficult to separate the effects of ingestion of water from those of carbohydrate, electrolytes, and other drink components. Nevertheless, there is good evidence that drinking appropriate amounts of water, especially cold water, can enhance exercise performance in many situations [12378].

In adolescents

There is a lack of studies concerning hydration status of young athletes exercising in the heat. To assess pre-exercise hydration status in young soccer players during a summer sports camp and to evaluate body water balance after soccer training sessions initial hydration status was assessed in 107 young male soccer players (age: 11-16 years) during the 2nd day of the camp. Seventy two athletes agreed to be monitored during two more
training sessions (3rd and 5th day of the camp) in order to calculate dehydration via changes in body weight, while water drinking was allowed ad libitum. Hydration status was assessed via urine specific gravity (USG), urine color and changes in total body weight. Mean environmental temperature and humidity were 27 ± 2°C and 57 ± 9 percent, respectively. According to USG values, 95 out of 107 of the players were hypohydrated (USG≥1.020), prior to practice. The prevalence of dehydration observed was maintained in both days, with 96 and 97 percent of the players being dehydrated, after the training sessions in the 3rd and 5th day, respectively. Despite fluid availability, 54 out of the 66 (82 %) dehydrated players reduced their body weight (-0.35 ± 0.04 kg) as a response to training, while 75 percent (47 out of the 63) reduced further their body weight (-0.22 ± 0.03 kg) after training in the 5th day. It was concluded that approximately 90 percent of the young soccer players who began exercising under warm weather conditions were hypohydrated; while drinking ad libitum during practice, did not prevent further dehydration in already dehydrated players [12379].

The purpose of one study was to determine the hydration status, fluid intake, and electrolyte losses of 21 male professional youth soccer players (age 17 years) training in a cool environment. Pretraining and posttraining measurements of body mass, urine (freezing-point osmolality method), and sweat concentration (flame-emission spectroscopy) were collected. Fourteen players were found to be hypohydrated before training. The amount of fluid lost due to exercise equated to a 2 percent loss in body mass, which equated to a gross dehydration loss of 0.5 percent. Overall, the soccer players replaced 46 ± 88 percent of sweat loss during training, and only 4 remained hypohydrated after training. No significant correlations between sweat loss and sweat concentrations of Na⁺ or K⁺ were found, but there was a significant correlation with Mg²⁺. The study found large variability in pretraining hydration status that the players were able to rehydrate during the training sessions. However, given the numbers starting training in a hypohydrated state, adequate hydration status before training should be considered by youth players, coaches, and sports-science support staff [12380].

Effects on environment and persons around for drinking habits

People tend to drink and eat more when others are present than when alone [De Castro. Physiol Behav 1994; 56: 445-55]. One study investigated the influence of the presence of other people on water consumption in adults living in the UK. Eight females and 11 males who consumed 74 ± 12 percent (Mean ± SD) of their meals and snacks alone (1) and 8 females and 11 males who had 80 ± 17 percent of their meals and snacks with other people present (2+) completed a 3-consecutive day dietary record for 1 weekend and 2 week days. Every item consumed, eating occasion (meal/snack), time, food preparation method, product brand, location of consumption and details of any people with them were recorded. Characteristics of subjects 1 and 2+ are: 31 ± 13 year, 41 ± 15 year; body mass 74 ± 17 kg, 75 ± 14 kg; height 171 ± 11 cm, 172 ± 8 cm; BMI 26 ± 7 kg/m², 25 ± 4 kg/m²; environmental temperature 10 ± 5°C, 10 ± 4°C, respectively. Comparisons between groups were determined by unpaired t-tests. Group 2+ consumed more water than group 1 subjects (2482 ± 691 mL/ day, 1681 ± 630 mL/day. This was because they consumed more water via drinks (non-water beverages: 1143 ± 565 mL, 731 ± 559 mL respectively, plain water: 791 ± 432 mL, 383 ± 347 mL respectively) as there was no difference between groups in total water intake from food (1; 506 ± 270 mL, 2+; 608 ± 152 mL respectively. There was a strong and significant correlation between water intake and other people present. The amount of water ingested with other people present increase by over 801 mL/ day than alone. This is similar to previous research [De Castro et al. Physiol Behav 1991; 51: 121-5] which found that eating alone decreased intake by 200 kcal/day [11516].

Effect of warm and cold climate
Olympic class sailing poses physiological challenges similar to other endurance sports such as cycling or running, with sport specific challenges of limited access to nutrition and hydration during competition. As changes in hydration status can impair sports performance, examining fluid consumption patterns and fluid/electrolyte requirements of Olympic class sailors is necessary to develop specific recommendations for these elite athletes. The purpose of one study was to examine if Olympic class sailors could maintain hydration status with self-regulated fluid consumption in cold conditions and the effect of fixed fluid intake on hydration status in warm conditions. In a cold condition study (CCS), 11 elite Olympic class sailors were provided ad libitum access to three different drinks. Crystal Light (control, C); Gatorade (experimental control, G); and customized sailing-specific Infinit (experimental, IN) (1.0:0.22 CHO:PRO), were provided on three separate training days in cold 7.1°C. A warm condition study (WCS) examined the effect of fixed fluid intake (11.5 mL/kg/h) of C, G and heat-specific experimental Infinit (INW)(1.0:0.074 CHO:PRO) on the hydration status of eight elite Olympic Laser class sailors in 19.5°C. Both studies used a completely random design. In CCS, participants consumed 802 ± 91, 924 ± 137 and 707 ± 152 mL of fluid in each group respectively. This did not change urine specific gravity, but did lead to a main effect for time for body mass, blood sodium, potassium and chloride with all groups lower post-training. In WCS, fixed fluid intake increased participant's body mass post-training in all groups and decreased urine specific gravity post-training. There was a main effect for time for blood sodium, potassium and chloride concentration, with lower values observed post-training. C blood sodium concentrations were lower than the INW group post-training with a trend towards significance in the G group. It was concluded that ad libitum fluid consumption in cold conditions was insufficient in preventing a decrease in body mass and blood electrolyte concentration post-training. However, when a fixed volume of 11.5 mL/kg/L was consumed during warm condition training, hydration status was maintained by preventing changes in body mass and urine specific gravity [13599].

Sweating

Participation in physical activity exposes individuals to a variety of factors that influence sweat losses; these include the duration and intensity of exercise, the environmental conditions and the type of clothing/equipment worn. Sometimes, these factors are standardized for a specific activity or event within a sport (e.g. the temperature of an air conditioned indoor stadium or the uniform worn by a sporting team). In other cases, these factors occur in a predictable manner (e.g. running speeds in a 10,000 m race are higher than in a marathon, Nordic skiing, and other outdoor winter sports are undertaken in colder environments than summer sports). Nevertheless, in most activities, there is considerable variability in exposure to the factors that contribute to sweat rates between participants. Individual characteristics, such as body weight, genetic predisposition, heat acclimatization state, and metabolic efficiency (economy at undertaking a specific exercise task) will influence sweat rates for a given activity. As a result, there is a large range in sweat rates and total sweat losses of individuals between and within activities, and in some cases even in the same event on a given day. For example, elite marathon runners may have higher sweating rates but similar total sweat losses (run for shorter duration) as recreational runners who finish the race at the rear of the field. In a soccer match, sweat rates will vary between players according to their position and playing style as well as the total time spent on the field. Likewise, American football players (large body mass and wearing protective clothing) will have markedly greater daily sweat losses than cross country runners training in the same hot environmental for the same duration. Individuals often achieve sweating rates from 0.5 to 2.0 L/h. The differences in sweat rates between individuals, different sports and climatic season demonstrate the difficulties in providing a single one size fits all recommendation [07249].
Muscular contractions produce metabolic heat that is transferred from the active muscles to blood and then the body core. Subsequent body core temperature elevations elicit physiologic adjustments that facilitate heat transfer from within the body core to the skin where it can be dissipated to the environment. Heat exchange between the skin and the environment is governed by biophysical properties dictated by the surrounding temperature, humidity and air motion, sky and ground radiation, and clothing. In temperate and cooler environments, the high capacity for dry heat loss (radiation and convection) reduces evaporative cooling requirements, so sweat losses are relatively small. As the environmental heat stress increases, there is a greater dependence on sweating for evaporative cooling. The wearing of heavy or impermeable clothing, such as a football uniform, greatly increases the heat stress and evaporative cooling requirements while exercising in temperate to hot environments. Likewise, wearing heavy or impermeable clothing while exercising in cold weather can elicit unexpectedly high sweat rates. If the exercise task is 20 percent efficient, then 80 percent of metabolic energy is converted to heat in the body. Therefore, high intensity exercise will require about 800 W (11.5 kcal/min) of metabolic energy to be dissipated to avoid heat storage. If secreted sweat drips from the body and is not evaporated, higher sweating will be needed to achieve the evaporative cooling requirements. Conversely, increased air motion (wind, movement velocity) will facilitate evaporation and minimize wasted (dripping) sweat. Heat acclimatization enhances an individual to achieve higher and more sustained sweating rates, if needed. Similarly, aerobic exercise training has a modest effect on enhancing sweating rate responses. Other factors, such as wet skin (e.g. from high humidity) and dehydration can act to suppress the sweating rate response [07249].

Sweat electrolyte losses depend on the total sweat losses and sweat electrolyte concentrations. Sweat sodium concentration averages about 35 mEq/L (range 10-70 mEq/L) but varies depending upon genetic predisposition, diet, sweating rate, and heat acclimatization state. Sweat concentrations of potassium averages 5 mEq/L (range 3-15 mEq/L), calcium averages 1 mEq/L (range 0.3-2 mEq/L), magnesium average 0.8 mEq/L (range 0.2-1.5 mEq/L), and chloride averages 30 mEq/L (range 5-60 mEq/L). Neither gender, maturation, nor aging appear to have marked effects on sweat electrolyte concentrations; although dehydration can increase the sweat concentrations of sodium and chloride. Sweat glands reabsorb sodium and chloride, but the ability to reabsorb these electrolytes does not increase proportionally with the sweating rate. As a result, the concentration of sweat sodium and chloride increases as a function of sweating rate. Heat acclimatization improves the ability to reabsorb sodium and chloride, thus heat acclimatized individuals usually have lower sweat sodium concentrations for any given sweating rate [07249].

Hydration assessment

Water gain occurs from consumption (liquids and food) and production (metabolic water), while water losses occur from respiratory, gastrointestinal, renal, and sweat losses. The volume of metabolic water produced during cellular metabolism (about 0.13 g/kcal) is approximately equal to respiratory water losses (about 0.12 g/kcal), so this results in water turnover with no net change in total body water. Gastrointestinal tract losses are small (100-200 mL/d) unless the individual has diarrhea. Sweating provides the primary avenue of water loss during exercise-heat stress. The kidneys regulate water balance by adjusting urine output, with minimum and maximum urine outputs of approximately 20 and 1000 mL/h, respectively. During exercise and heat stress, both glomerular filtration and renal blood flow are markedly reduced, resulting in decreased urine output. Therefore, when fluids are over consumed during exercise (hyperhydration), there may be a reduced ability to produce urine to excrete the excess volume. With intermittent activities these effects may not be as strong.
on reducing urine production. Over a protracted period (e.g. 8-24 h), if adequate fluid and electrolytes are consumed, the water losses will usually be fully replaced to reestablish the "normal" total body water (TBW). TBW is regulated within 0.2 to 0.5 percent of daily body mass. TBW averages about 60 percent of body mass, with a range from approximately 45 to 75 percent. These differences are primarily due to body composition; fat-free mass is about 70 to 80 percent water, while adipose tissue contains about 10 percent water. These water content relationships are independent of age, sex and race. Therefore, an average 70-kg person has approximately 42 L of total body water, with a range of 31–51 L. Trained athletes have relatively high TBW values by virtue of having a high muscle mass and low body fat and a small aerobic training effect. Additionally, individuals who load glycogen may experience a small increase in TBW, but this is not always observed. Furthermore, the surplus water associated with typical muscle glycogen increases is minor (about 200 mL) when considering the small absolute muscle mass involved and assuming 3 mL water per gram glycogen. The precise fate of water liberated as glycogen is utilized is unknown, but the fact that any water bound to glycogen is part of the starting TBW pool suggests it is of little potential consequence to fluid intake recommendations [07249].

When assessing an individual’s hydration status, there is no one TBW that represents euhydration, and determinations need to be made of body water fluctuations beyond a range that have functional consequences. Ideally, the hydration biomarker should be sensitive and accurate enough to detect body water fluctuations of about 3 percent of TBW (or water content change sufficient to detect fluctuations of 2 percent body weight for the average person). In addition, the biomarker should also be practical (time, cost, and technical expertise) to be used by individuals and coaches. Individuals can determine their hydration status by using several simple biomarkers (urine and body weight) that by themselves have marked limitations; but when these indicators are used together in the proper context, they can provide valuable insight. The use of first morning body weight measurement after voiding, in combination with a measure of urine concentration should allow sufficient sensitivity (low false negative) to detect deviations in fluid balance. Urine biomarkers of hydration status can allow discrimination of whether an individual is euhydrated or dehydrated. Urine specific gravity (USG) and osmolality are quantifiable, whereas urine color and urine volume are often subjective and might be confounded. USG of > 1.020 is indicative of being euhydrated. Osmolality is more variable, but values > 700 mOsmol/kg are indicative of being euhydrated. However, urine values can provide misleading information regarding hydration status if obtained during rehydration periods. For example, if dehydrated persons consume large volumes of hypotonic fluids, they will have copious urine production long before euhydration is reestablished. Urine samples collected during this period will be light in color and have USG and osmolality values that reflect euhydration when in fact the person remains dehydrated. This emphasizes the need to use either first morning urine samples, or samples after several hours of stable hydration status, to allow valid discrimination between euhydration and dehydration. Body weight (BW) measurements provide another simple and effective tool to assess fluid balance. For well-hydrated persons, who are in energy balance, a first morning (after urinating) nude BW will be stable and fluctuate by < 1 percent. At least three consecutive morning nude BW measurements should be made to establish a baseline value, which approximates euhydration, in active men consuming food and fluid ad libitum. Women may need more BW measurements to establish a baseline value, because their menstrual cycle influences body water status. For example, luteal phases can increase body water and BW by >2 kg. Lastly, first morning BW is influenced by changes in eating and bowel habits [07249].

Acute changes in BW during exercise can be used to calculate sweating rates and perturbations in hydration status that occur in different environments. This approach assumes that 1 mL of sweat loss represents a 1-g loss in body weight (i.e. specific gravity of
sweat is 1.0 g/mL). The before-exercise BW measures are used with the postexercise BW corrected for urine losses and drink volume. When possible, nude weights should be used to avoid corrections for sweat trapped in the clothing. Other nonsweat factors contributing to BW loss during exercise include respiratory water and carbon exchange. Ignoring those two factors will over estimate sweat rate modestly (5-15 %) but generally do not require correction for exercise durations <3 h. If proper controls are made, BW changes can provide a sensitive estimate of acute TBW changes to access hydration changes during exercise [07249].

Hydration status is not easily measured, but acute changes in hydration status are often estimated from body mass change. Changes in body mass are also often used as a proxy measure for sweat losses. There are, however, several sources of error that may give rise to misleading results, and the aim in one paper was to quantify these potential errors. Respiratory water losses can be substantial during hard work in dry environments. Mass loss also results from substrate oxidation, but this generates water of oxidation which is added to the body water pool, thus dissociating changes in body mass and hydration status: fat oxidation actually results in a net gain in body mass as the mass of carbon dioxide generated is less than the mass of oxygen consumed. Water stored with muscle glycogen is presumed to be made available as endogenous carbohydrate stores are oxidized. Fluid ingestion and sweat loss complicate the picture by altering body water distribution. Loss of hypotonic sweat results in increased osmolality of body fluids. Urine and faecal losses can be measured easily, but changes in the water content of the bladder and the gastrointestinal tract cannot. Body mass change is not always a reliable measure of changes in hydration status and substantial loss of mass may occur without an effective net negative fluid balance [07250].

It was evaluated the change in body mass including fat mass and skeletal muscle mass in one ultracyclist whilst cycling 1,000 km in 48 hours at a constant intensity of about 48 percent VO\text{2}\text{max}, corresponding to a heart rate frequency of 105 ± 5 bpm. A 1 kg fat mass decrease resulted, with the largest decrease occurring between the 12th and the 24th hour. No steady state in metabolism was observed and no regular decrease of subcutaneous adipose tissue resulted. This result is backed up by the nuclear magnetic resonance (NMR) urine analysis. Body water increase with simultaneous dehydration is possibly due to endocrine-induced renal water retention, in order to maintain metabolism processes that are required for energy supply and blood flow during very prolonged exercise. Both applied methods, the anthropometric and the bioelectrical impedance analysis, analyse fluid accumulation – especially in the skinfolds of the lower extremities – apparently incorrectly as an increase in body mass and not as an increase in fluids [11381].

**Dehydration**

For the past 30 years, these have been two of the basic tenets of sports nutrition. Recently, however, both these universally regarded “truths” have been challenged. Firstly, it is now know that excessive intake of fluid during endurance events can lead to exercise-associated hyponatraemic encephalopathy (EAHE) or “water intoxication.” There have been a number of deaths reported from this syndrome. Drinking too much water can lead to fluid retention and EAHE in those who also have the syndrome of inappropriate antidiuretic hormone secretion (SIADH). Endurance athletes have been encouraged for the past three decades to not wait until they were thirsty, but to drink large amounts of water or sports drinks to prevent dehydration and heat illness. However, excessive fluid overload is the major danger in endurance exercise, not dehydration and heat stroke, and that the old adage of drink when you are thirsty is actually correct [13596].
It is purported that exercise-induced dehydration (EID), especially if \( \geq 2 \) percent of body-weight, impairs endurance performance (EP). The recommendation to limit dehydration to 2 percent bodyweight during exercise is based from results of studies that used exercise protocols where athletes were forced to exercise at fixed-work rates until exhaustion or at least during part of the exercise protocols. These research designs have a poor reliability or possess a very low ecological validity, thereby suggesting that they should not be used in the establishment of fluid intake guidelines, especially those designed for athletes. In fact, several studies have shown that athletes’ exercise intensity during racing conditions never remains constant but rather constantly varies throughout either on a macro- or micro-scale. There is no sporting event where athletes are required to exercise until exhaustion. Finally, optimal endurance performance can only be achieved when the knowledge of the distance or time to be completed during an exercise bout is known. Using the meta-analytic procedure, one study compared the findings of laboratory-based studies that examined the impact of EID upon EP using either ecologically valid (EV) (timetrial exercise) or non-ecologically valid (NEV) (clamped-intensity exercise) exercise protocols. EP outcomes were put on the same scale and represent percentage changes in power output between euhydrated and dehydrated exercise tests. Random effects model meta-regressions and weighted mean effect summaries, mixed-effects model analogue to the ANOVAs and magnitude-based effect statistics were used to delineate treatment effects. Fifteen research articles were included, producing 28 effect estimates, representing 122 subjects. Compared with euhydration, EID increased \((0.09 \pm 2.60 \% )\), not statistically significant, EP under time-trial exercise conditions, whereas it significantly reduced it \((1.91 \pm 1.53 \% )\) with NEV exercise protocols. Only with NEV exercise protocols did EID \( \geq 2 \) percent body weight impair EP. Evidence thus indicates that exercise-induced dehydration \( \leq 4 \) percent bodyweight is very unlikely to impair EP under real-world exercise conditions (time-trial type exercise) and; under situations of fixed-exercise intensity, which may have some relevance for military and occupational settings, EID \( \geq 2 \) percent bodyweight is associated with a reduction in endurance capacity. The 2 percent bodyweight loss rule has been established from findings of studies using NEV exercise protocols and does not apply to out-of-doors exercise conditions. Athletes are therefore encouraged to drink according to thirst during exercise [13597].

To determine the effects of exercise heat-induced two percent dehydration (DEH) and euhydration (EUH) with a six percent carbohydrate-electrolyte solution (CES) compared with placebo EUH (P EUH) on basketball skills in skilled young players. Fifteen 12- to 15-year-old boys underwent three separate 2-h exercise heat exposures (double blind, random order): 2 percent DEH by limiting fluid intake during exercise in the heat and basketball skill drills, EUH (no net weight change) with a 6 percent CES, and EUH with a flavored water placebo (P EUH). After recovery, subjects performed an orchestrated sequence of continuous basketball drills designed to simulate a game (12-min quarters + a 10-min halftime). Performance measures and component drills inherent to basketball included various individual and combined shooting percentages (3-point, 15-foot, free-throw shots), sprint (suicides, court widths), lateral movement (zigzags, lane slides), and defensive drill (combining lateral and front-to-back movement) times. Compared with P EUH, combined shooting percentage was improved by 2 percent DEH and improved by CES intake. Total sprint times showed a similar effect. Total lateral movement times were impaired by 2 percent DEH. CES improved total defensive drill times compared with 2 percent DEH. It was concluded that deterioration in basketball skill performance accompanies two percent dehydration in skilled 12- to 15-year-old basketball players. Additionally, EUH with a 6 percent CES significantly improves shooting performance and on-court sprinting over EUH with water [06252].

Individuals often start an exercise task with normal total body water and dehydrate over an extended duration; however, in some sports the person might initiate the exercise task
dehydrated such as when the interval between exercise sessions is inadequate for full rehydration or when initial body weight is an issue. For example, in weight-class sports (e.g. boxing, power lifting, wrestling) individuals may purposely dehydrate to compete in lower weight classes. In addition, some individuals undertaking twice a day training, or prolonged daily sessions of exercise in hot conditions, may also carry a fluid deficit from their previous workout into the next. Water deficit without proportionate sodium chloride loss is the most commonly seen form of dehydration during exercise in the heat. If large sodium chloride deficits occur during exercise then the extracellular fluid volume will contract and cause “salt depletion dehydration.” Regardless of the dehydration method, for any water deficit, there is similarity in altered physiologic function and performance consequences. Dehydration increases physiologic strain as measured by core temperature, heart rate and perceived exertion responses during exercise-heat stress. The greater the body water deficit, the greater the increase in physiologic strain for a given exercise task. Dehydration >2 percent of body weight degrades aerobic exercise and cognitive/mental performance in temperate-warm-hot environments. Greater levels of dehydration will further degrade aerobic exercise performance. The critical water deficit (>2 % BW for most individuals) and the magnitude of performance decrement are likely related to the environmental temperature, exercise task, and the individual’s unique biological characteristics (e.g. tolerance to dehydration). Therefore, some individuals will be more or less tolerant to dehydration. Dehydration (3 % BW) has marginal influence on degrading aerobic exercise performance when cold stress is present. Dehydration (3-5 % BW) probably does not degrade either muscular strength or anaerobic performance. Physiologic factors that contribute to dehydration-mediated aerobic exercise performance decrements include increased body core temperature, increased cardiovascular strain, increased glycogen utilization, altered metabolic function, and perhaps altered central nervous system function. Though each factor is unique, evidence suggests that they interact to contribute in concert, rather than in isolation, to degrade aerobic exercise performance. The relative contribution of each factor may differ depending on the specific activity, environmental conditions, heat acclimatization status and athlete prowess, but elevated hyperthermia probably acts to accentuate the performance decrement. Cognitive/mental performance, which is important where concentration, skilled tasks and tactical issues are involved, is also degraded by dehydration and hyperthermia. The evidence is stronger for a negative effect of hyperthermia than that of mild dehydration on degrading cognitive/mental performance, but the two are closely linked when performing exercise in warm-hot weather [07249].

To determine the effect of a 48-h period of either fluid restriction (FR), energy restriction (ER), or fluid and energy restriction (F + ER) on 30-min treadmill time trial (TT) performance in temperate conditions. Thirteen males participated in four randomized 48-h trials (mean 21 years). Control (CON) participants received their estimated energy and water requirements. For FR, participants received their energy requirements and 193 mL/day water to drink, and for ER, participants received their water requirements and 290 kcal/day. F + ER was a combination of FR and ER. After 48 h, participants performed a 30-min treadmill TT in temperate conditions. A separate investigation (n=10) showed the TT to be highly reproducible (CV 1.6 %). Body mass loss (BML) was 0.6 percent (CON), 3.2 percent (FR), 3.4 percent (ER), and 3.6 percent (F + ER). Compared with CON less distance was completed on ER (10.3 %) and F + ER (15.0 %). Although less distance was completed on FR (2.8 %), this was not significantly different from CON. These results show a detrimental effect of a 48-h period of ER but no significant effect of FR on 30-min treadmill TT performance in temperate conditions. Therefore, these results do not support the popular contention that modest hypohydration (2-3 % BML) significantly impairs endurance performance in temperate conditions [07251].
One article summarizes a case of ischemic colitis suffered by a triathlete during an Ironman competition. Exercise results in a significant reduction in splanchnic blood flow to help maintain cardiovascular function. When dehydration and heat stress accompany exercise, blood flow to the splanchnic vasculature is further reduced, increasing the risk of local ischemia and tissue injury. Right hemicolectomy involving a 16-cm segment of ischemic large intestine and appendectomy the day following the race. The case study highlights one of the risks associated with dehydration during prolonged exercise in the heat. Of particular interest are practical interventions to reduce health and performance issues. Poor hydration and nutrition practices during intense exercise can affect gut function, impair performance, and jeopardize health. Optimal intake of fluid, carbohydrate, and salt will enhance performance and reduce risk to health [07252].

To determine the effect of 1, 2, 3, and 4% dehydration (DEH) versus euhydration (EUH) on basketball performance in adult male players 17-17 to 28-year-old male basketball players completed 3 h of interval treadmill walking (40 degrees C and 20 % relative humidity) with or without fluid replacement. Subjects completed six trials in random order: EUH with a carbohydrate-electrolyte solution (CES), EUH control (flavored water with 0 percent carbohydrate and 18 mM sodium), 1 percent DEH, 2 percent DEH, 3 percent DEH, and 4 percent DEH. After a 70-min recovery period, subjects performed a sequence of continuous basketball drills designed to simulate a fast-paced game. Measures of overall skill performance during the 80-min game included total time to complete basketball-specific movement drills (sprinting, defensive slides, sprinting-defensive slides combination, and repetitive jumping drills) and 2) total number of shots (foul-line and baseline jump shots, layups, three-point, 15-ft, free throws) made per game. Performance during all timed and shooting drills declined progressively as % DEH increased. Total time to complete basketball-specific movement drills was significantly slower and fewer shots were made during DEH versus EUH control. There were no significant differences in performance between CES and EUH control. It was concluded that basketball players experienced a progressive deterioration in performance as DEH progressed from 1 to 4 percent. The threshold, or percent DEH at which the performance decrement reached statistical significance, was 2 percent for combined timed and shooting drills [07253].

While in vitro work has revealed that dehydration and hyperthermia can elicit increased cellular and oxidative stress, in vivo research linking dehydration, hyperthermia, and oxidative stress is limited. The purpose of one study was to investigate the effects of exercise-induced dehydration with and without hyperthermia on oxidative stress. Seven healthy male, trained cyclists (power output (W) at lactate threshold (LT): 199 ± 19 W) completed 90 min of cycling exercise at 95 percent LT followed by a 5-km time trial (TT) in 4 trials: (i) euhydration in a warm environment (EU-W, control), (ii) dehydration in a warm environment (DE-W), (iii) euhydration in a thermoneutral environment (EU-T), and (iv) dehydration in a thermoneutral environment (DE-T). Oxidized glutathione (GSSG) increased significantly postexercise in dehydration trials only, and while not significant, total glutathione (TGSH) and thiobarbituric acid reactive substances (TBARS) tended to increase postexercise in dehydration trials. Monocyte heat shock protein 72 (HSP72) concentration was increased while lymphocyte HSP32 concentration was decreased for all trials. Exercise-induced dehydration led to an increase in GSSG concentration while maintenance of euhydration attenuated these increases regardless of environmental condition. Additionally, we found evidence of increased cellular stress (measured via HSP) during all trials independent of hydration status and environment. Finally, both 90-min and 5-km TT performances were reduced during only the DE-W trial, likely a result of combined cellular stress, hyperthermia, and dehydration. These findings highlight the importance of fluid consumption during exercise to attenuate thermal and oxidative stress during prolonged exercise in the heat [11517].
Exercise-associated collapse

Exercise-associated collapse (EAC) commonly occurs after the completion of endurance running events. EAC is a collapse in conscious athletes who are unable to stand or walk unaided as a result of light headedness, faintness and dizziness or syncope causing a collapse that occurs after completion of an exertional event. Although EAC is perhaps the most common aetiology confronted by the medical provider attending to collapsed athletes in a finish-line tent, providers must first maintain vigilance for other potential life-threatening aetiologies that cause collapse, such as cardiac arrest, exertional heat stroke or exercise-associated hyponatraemia. Previously, it has been believed that dehydration and hyperthermia were primary causes of EAC. On review of the evidence, EAC is now believed to be principally the result of transient postural hypotension caused by lower extremity pooling of blood once the athlete stops running and the resultant impairment of cardiac baroreflexes. Once life-threatening aetiologies are ruled out, treatment of EAC is symptomatic and involves oral hydration and a Trendelenburg position – total body cooling, intravenous hydration or advanced therapies is generally not needed [11518].

To investigate which of two commonly used treatment protocols for exercise-associated postural hypotension (EAPH) resulted in earlier discharge from the medical facility a randomised clinical field trial was undertaken at two Ironman Triathlon competitions and one ultra-distance footrace. All collapsed athletes admitted to the medical facilities were considered for the trial. Following clinical assessment and special investigations to confirm the diagnosis of EAPH, 28 athletes were randomly assigned to an oral fluid and Trendelenburg position (OT=14) or an intravenous fluid (IV=14) treatment group. Following admission fluid intake was recorded, and all athletes were assessed clinically (blood pressure, heart rate, level of consciousness) every 15 min until discharge criteria were met. The main measure of outcome was the time to discharge (min). On admission, subjects in the OT and IV groups were similar with respect to age, systolic blood pressure, heart rate and serum sodium concentration. There were no significant differences in heart rate, systolic and diastolic blood pressure between groups and over time until discharge. The fluid intake during the treatment period was significantly greater in the IV group (IV 1045 ± 185 mL, OT 204 ± 149 ml). The average time to discharge for the OT group (58 ± 23 min) was similar to that of the IV group (52.5 ± 18 min). It was concluded that endurance athletes with EAPH can be treated effectively using the Trendelenburg position and oral fluids and the administration of intravenous fluids does not reduce the time to discharge. The findings of this study support the hypothesis that EAPH is a result of venous pooling due to peripheral vasodilatation, rather than dehydration [11519].

Hyperhydration

Hyperhydration can be achieved by overdrinking combined with an agent that “binds” water within the body. These binding agents include glycerol and hypertonic drinks that can induce hyperhydration for varied durations. Simple overdrinking will usually stimulate urine production and body water will rapidly return to euhydration within several hours, however, this compensatory mechanism (urine production) is less effective during exercise and there is a risk of dilutional hyponatremia. Likewise, overconsumption of fluids with most hyperhydration binding agents will still elevate urine output well above normal levels. Hyperhydration does not provide any thermoregulatory advantages, but can delay the onset of dehydration, which may be responsible for any small performance benefits that are occasionally reported [07249].

Muscle problems in hypohydration
Skeletal muscle cramps are believed associated with dehydration, electrolyte deficits and muscle fatigue, and are common in non-heat-acclimatized football players, tennis matches, long cycling races, late in tropical triathlons, soccer and beach volleyball. Muscle cramps can also occur in winter activities-in cross-country ski-racers and icehockey goalies. Persons susceptible to muscle cramps are believed to be often profuse sweaters with large sweat sodium losses. Triathlon athletes experiencing muscle cramps, however, have been reported to not have clinically significant different serum electrolyte concentrations than counterparts without cramps [07249].

Rhabdomyolysis (syndrome causing release of skeletal muscle contents) is most often observed with novel, strenuous, overexertion and clinical evidence suggests that dehydration can increase the consequences of rhabdomyolysis. For example, it appears that dehydration increases the likelihood or severity of acute renal failure associated with rhabdomyolysis. A cluster of exertional rhabdomyolysis cases provides evidence that dehydration, combined with heat stress and novel training, can induce serious health problems [07249].

Exercise associated hyponatremia was first reported at the comrades marathon. Later, exercise-associated hyponatremia was reported in endurance runners, and since that time a number of participants from a variety of occupational and recreational activities have been hospitalized for this condition, with several having died. Symptomatic hyponatremia can occur when plasma sodium rapidly drops to about 130 mmol/L and below. The lower the plasma sodium falls, the faster it falls, and the longer it remains low, the greater the risk of dilutional encephalopathy and pulmonary edema. Some individuals have survived plasma sodium levels as low as 109 mmol/L and others have died with initial (in hospital) levels over 120 mmol/L. With plasma sodium <125 mmol/L and falling, symptoms become increasingly severe and include headache, vomiting, swollen hands and feet, restlessness, undue fatigue, confusion and disorientation (due to progressive encephalopathy), and wheezy breathing (due to pulmonary edema). When plasma sodium falls well below 120 mmol/L, the chances increase for severe cerebral edema with seizure, coma, brainstem herniation, respiratory arrest, and death. Contributing factors to exercise-associated hyponatremia include overdrinking of hypotonic fluids and excessive loss of total body sodium. In marathoners, symptomatic hyponatremia is more likely to occur in smaller and less lean individuals who run slowly, sweat less, and drink heavily-water and other hypotonic fluids-before, during, and after the race. In tropical triathlons, some participants may have been both dehydrated and hyponatremic based upon clinical observations. In general, symptomatic hyponatremia in events that last >4 h is from overdrinking before, during and sometimes even after the event. In longer ultraendurance events, sodium losses can induce hyponatremia to levels associated with the onset of symptoms regardless if the individual is over- or underdrinking, so replacing some of the sodium losses is warranted. Exercise-associated hyponatremia occurs occasionally in tennis players who drink too much water to treat or try to prevent heat cramps, or when a cramping player is given hypotonic fluid intravenously [07249].

**Modifying factors for hypohydration**

Women typically have lower sweating rates and electrolyte losses than men. The lower sweating rates are primarily because they have smaller body size and lower metabolic rates when performing a given exercise task. In addition, women seem to have less wasted sweat when their skin is wet. Gender differences in renal water and electrolyte retention are subtle and probably not of consequence. The diuretic response to a water load can be greater in women than men, suggesting that women turn water over more quickly than men. Women show reduced arginine vasopressin (AVP) responses to osmotic stimuli, which should result
in elevated renal water and electrolyte losses. Paradoxically, within women, both endogenous estrogens and exogenously administered estrogens appear to increase AVP release and both estrogens and progesterone enhance renal water and electrolyte retention. Women appear to be at greater risk than men to develop symptomatic hyponatremia when competing in marathon and ultra marathon races. While the explanation for this increased risk may be due to a number of biological and psychosocial factors, the cause for greater risk for hyponatremia has not been established with certainty. Although the kidney is important in the pathogenesis of hyponatremia, the target organs for morbidity and mortality are the brain and lungs. During AVP-induced hyponatremia, animal studies have shown significantly greater sodium transport in the male rat versus the female rat brain, suggesting impairment of the Na⁺-K⁺-ATPase pump activity in the female brain. Therefore, this might aggravate hyponatremia induced cerebral edema. Likewise, sex hormones have been suggested to impair Na⁺-K⁺-ATPase pump activity in the female brain and account for women having increased morbidity and mortality from postoperative hyponatremia [07249].

Older (ages >65 years) persons are generally adequately hydrated. However, there is an age-related blunting of thirst response to water deprivation, making older persons more susceptible to becoming dehydrated. Older adults have an age-related increase in resting plasma osmolality and are slower to restore body fluid homeostasis in response to water deprivation and exercise than younger adults. If given sufficient time and access to water and sodium, older adults will adequately restore body fluids, indicating appropriate, albeit sluggish, control of body fluids. Older persons are also slower to excrete water following fluid loads. This slower water and sodium excretion increases sodium retention and can lead to increases in blood pressure. While thirst sensitivity to a given extracellular fluid loss is reduced in older adults, osmoreceptor signaling remains intact. The osmotic and volume stimuli that results from dehydrating, impart important drives for thirst and drinking in older adults. Thus, older adults should be encouraged to rehydrate during or after exercise, but they should also consider the risks of excess water (i.e. hyponatremia) or sodium ingestion (i.e. hypertension) because they may be slower to excrete both the water and electrolytes. Prepubescent children have lower sweating rates than adults, and with values rarely exceeding 400 mL/h. These lower sweating rates are probably the result of smaller body mass and thus lower metabolic rate. Sweat electrolyte content is similar or slightly lower in children than adults [07249].

Carbohydrate-electrolyte solution (CES)
One study examined the effects of a carbohydrate-electrolyte drink on voluntary fluid intake, affect and self-selected intensity during recreational exercise after fluid restriction. In a randomised counterbalanced design, ten physically active adults were dehydrated via a 24-h period of fluid restriction before completing two 20-min bouts of cardiovascular exercise, 20-min of resistance exercise and 20 min on a cycle ergometer at a self-selected intensity with ad libitum access to water (W) or a carbohydrate-electrolyte solution (CES). Fluid restriction induced hyponatremia of about 1.2 percent initial body mass. Fluid intake during exercise was greater with CES and resulted in more adequate hydration. Plasma glucose concentrations and pleasure ratings were greater with CES than W. Mean power output during exercise performed at a self-selected intensity was 5.6 percent greater with CES. In physically active adults performing a “real-life” recreational exercise simulation, CES resulted in more adequate hydration and an enhanced affective experience that corresponded with an increase in self-selected exercise intensity [13595].

Diet
Meal consumption is critical to ensure full hydration on a day-to-day basis. Eating food
promotes fluid intake and retention. Sweat electrolyte (e.g. sodium and potassium) losses need to be replaced to reestablish total body water and this can be accomplished during meals with most persons. Diet macronutrient composition has a minor influence on urine losses during rest and probably has even a smaller influence during exercise. Therefore, diet macronutrient composition does not measurably alter daily fluid needs for individuals [07249].

Caffeine is contained in many beverages and foods and recent evidence suggests if consumed in relatively small doses (<180 mg/d) it will likely not increase daily urine output or cause dehydration. The influence of caffeine consumption on urine output during exercise or in dehydrated individuals is not well documented, but urine production is already decreased by dehydration, exercise and heat stress. Therefore, it is doubtful that caffeine consumption during exercise would elevate urine output and induce dehydration during exercise. Since alcohol can act as a diuretic (particularly at high doses) and increase urine output, it should be consumed in moderation, particularly during the postexercise period when rehydration is a goal [07249].

**Fluid replacement before exercise**

The goal of prehydrating is to start the physical activity euhydrated and with normal plasma electrolyte levels. If sufficient beverages are consumed with meals and a protracted recovery period (8-12 h) has elapsed since the last exercise session, then the person should already be close to being euhydrated. However, if the person has suffered substantial fluid deficits and has not had adequate time or fluids/electrolytes volumes to reestablish euhydration, then an aggressive prehydration program may be merited. The prehydration program will help ensure that any previously incurred fluid-electrolyte deficit is corrected prior to initiating the exercise task. When hydrating prior to exercise the individual should slowly drink beverages (for example, 5-7 mL/kg per body weight) at least 4 h before the exercise task. If the individual does not produce urine, or the urine is dark or highly concentrated, she or he should slowly drink more beverage (for example, another 3-5 mL/kg) about 2 h before the event. By hydrating several hours prior to exercise there is sufficient time for urine output to return towards normal before starting the event. Consuming beverages with sodium (20-50 mEq/L) and/or small amounts of salted snacks or sodium-containing foods at meals will help to stimulate thirst and retain the consumed fluids. Attempting to hyperhydrate with fluids that expand the extra- and intracellular spaces (e.g. water and glycerol solutions) will greatly increase the risk of having to void during competition and provides no clear physiologic or performance advantage over euhydration. In addition, hyperhydration can substantially dilute and lower plasma sodium before starting exercise and therefore increase the risk of dilutional hyponatremia, if fluids are aggressively replaced during exercise. Enhancing palatability of the ingested fluid is one way to help promote fluid consumption, before, during, or after exercise. Fluid palatability is influenced by several factors including temperature, sodium content and flavoring. The preferred water temperature is often between 15 and 21 °C, but this and flavor preference varies greatly between individuals and cultures [07249].

**Fluid replacement during exercise**

The goal of drinking during exercise is to prevent excessive dehydration (92 % BW loss from water deficit) and excessive changes in electrolyte balance to avert compromised exercise performance. The amount and rate of fluid replacement depends upon the individual sweating rate, exercise duration, and opportunities to drink. Individuals should periodically drink (as opportunities allow) during exercise, if it is expected they will become excessively dehydrated. Care should be taken in determining fluid replacement rates, particularly in
prolonged exercise lasting greater than 3 h. The longer the exercise duration the greater the cumulative effects of slight mismatches between fluid needs and replacement, which can excessive dehydration or dilutional hyponatremia. It is difficult to recommend a specific fluid and electrolyte replacement schedule because of different exercise tasks (metabolic requirements, duration, clothing, equipment), weather conditions, and other factors (e.g. genetic predisposition, heat acclimatization and training status) influencing a person’s sweating rate and sweat electrolyte concentrations. It is recommended that individuals should monitor body weight changes during training/competition sessions to estimate their sweat lost during a particular exercise task with respect to the weather conditions. This allows customized fluid replacement programs to be developed for each person’s particular needs; however, this may not always be practical. Fluid and electrolyte replacement strategies will be vastly different for a large football player in early season summer practice when contrasted with a petite marathoner running at a 6-h pace. A possible starting point suggested for marathon runners (who are euhydrated at the start) is they drink ad libitum from 0.4 to 0.8 L/h, with the higher rates for faster, heavier individuals competing in warm environments and the lower rates for the slower, lighter persons competing in cooler environments. For smaller runners, drinking at 0.8 L/h resulted in over-consumption (weight gain, light shaded areas), and for larger runners, drinking at 0.4 L/h resulted in excessive dehydration (3 % body weight loss). The composition of the consumed fluids can be important. It has been recommended that these types of fluid replacement beverages might contain about 20-30 meq/L sodium (chloride as the anion), about 2-5 meq/L potassium and 5-10 percent carbohydrate. The need for these different components (carbohydrate and electrolytes) will depend on the specific exercise task (e.g. intensity and duration) and weather conditions. The sodium and potassium are to help replace sweat electrolyte losses, while sodium also helps to stimulate thirst, and carbohydrate provides energy. These components also can be consumed by nonfluid sources such as gels, energy bars, and other foods. Carbohydrate consumption at a rate of 30-60 g/h has been demonstrated to maintain blood glucose levels and sustain exercise performance. The greatest rates of carbohydrate delivery are achieved with a mixture of sugars (e.g. glucose, sucrose, fructose, maltodextrine). If both fluid replacement and carbohydrate delivery are going to be met with a single beverage, the carbohydrate concentration should not exceed 8 percent, or even be slightly less, as highly concentrated carbohydrate beverages reduce gastric emptying. Finally, caffeine consumption might help to sustain exercise performance and likely will not alter hydration status during exercise [07249].

Drinking pattern during different parts of activity

Energy drinks are intended for people who work hard, both physically and mentally, particularly young people engaged in an active lifestyle. To assess the intake of energy drinks in a student group, during examinations and throughout an academic year a survey was performed on 92 students attending the Faculty of Human Nutrition and Consumer Sciences and those from the Faculty of Physical Education in Warsaw. Students consumed many more energy drinks during examinations (1424 ± 1577 mL/week) than during the rest of the academic year (441 ± 579 mL/week). About 30 percent more subjects from UPE drank such drinks, throughout both examinations and the academic year, compared to those from WULS. On average, most students drunk less than one can per day. During exams, only 49 percent students consumed an average of less than 125 ml of energy drinks per day, whereas this rose to 84 percent during the academic year. The most popular brands were Tiger, Red Bull and Burn. It is important that due care and attention is exercised in consuming high amounts of energy drinks as they contain bioactive substances, including caffeine, inositol, taurine, glucuronolactone and vitamins of the group B. These all have specific effects on the body and can be a cause for concern if their intake is high [13601].

1630
Different commercial available sport drinks

To determine the effectiveness of 3 commonly used beverages in restoring fluid and electrolyte balance, 8 volunteers dehydrated by 1.9 ± 0.2 percent of body mass by intermittent exercise in the heat, then ingested a carbohydrate-electrolyte solution (Gatorade), carbonated water/apple-juice mixture (Apfelschorle), and San Benedetto mineral water in a volume equal to 150 percent body-mass loss. These drinks are all are perceived to be effective rehydration solutions, and their effectiveness was compared with the rehydration effectiveness of Evian mineral water, which is not perceived in this way by athletes. Four hours after rehydration, the subjects were in a significantly lower hydration status than the pretrial situation on trials with two but were in the same hydration status as before the dehydrating exercise on Gatorade. Sodium balance was negative on all trials throughout the study; only with Apfelschorle did subjects remain in positive potassium balance. In this scenario, recovery of fluid balance can only be achieved when significant, albeit insufficient, quantities of sodium are ingested after exercise. There is a limited range of commercially available products that have a composition sufficient to achieve this, even though the public thinks that some of the traditional drinks are effective for this purpose [07254].

The purpose of one study was to identify and to compare the effects of ingesting liquids during a 16-km military march under moderate environmental conditions. Twenty-six volunteer male subjects were randomly divided into two groups. Group GW received water (n=12), and group GP received an electrolytic carbohydrate solution (n=14). Blood and urine samples were obtained immediately before and after the march. No significant differences between the drinks were found for any of the measured variables. However, important results (p < 0.05) were observed by comparing variables before and after exercise. The variables included sodium, hematocrit, red blood cell, hemoglobin, and lactic acid levels and body weight (group GW) and sodium, potassium, hematocrit, red blood cell, hemoglobin, and lactic acid levels (group GP). Under the environmental conditions and hydration procedures applied, the results of this study showed similarities in the behavior of the variables, regardless of the kind of beverage consumed [07255].

Sports drinks versus water

Sports drinks are often used before, during and after tennis tournaments, but their ability to influence physiological and psychological variables and the characteristics of tennis match play remains uncertain. The objective of this study was to evaluate the impact of ingesting specially formulated pre-exercise, endurance and recovery sports drinks on glycaemia and performance indices during a simulated tennis tournament. Eight well-trained male tennis players performed two 3-match round-robin tennis tournaments while ingesting sports drinks (SPDs) or placebos (PLAs) before, during and after each match (crossover study design). Before the first tournament, match and drink order were randomized (SPDs or PLAs first) and players were placed under controlled nutritional and hydration conditions. Glycaemia, heart rate response, rate of perceived exertion and notational/match analysis were assessed during each match. SPDs maintained higher glycaemia levels during match 2 and 3 of the tennis tournament compared with PLAs. Moreover, higher mean heart rates and stroke frequencies concomitantly with lower rates of perceived exertion were recorded throughout the duration of the tournament, when players used the SPDs. During a 3-match tennis tournament, SPDs allow higher stroke frequency during play, with decreased rates of perceived exertion [13598].

Effect of osmolality

It was investigated the effects of two carbohydrate-based sports drinks on fluid intake and immunoendocrine responses to cycling. Six well-trained male cyclists completed trials on three separate days that involved cycling at 60 percent VO_{2peak} for 90 min in hot conditions.
During each trial, the subjects consumed ad libitum an isotonic sports drink (osmolality 317 mOsm/kg), a hypotonic sports drink (osmolality 193 mOsm/kg) or plain water. The cyclists consumed significantly more of the isotonic drink and hypotonic drink compared with water. Compared with water, body mass decreased significantly less after consuming the hypotonic drink but not the isotonic drink. Blood glucose concentration was significantly higher at the end of the isotonic and hypotonic drink trials compared with the water trial. Neutrophil count and the plasma concentrations of catecholamines, interleukin 6 (IL-6), myeloperoxidase, calprotectin and myoglobin increased significantly during all three trials. IL-6 and calprotectin were significantly lower following the hypotonic drink trial compared with the water trial. In conclusion, hypotonic sports drinks are appealing for athletes to drink during exercise, and may help to offset fluid losses and attenuate some inflammatory responses to exercise [13600].

**Dehydration despite favorable conditions for fluid intake**

Study investigated the relationship between runners' perceptions of fluid needs and drinking behavior under conditions of compensable heat stress (ambient temperature 21 degrees C; relative humidity 77 %). Eighteen experienced runners (15 men, 41 years, and 3 women, 42 years) were given ad libitum access to a sports drink (6 % carbohydrate-electrolyte solution) at miles 2, 4, 6, and 8. After the run (76 min), subjects completed questionnaires that required them to estimate their individual fluid intake and sweat loss. Dehydration averaged 1.9 ± 0.8 percent of initial body weight (a mean sweat loss of 22 ± 5 mL/kg and h). Subjects replaced only 31 percent of sweat loss and underestimated their sweat loss by 43 percent. Subjects' self-estimations of fluid intake were not significantly different from actual fluid intake and were significantly correlated. The data indicate that even under favorable conditions, experienced runners voluntarily dehydrate possibly because they are unable to accurately estimate sweat loss and consequently cannot subjectively judge how much fluid to ingest to prevent dehydration. This conclusion suggests that runners should not depend on self-assessment to maintain adequate hydration, underscores the need for runners to enhance their ability to self-assess sweat losses, and suggests that a predetermined regimen of fluid ingestion might be necessary if they wish to maintain more optimal hydration [07256].

**Gastric emptying**

This study examined gastric emptying, core temperature, and sprint performance during prolonged intermittent shuttle running in 30 degrees C when ingesting a carbohydrate-electrolyte solution (CES) or flavored water (FW). Nine male soccer players performed 60 min of shuttle running, ingesting fluid before exercise and every 15 min during exercise. Gastric emptying was measured using a double-sampling aspiration technique, and intestinal temperature was monitored via ingested capsules. There were no differences between trials in the total fluid volume emptied from the stomach during each exercise period (P = 0.054). The volume emptied every 15 min was 244 ± 67 mL in the CES trial and 273 ± 66 mL in the FW trial. Intestinal temperature was significantly higher during exercise in the CES trial, and cumulative sprint time was shorter. Sprint performance was enhanced by the ingestion of a CES, which resulted in elevated core temperatures, and the rate of gastric emptying remained similar between solutions [07257].

**Fluid restriction increases GI permeability**

The purpose of one study was to determine gastrointestinal (GI) permeability during prolonged treadmill running (60 min at 70 % VO2max) with and without fluid intake (3 ml/kg body mass/10 min). Twenty runners (11 males, 9 females; age 22 years) completed four
experiments: 1) rest, 2) running with no fluid (NF), 3) running with ingestion of a 4% glucose solution (GLU), and 4) running with ingestion of a water placebo (PLA). To determine GI permeability, subjects also drank a solution containing 5 g sucrose (S), 5 g lactulose (L), and 2 g rhamnose (R) immediately prior to each trial. Gastroduodenal permeability was determined by urinary S excretion, while small intestinal permeability was determined by the L/R excretion ratio. Percent body mass loss (i.e. dehydration) was negligible during rest, GLU and PLA, while NF resulted in a 1.5 percent loss of body mass. Gastroduodenal and intestinal permeability were significantly increased in NF compared to rest. There were no other differences in GI permeability. These results indicate that fluid restriction during 1 h of steady-state running increases GI permeability above resting levels [07264].

Effects of hypohydration on performance

Although many studies have attempted to examine the effect of hypohydration on strength, power, and high-intensity endurance, few have successfully isolated changes in total body water from other variables that alter performance (e.g., increased core temperature), and none have documented the influence of hypohydration on an isotonic, multiset, multirepetition exercise bout typical of resistance exercise training. Further, no investigations document the effect of hypohydration on the ability of the central nervous system to stimulate the musculature, despite numerous scientists suggesting this possibility. The purposes of one study were to examine the isolated effect of hydration state on strength, power, and the performance of acute resistance exercise, and central activation ratio (CAR). Seven healthy resistance-trained males (age 23 years) completed three resistance exercise bouts in different hydration states: euhydrated (EU), hypohydrated by approximately 2.5 percent body mass (HY25), and hypohydrated by approximately 5.0 percent body mass (HY50). Investigators manipulated hydration status via exercise-heat stress and controlled fluid intake 1 d preceding testing. Body mass decreased 2.4 ± 0.4 and 4.8 ± 0.4 percent during HY25 and HY50, respectively. No significant differences existed among trials in vertical jump height, peak lower-body power (assessed via jump squat), or peak lower-body force (assessed via isometric back squat). CAR tended to decrease as hypohydration increased. When evaluated as a function of the percentage of total work completed during a six-set back squat protocol, hypohydration significantly decreased resistance exercise performance during sets 2-3 and 2-5 for HY25 and HY50, respectively. These data indicate that hypohydration attenuates resistance exercise performance; the role of central drive as the causative mechanism driving these responses merits further research [07258].

Drinking pattern during sports

Although expert groups have developed guidelines for fluid intake during sports, there is debate about their real-world application. It was reviewed the literature on self-selected hydration strategies during sporting competitions to determine what is apparently practical and valued by athletes. It was found few studies of drinking practices involving elite or highly competitive athletes, even in popular sports. The available literature revealed wide variability in fluid intake and sweat losses across and within different events with varied strategies to allow fluid intake. Typical drinking practices appear to limit body mass (BM) losses to about 2 percent in non-elite competitors. There are events, however, in which mean losses are greater, particularly among elite competitors and in hot weather, and evidence that individual participants fail to meet current guidelines by gaining BM or losing >2 percent BM over the competition activity. Substantial (>5%) BM loss is noted in the few studies of elite competitors in endurance and ultra-endurance events; while this may be consistent with winning outcomes, such observations cannot judge whether performance was optimal for that individual. A complex array of factors influence opportunities to drink during continuous
competitive activities, many of which are outside the athlete's control: these include event rules and tactics, regulated availability of fluid, need to maintain optimal technique or speed, and gastrointestinal comfort. Therefore, it is questionable, particularly for top competitors, whether drinking can be truly ad libitum (defined as "whenever and in whatever volumes chosen by the athlete"). While there are variable relationships between fluid intake, fluid balance across races, and finishing times, in many situations it appears that top athletes take calculated risks in emphasizing the costs of drinking against the benefits. However, some non-elite competitors may need to be mindful of the disadvantages of drinking beyond requirements during long events. Across the sparse literature on competition hydration practices in other sports, there are examples of planned and/or ad hoc opportunities to consume fluid, where enhanced access to drinks may allow situations at least close to ad libitum drinking. However, this situation is not universal and, again, the complex array of factors that influence the opportunity to drink during an event is also often beyond the athletes' control. Additionally, some competition formats result in athletes commencing the event with a body fluid deficit because of their failure to rehydrate from a previous bout of training/competition or weight-making strategies. Finally, since fluids consumed during exercise may also be a source of other ingredients (e.g., carbohydrate, electrolytes, or caffeine) or characteristics (e.g., temperature) that can increase palatability or performance, there may be both desirable volumes and patterns of intake that are independent of hydration concerns or thirst, as well as benefits from undertaking a "paced" fluid plan. Further studies of real-life hydration practices in sports including information on motives for drinking or not, along with intervention studies that simulate the actual nature of real-life sport, are needed before conclusions can be made about ideal drinking strategies for sports. Different interpretations may be needed for elite competitors and recreational participants [13594].

**Hydration in marathon**

There is a large and growing body of scientific evidence that documents the benefits of ingesting salt and glucose (carbohydrates) during prolonged exercise. Those benefits include maintenance of cardiovascular function, enhanced carbohydrate oxidation, blunted decline in plasma sodium concentration and improved performance. The consumption of approximately 1 g of carbohydrate per kilogram of bodyweight per hour appears sufficient to improve performance in prolonged exercise. Research also indicates that approximately 450 mg of sodium per hour is the minimum amount required to maintain plasma volume and slow the decline in plasma sodium concentration that can accompany prolonged exercise in some runners. Adequate carbohydrate and electrolyte intake can be achieved by consuming a well formulated sports drink at regular intervals during exercise, in volumes designed to minimise dehydration. For marathon runners, this could range from approximately 400 mL to >1.5 L per hour, depending upon individual sweating rates [07259].

**Water and carbohydrates**

One study investigated water absorption and blood volume changes after drinking water and 3 percent and 6 percent carbohydrate drinks. Nine healthy male volunteers completed this crossover study after giving informed consent to participate. On three separate occasions they consumed 500 ml of Evian water (W), a 3 percent carbohydrate drink (3 % CHO) or a 6 percent carbohydrate drink (6 % CHO) to which had been added 10.00 ± 0.01 g of deuterium oxide. Blood samples were collected 10 and 5 min before drink ingestion and at 2, 5, 10, 15, 20, 30, 45 and 60 min after drink ingestion. In addition, subjective feeling questionnaires were completed at intervals throughout and subjects urinated before and after the study period. Blood samples were analysed for hematocrit and haemoglobin, glucose and deuterium concentrations. There was a significant increase in blood glucose concentration...
after consumption of both the 3 percent CHO and 6 percent CHO drinks, with the increase being greater after consumption of the 6 percent CHO drink. Both the 3 percent CHO and 6 percent CHO drinks promoted a significant blood and plasma volume expansion after their consumption whereas there was no change in blood and plasma volumes when water was consumed. Blood deuterium enrichment increased after consumption of all three drinks, but there was no significant difference between drinks. The results suggest that when a significant expansion of blood volume is desired, this can be achieved by consumption of the 3 percent CHO or 6 percent CHO drink rather than with plain water consumption. This is likely to be due to the sodium and carbohydrate content of these drinks [10508].

Carbohydrate (CHO) ingestion results in positive effects on exercise capacity, repeated sprint speed and sport-specific skill performance during intermittent exercise. One study evaluated the effects of lower concentrations of CHO on performance during intermittent exercise. Ten healthy males completed four 15-min periods of intermittent running, consisting of maximal sprinting interspersed with periods of running and walking. This was followed by the multistage shuttle-running fitness test to volitional exhaustion. Subjects consumed 3 ml/kg body mass of plain water, a 3 percent CHO solution or a 6 percent CHO solution before exercise and a further 1.5 ml/kg body mass of the appropriate drink after each 15-min block. A trial with no fluid ingestion was also completed. Sprint time, heart rate, core temperature, perceived exertion and thermal stress were recorded at regular intervals. Cognitive function was assessed using a computer-based test battery. Time to exhaustion in the multistage shuttle-running fitness test was 9.9 ± 2.0 min, 10.2 ± 2.0 min, 10.9 ± 1.9 min and 11.2 ± 1.8 min in the no fluid, water, 3 percent and 6 percent CHO trials, respectively. Core temperature reached 38.9 ± 0.4°C during the no fluid trial, 38.5 ± 0.4°C in the water trial and 38.6 ± 0.3°C at the end of the CHO trials. There was no change in response times to a visual search task in the no fluid and water trials, but CHO ingestion improved response times after exercise (3 % CHO; 6 % CHO). These data suggest that drinks containing lower CHO concentrations can produce improvements in exercise capacity and motor response times similar to those observed with the ingestion of the amounts of CHO found in many sports drinks. The study was carried out in relation to the product Powerade and was funded in part by The Coca-Cola Company [10509].

**Water and electrolytes**

The purpose of one study was to summarize water, carbohydrate (CHO), and electrolyte absorption from carbohydrate-electrolyte (CHO-E) solutions based on all of the triple-lumen-perfusion studies in humans since the early 1960s. The current statistical analysis included 30 reports from which were obtained information on water absorption, CHO absorption, total solute absorption, CHO concentration, CHO type, osmolality, sodium concentration, and sodium absorption in the different gut segments during exercise and at rest. Mean differences were assessed using independent-samples t tests. Exploratory multiple-regression analyses were conducted to create prediction models for intestinal water absorption. The factors influencing water and solute absorption are carefully evaluated and extensively discussed. The authors suggest that in the human proximal small intestine, water absorption is related to both total solute and CHO absorption; osmolality exerts various impacts on water absorption in the different segments; the multiple types of CHO in the ingested CHO-E solutions play a critical role in stimulating CHO, sodium, total solute, and water absorption; CHO concentration is negatively related to water absorption; and exercise may result in greater water absorption than rest. A potential regression model for predicting water absorption is also proposed for future research and practical application. In conclusion, water absorption in the human small intestine is influenced by osmolality, solute absorption, and the anatomical structures of gut segments. Multiple types of CHO in a CHO-E solution
facilitate water absorption by stimulating CHO and solute absorption and lowering osmolality in the intestinal lumen [10433].

The aim of one study was to determine the effects of postexercise ingestion of different-molecular-weight glucose polymer solutions on subsequent high-intensity interval-running capacity. In a repeated-measures design, 6 men ran for 60 min in the morning at 70 percent VO$_2$max. Immediately post- and at 1 and 2 hr postexercise, participants consumed a 15 percent low-molecular-weight (LMW) or high-molecular-weight (HMW) carbohydrate solution, at a rate of 1.2 g of carbohydrate/kg body mass, or an equivalent volume of flavored water. After recovery, participants performed repeated 1-min intervals at 90 percent VO$_2$max interspersed with 1 min active recovery (walking) until volitional exhaustion. Throughout the 3-hr recovery period, plasma glucose concentrations were significantly higher during the HMW and LMW conditions than with flavored water, although there was no difference between HMW and LMW conditions. Exercise capacity was 13 and 11 min longer with HMW and LMW solutions, respectively, than with flavored water (30 ± 9 min). There was no substantial difference in exercise capacity between LMW and HMW solutions. Although this magnitude of difference is most likely trivial in nature, the uncertainty allows for a possible small substantial enhancement of physiological significance, and further research is required to clarify the true nature of the effect [10510].

Solutions containing multiple carbohydrates utilizing different intestinal transporters (glucose and fructose) show enhanced absorption, oxidation, and performance compared with single-carbohydrate solutions, but the impact of the ratio of these carbohydrates on outcomes is unknown. In a randomized double-blind crossover, 10 cyclists rode 150 min at 50 percent peak power, then performed an incremental test to exhaustion, while ingesting artificially sweetened water or one of three carbohydrate-salt solutions comprising fructose and maltodextrin in the respective following concentrations: 4.5 and 9 percent (0.5-Ratio), 6 and 7.5 percent (0.8-Ratio), and 7.5 and 6 percent (1.25-Ratio). The carbohydrates were ingested at 1.8 g/min and naturally $^{13}$C-enriched to permit evaluation of oxidation rate by mass spectrometry and indirect calorimetry. Mean exogenous carbohydrate oxidation rates were 1.04, 1.14, and 1.05 g/min (coefficient of variation 20 %) in 0.5-, 0.8-, and 1.25-Ratios, respectively, representing likely small increases in 0.8-Ratio of 11 percent and 10 percent relative to 0.5- and 1.25-Ratios, respectively. Comparisons of fat and total and endogenous carbohydrate oxidation rates between solutions were unclear. Relative to 0.5-Ratio, there were moderate improvements to peak power with 0.8- and 1.25-Ratio but unclear with water Increases in stomach fullness, abdominal cramping, and nausea were lowest with the 0.8-followed by the 1.25-Ratio solution. At high carbohydrate-ingestion rate, greater benefits to endurance performance may result from ingestion of 0.8- to 1.25-Ratio fructose-maltodextrin solutions. Small perceptible improvements in gut comfort favor the 0.8-Ratio and provide a clearer suggestion of mechanism than the relationship with exogenous carbohydrate oxidation [10511].

Alcoholic beverage for treatment of dehydration

Beer for water replacement
To investigate the effect of manipulating the alcohol and sodium content of beer on fluid restoration following exercise 7 male volunteers exercised on a cycle ergometer until 1.96 ± 0.25 percent body mass (mean ± SD) was lost. Participants where then randomly allocated a different beer to consume on four separate occasions. Drinks included a low alcohol beer (2.3 % ABV) [LightBeer], a low alcohol beer with 25 mmol/L of added sodium [LightBeer+25], a full strength beer (4.8 % ABV) [Beer] or a full strength beer with 25 mmol/L of added sodium [Beer+25]. Volumes consumed were equivalent to 150 percent of body mass loss.
during exercise and were consumed over a 1h period. Body mass and urine samples were obtained before and hourly for 4h after beverage consumption. Significantly enhanced net fluid balance was achieved following the LightBeer+25 trial (-1.02 ± 0.35 kg) compared to the Beer (-1.59 ± 0.32 kg) and Beer+25 (-1.64 ± 0.28 kg) treatments. Accumulated urine output was significantly lower in the LightBeer+25 trial (1477 ± 485 mL) compared to the Beer+25 (2101 ± 482 mL) and Beer (2175 ± 372 mL) trials. It was concluded that a low alcohol beer with added sodium offers a potential compromise between a beverage with high social acceptance and one which avoids the exacerbated fluid losses observed when consuming full strength beer [13603].

**Dehydration plus alcohol**

One study investigated the impact of mild-moderate dehydration on alcohol-induced deteriorations in cognitive functions. Sixteen healthy males participated in a single-blind, placebo-controlled cross-over design study involving 4 experimental trials (separated by ≥7 d). In each trial, participants were dehydrated by 2.5 percent body mass through exercise. After 1 h recovery in a thermo-neutral environment (22 ± 2°C, 60-70 % relative humidity) 4 tasks from the Cambridge Neuropsychological Test Automated Battery (CANTAB) were administered to the participants (test 1). In two of the trials, participants were provided with water equivalent to either 50 or 150 percent body mass loss and given salt (NaCl) capsules (50 mmol/L). A set volume of alcohol or placebo was then consumed in each trial, incorporating the conditions: dehydration-placebo (DP), dehydration-alcohol (DA), partial rehydration-alcohol (PA), and full rehydration-alcohol (FA). The same 4 CANTAB tasks were then re-administered (test 2). Subjective ratings of mood and estimates of alcohol intoxication and driving impairment were also recorded in each trial. Alcohol consumption caused deterioration on 3 of the 4 CANTAB measures (e.g. choice reaction time, executive function and response inhibition). This reduction in performance was exacerbated when participants were dehydrated compared to trials where full rehydration occurred. Subjective ratings of impairment and intoxication were not significantly different between any of the trials where alcohol was consumed; however ratings for alcohol trials were significantly higher than in the placebo trial. These findings suggest that rehydration after exercise that causes fluid loss can attenuate alcohol-related deterioration of cognitive functions. This may pose implications for post match fluid replacement if a moderate amount of alcohol is also consumed [13604].

**Oral salt supplementation**

During long-distance exercise, it can become difficult to continue to provide good nutrition support. The aim was to evaluate the potential of sausage “Saucisport” (water: 71 %, proteins: 17 %, carbohydrates: 3 %, fat: 6 %, other: 3 %) to maintain metabolic balance and performance during long-distance exercise. Ten male long-distance runners (mean ± SD: age 34 ± 14 years, height 177 ± 6 cm, weight 71 ± 6 kg, VO2max 56 ± 8 ml/min/kg) performed two sessions on treadmill (4 h at 65-70 % VO2 max). These two exercises were separated by 10 days. Subjects were randomized to receive “standard” or oral salt supplementation. During rectangular test, different parameters were recorded every hour (T0, T1, T2, T3, T4) and 1 h postexercise (T5): ventilation, respiratory quotient, oxygen consumption, heart rate, insulin, glucose and free fatty acids. Analysis of respiratory and cardiovascular parameters did not show any difference between salt and sugar supplementation, although in general the values observed in sweet supplementation are slightly higher than those in salt supplementation. Evolution of insulin during the stress test was comparable in both types of supplementation. Blood glucose values were higher after 3 h and 4 h of effort under “standard” supplementation, but were not different at the end of the exercise test. The study
shows that the sausage Saucisport® does not alter the physiological parameters, metabolic balance or performance during long-distance exercise [11520].

Exercise-associated hyponatremia

Exercise-associated hyponatremia has been described after sustained physical exertion during marathons, triathlons, and other endurance athletic events. As these events have become more popular, the incidence of serious hyponatremia has increased and associated fatalities have occurred. The pathogenesis of this condition remains incompletely understood but largely depends on excessive water intake. Furthermore, hormonal (especially abnormalities in arginine vasopressin secretion) and renal abnormalities in water handling that predispose individuals to the development of severe, life-threatening hyponatremia may be present. Studies have shown that endurance athletes not uncommonly develop hyponatremia at the end of the race, usually in the absence of clear central nervous system symptoms. For example, in the 2002 Boston Marathon, it was found that 13 percent of 488 runners studied had hyponatremia (defined as a serum sodium concentration of 135 mmol/L or less) and 0.6 percent had critical hyponatremia (serum sodium concentration of 120 mmol/L or less). In another study it was investigated 330 athletes who finished an ultramarathon race. In this study, 58 (18 %) were hyponatremic (defined as a serum sodium <135 mmol/L) and 11 had severe hyponatremia (serum sodium <130 mmol/L). Studies of other endurance events have reported the incidence of hyponatremia to be up to 29 percent. These incidence rates may be overestimations as a result of sampling biases. The majority of these athletes are asymptomatic or mildly symptomatic (nausea, lethargy). However, severe manifestations such as cerebral edema, noncardiogenic pulmonary edema, and death can occur. There have been at least 8 reported deaths from EAH (exercise-associated hyponatremia). Many of these reports relate to a series of fatalities in the military between 1989 and 1996. During this period, military recruits were encouraged to ingest 1.8 L of fluid for every hour they were exposed to temperatures above 30°C. Several risk factors have been linked with the development of EAH. The major risk factor seems to be overhydration or excessive fluid consumption during activity. This first was suggested by Noakes et al. in their original publication in 1985 and confirmed in this group’s later studies. The chronological history of the incidence of EAH also points to the primary role of overhydration in the pathogenesis. Before 1981, athletes were encouraged to drink heavily during exertion to avoid dehydration. With the description of EAH in South Africa and New Zealand in 1985, new fluid consumption guidelines that restricted overzealous fluid intake for endurance events in these countries were promoted. Concomitant with these recommendations, the incidence of EAH fell in both of these regions. Similar observations were made after the US military revised its guidelines for fluid consumption during training activities after the incidence of EAH increased. With an upper limit of fluid consumption set at 1.0 to 1.5 L/h, the incidence of EAH in the US military fell. In a study of runners in the Boston Marathon, it was found significant correlations between fluid intake and the incidence of hyponatremia. Specifically, a fluid intake of >3 L, a postrace weight greater than prerace weight, self-reported water loading (increased fluid consumption above baseline in preparation for the marathon), and self-reported fluid intake during the race all were found to be significant predictors for the development of hyponatremia. Substantial weight gain during the duration of the activity seemed to be the most important predictor of hyponatremia and correlated well with increased fluid intake. Also in another study it was found correlations between intrarace weight gain and hyponatremia: 73 percent of patients who were found to be severely hyponatremic had either gained or maintained weight during the race. In a large study it was investigated the changes in serum sodium concentration associated with changes in body weight in 2135 endurance athletes. The mean ± SD serum sodium was 136 ± 6 mmol/L for
athletes who gained weight during the race, 141 ± 4 mmol/L for those with minimal weight gain, and 141 ± 4 mmol/L for those who lost weight during the race. The authors estimated that athletes who gained >4 percent body weight during exercise had a 45 percent probability of developing hyponatremia. Importantly, 70 percent of individuals who gained weight during exercise did not develop hyponatremia, pointing to other important factors in the pathogenesis. It has not been possible to find a correlation in the type of fluids consumed (water versus electrolyte-containing solutions) and the subsequent development of hyponatremia. Other studies also have shown that the consumption of a carbohydrate/electrolyte-containing sports drink does not protect against the development of hyponatremia. This likely reflects the relative hypotonicity of most of the commercial sports drinks in which the sodium concentration typically is 18 mmol/L. Gender likely plays a role in the risk for development of EAH, with female athletes more likely than male athletes to develop hyponatremia during endurance events. Of 26 cases of EAH reported after the San Diego Marathon, 23 occurred in women. Hyponatremia was three times more common in women than in men in the 1997 New Zealand Ironman triathlon. It was also found that hyponatremia developed more commonly in women in the Boston Marathon. However, in that study, when these results were corrected for body mass index, racing time, and weight change, the difference did not reach statistical significance, suggesting that body size and duration of exercise may explain the gender differences. Furthermore, the incidence of hyponatremia in US military recruits reflects the gender distribution of this cohort and is not skewed to women. Some investigators also have suggested that women adhere more stringently to hydration recommendations during exercise and therefore consume more fluids. The finding of a gender association for the risk for symptomatic hyponatremia also has been seen in the postoperative state. The development of hyponatremia also has been correlated with the number of marathons run, the training pace, and the race duration. Those who have run fewer marathons (less experienced runners), have slower training paces, and have longer race times (especially >4 h) each were shown independently to have a significantly higher risk for developing hyponatremia. Longer race times likely correlate with increased water consumption and increased sodium losses. For example, participants who developed hyponatremia in the 1998 and 1999 San Diego Marathons had an average finishing time of 5 h and 38 min, and many of these individuals admitted to drinking as much fluid as possible during and after the event. A low body mass index also was shown to be a significant risk factor, perhaps as a result of the ingestion of larger amounts of fluid in proportion to size and total body water (TBW) [07260].

It has been suggested recently that financial links between manufacturers of sports drinks and professional Sports Science organisations in North America have suppressed information on the existence and ways of preventing an epidemic of exercise-associated hyponatraemia (EAH). One article reviewed evidence for the prevalence of both biochemical and clinical hyponatraemia. It concludes that a limited number of cases of EAH occur after ultra-long distance events, particularly when performed under cold and wet conditions, and that some eight deaths have been associated with EAH since 1985. However, this information has been widely reported, both in North America and in other parts of the world. Claims of an “epidemic” seem unwarranted, and there is no solid evidence supporting the claim that information has been suppressed because of ties between sports scientists and sports drink manufacturers [11521].

_Use of NSAID_

Medications also may play a significant role in the hyponatremia that is found in endurance athletes, but this largely is unproved. Nonsteroidal anti-inflammatory drug (NSAID) use is common among marathon runners, being used in 50 to 60 percent of men and women,
respectively. NSAID are known to potentiate the effects of arginine vasopressin (AVP) by inhibiting renal prostaglandin synthesis via the COX-2 isoform of cyclo-oxygenase. Furthermore, NSAID decrease the GFR when given to those with effective volume depletion, such as exercising endurance athletes. These effects may impair the urine-diluting capacity of the kidney. Despite these theoretical considerations, it has not been possible to associate the use of NSAID with the development of hyponatremia in the runners who were studied in the 2002 Boston Marathon. Other studies also have not been able to ascribe conclusively to NSAID use the development of hyponatremia, although several of these studies were underpowered to do so. However, one study in 330 triathletes demonstrated a significant association of NSAID use and the development of hyponatremia. In this study, the incidence of NSAID use in athletes was 30 percent, and NSAID use was highly associated with the development of hyponatremia, as well as higher plasma potassium and creatinine levels. Several other, smaller studies and case reports also have suggested a potentiating role for NSAID use. Therefore, the role of NSAID in the development of EAH remains controversial but in some runners likely is a potentiating factor. Whether other medications, such as selective serotonin reuptake inhibitors or thiazide diuretics, that are associated with hyponatremia in nonathletes can potentiate the development of EAH is not known. It is important to recognize that these risk factors do not suggest causation or even an independent association with the development of hyponatremia. However, they do offer important clues to the pathogenesis of the condition [07260].

Pathophysiology

Normally, renal and hormonal systems maintain the plasma osmolality within tight limits with variability of no more than 1 to 2 percent. These tight limits reflect the physiologic importance of osmolality regulation on cell volume and function. The development of hyponatremia (usually, in the setting of hypo-osmolality) reflects either defects in these hormonal and renal control mechanisms or water ingestion that overwhelms them. In the specific instance of EAH, defects in renal diluting mechanisms, hormonal control of water excretion, excessive sodium losses, and excessive water intake all contribute to the development of hypo-osmolality. Current evidence strongly supports that EAH is, in large part, dilutional in nature. In the majority of athletes who develop hyponatremia, there is an increase in TBW relative to that of total body exchangeable sodium. This seems to occur by the ingestion of hypotonic fluids (water or sports drinks) in excess of sweat, urine, and insensible (mainly respiratory and gastrointestinal) losses. In a seminal study, it was described a linear relationship with a negative slope between the serum sodium after racing and the degree of weight change in 2135 athletes. The primary cause of this weight gain during exercise must be the consumption of fluids during exercise. This consumption of fluids during exercise can be driven by thirst or through conditioned behavior. Some have hypothesized that in some athletes, the thirst drive may be excessive, but, more likely, the excessive fluid intake during exercise reflects conditioned behavior that is based on recommendations to drink fluid during exercise to avoid dehydration as well as the wide availability of fluids along the race course. This hypothesis is supported by data, that the incidence of EAH was rare or nonexistent before 1981, when recommendations for fluid intake during exercise were conservative. EAH was seen only after recommendations for more aggressive hydration were promulgated. Occasionally, some athletes may drink up to 3 L/h in an attempt to produce dilute urine to escape detection of banned drugs in the urine. Finally, some athletes may drink large volumes of fluid in the days leading up to a marathon in an attempt to ward off dehydration. This was the case for one female runner who drank 10 L of fluid on the evening before a marathon and then experienced postrace hyponatremia. However, excessive fluid consumption is not the sole explanation for the development of EAH. In one study hyponatremia did not develop in 70 percent of the athletes who overconsumed fluids and had an increase in TBW. This indicates that other important factors must be operational in the
pathogenesis of EAH. The importance of other factors also is highlighted by the fact that the maximum water excretory capacity of the kidneys is between 750 and 1500 mL/h. In combination with fluid losses from sweating and insensible losses (which may be in excess of 500 mL/h), most athletes should be able to consume fluids in excess of 1500 mL/h before retaining weight and increasing TBW. This amount of fluid consumption is at the upper limit of what most athletes would consume during an activity. Therefore, either defects in renal water excretion and/or significant sodium losses or failure to mobilize exchangeable sodium stores may occur in athletes who develop EAH. Furthermore, some athletes develop hyponatremia without appreciable gains in total body weight. These athletes may have significant sodium losses or also may have gained net body free water as a result of the metabolism of glycogen and triglycerides and not as a result of ingestion. However, the contribution of fuel metabolism or metabolic water production to TBW likely is small. During treadmill running at 74 percent of maximal oxygen consumption, metabolic water production averages 144 g/h (in contrast, sweat loss during this time was 1200 g/h). There is a possibility that water that is stored with glycogen can be released with glycogen breakdown. This may be an important component in the cause of hyponatremia that occurs without weight gain because each kilogram of glycogen can contain upwards of 3 kg of associated water.

Arginine vasopressin (AVP)

Data on the levels of AVP during exercise are conflicting. Unfortunately, systematic measurement of AVP levels or free water clearances in athletes who present with hyponatremia has not been done except in isolated cases. There are several potential pathways for stimulation of AVP release in exercising athletes. Controlled laboratory studies have demonstrated that as exercise intensity increases above 60 percent of maximal oxygen consumption, there are concomitant increases in AVP levels. Nonspecific stresses that are experienced by athletes and caused by factors such as pain, emotion, or physical exercise have been thought to cause nonosmotic release of AVP. However, it is difficult to determine whether this effect is mediated by a specific pathway or is due to a secondary stimulus, such as hypotension or nausea, that may occur in exercising athletes. AVP production also may be stimulated appropriately in athletes who develop volume depletion. However, the level of volume depletion that is required to stimulate AVP production in the absence of hyperosmolality is in excess of 7 to 8 percent of body volume. These levels of volume depletion typically are not seen in athletes (e.g., in the 2001 South African Ironman Triathlon, only 7 percent of finishers had a net body weight loss >5%). Furthermore, the majority of athletes with EAH finish events with an increase in body weight and possibly an expanded plasma volume. Exposure to heat also can lead to the secretion of AVP. However, this effect of temperature may be influenced secondarily by changes in effective arterial volume that occur with heat-induced vasodilation. Despite these considerations, in some athletes during prolonged exercise, plasma AVP levels may not be suppressed maximally despite maintenance or even excess of plasma volume. This has been described in studies of hikers who developed hyponatremia in the Grand Canyon and in an army recruit during a prolonged field march. In one study it was also described median AVP levels that were significantly higher in athletes who developed hyponatremia in the 1997 New Zealand Ironman Triathlon. An intriguing link between exercise and the nonosmotic stimulation of AVP release may be related to the release of inflammatory cytokines by the exercising and injured skeletal muscle. As glycogen stores are depleted, rhabdomyolysis or lesser degrees of muscle injury can occur with the release of inflammatory cytokines such as IL-6. Independent of rhabdomyolysis, studies have shown that exercise primes an array of pro- and anti-inflammatory and growth factor expressions within circulating leukocytes. A particular athlete may be predisposed to EAH on the basis of the single-nucleotide polymorphism profile and specific inflammatory response to exercise. Conversely, IL-6 in a rat sepsis model has been
shown to reduce the expression of aquaporin-2, the downstream target of AVP and ultimate regulator of water diuresis. How these factors interact to cause EAH is not known but should be an avenue of research. Consistent with the probable role of AVP in EAH, athletes who have finished races with hyponatremia have also been demonstrated, in some cases, to have inappropriately elevated urine osmolality. In this setting, even small increases in plasma AVP levels can cause significant water retention and hyponatremia, especially in combination with excessive water intake. Furthermore, gastrointestinal blood flow and water absorption from the stomach and intestine may be impaired during exercise. When the athlete stops activity, water absorption may increase rapidly and significantly. In the setting of elevated AVP levels, this rapid absorption of large quantities of water or hypotonic fluids can lead to significant falls in serum sodium.

**Sweating**

Although overdrinking clearly is the most important causative factor in the development of EAH, there is a variable and important contribution of sodium loss from sweating. The concentration of sodium in sweat varies widely but is usually 15 to 65 mEq/L, with highly fit athletes generally excreting sweat with sodium concentrations <40 mEq/L. The volume of sweat during exercise also varies widely, from approximately 250 ml/h to >2 L/h, again being less in more fit athletes. This loss of a substantial amount of hypotonic fluid may seem to protect against the development of hyponatremia. However, these losses are replaced by the ingestion of more hypotonic fluids (water or sports drinks), and the extracellular volume loss in sweat may serve as a stimulus for antidiuretic hormone (ADH) secretion. In fact, mathematical models demonstrate that the magnitude of sweat sodium loss is insufficient to produce EAH. For example, in a 90-km ultramarathon race, an athlete may lose approximately 8.6 L of sweat. Assuming sweat sodium concentrations of either 25 or 50 mmol/L and that all fluid losses were replaced by water, the resulting sodium deficits would be 215 and 430 mmol, respectively. For a 70-kg athlete, the resulting serum sodium concentration would be either 135 or 130 mmol/L, respectively. However, for longer duration events and for those with high sweat sodium concentrations (>75 mmol/L), a sufficient sweat sodium deficit can occur for athletes to finish the race both dehydrated and hyponatremic. This is supported by the finding that some athletes finish races with net weight loss and hyponatremia. Furthermore, one case report of a patient who had cystic fibrosis (patients with cystic fibrosis excrete large amounts of sodium in their sweat) and developed EAH points to the possibility that some people may be genetically predisposed to EAH as a result of high sweat sodium.

**Clinical features**

The clinical manifestations of EAH range from no or minimal symptoms to severe encephalopathy, seizures, respiratory distress, and death. In general, the degree of clinical symptoms is related not to the absolute measured level of serum sodium but to both the rate and the extent of the drop in extracellular tonicity. However, individual variability in the clinical manifestations of hyponatremia is great. It seems that the majority of runners with EAH have mild (weakness, dizziness, headache, nausea/vomiting) or no symptoms (usually associated with serum sodium values ranging from 134 to 128 mmol/L). In athletes with serum sodium values <126 mmol/L, there is a higher likelihood of severe clinical manifestations such as cerebral edema, altered mental status, seizures, pulmonary edema, coma, and death. It was examined the clinical manifestations of 21 hyponatremic runners who finished the Houston Marathon in 2000. These clinical manifestations were compared with those of runners who did not have hyponatremia and presented to the medical tent at the conclusion of the race. The only symptom that was more common in the hyponatremic group was vomiting. Other
symptoms such as headache, nausea, dizziness, and lightheadedness could not distinguish hyponatremia from other causes, attesting to the nonspecific nature of signs and symptoms that are associated with hyponatremia. A common scenario for medical personnel who staff endurance athletic events is the care of the “collapsed athlete.” Several studies have examined the incidence of hyponatremia in this cohort, and a range of 6 to 30 percent of these athletes had serum sodium values below normal. The wide range of incidence likely reflects differences in fluid replacement guidelines that were prevalent at the time and place of the study. It is critically important to realize that a postrace venous serum sodium measurement may underestimate significantly the severity of hyponatremia. This occurs for three reasons:

1. Water may be retained in the gastrointestinal tract during the athletic event only to be absorbed rapidly in the postrace period. If AVP levels are elevated, this retained water can lower rapidly the serum sodium when reabsorbed into the circulation.
2. Arterial sodium concentration can be significantly lower than the venous sodium concentration, with this difference being accentuated with more rapid absorption of water (there may be as much as a 4-mM difference between arterial and venous sodium concentrations when water is ingested rapidly). Because it is the arterial sodium concentration that determines the risks for acute central nervous system symptoms, runners with a large amount of retained water in the gastrointestinal tract may be at higher risk for cerebral edema than their venous serum sodium concentration would indicate. Therefore, in athletes with low body mass, mildly depressed venous sodium concentrations, and recent large water intakes, the risk for deterioration secondary to worsening hyponatremia may go unrecognized.
3. There may be transient rises in venous sodium concentration at the end of a race (especially if sprinting) as muscle lactic acid accumulates and leads to a shift of water intracellularly. This transient rise in serum sodium can be as high as 10 mM and may mask significant hyponatremia.

Prevention of EAH

Because EAH primarily develops by consumption of fluid in excess of urinary and sweat losses, most efforts at prevention have been focused on education about the risks of the overconsumption of fluids. In many respects, EAH can be viewed as an iatrogenic condition because of the prevailing view that exercising athletes should drink as much fluid as tolerable during a race. Given that there is a wide variation of sweat production and renal water excretory capacity both between individual athletes and in the same individual depending on ambient conditions during the race, universal guidelines for prevention are not feasible. However, several general recommendations for the prevention of EAH have been made. The first is to drink only according to thirst and no more than 400 to 800 ml/h. The higher rates of fluid intake would be recommended for runners with higher rates of exertion (e.g., heavier runners, warmer conditions, longer times of exertion). This rate of fluid intake is well below the levels of intake that are seen in athletes who develop EAH (up to 1.5 L/h water) but above the level that would be associated with dehydration. The second recommendation is to use the USA Track and Field guidelines or other methods to estimate hourly sweat losses during exercise and avoid consuming amounts that are greater than this amount during endurance events. This is facilitated by serial measurements of weights during and after exercise with the goal to maintain weight or even finish exercise with a slighter lower weight. However, this is difficult, time-consuming, and less likely to be followed by casual athletes. Currently, there is insufficient evidence to support the suggestion that ingestion of sodium prevents or decreases the risk for EAH; neither is there any evidence that consumption of sports drinks (electrolyte-containing hypotonic fluids) can prevent the development of EAH. Again, most commercial sports drinks are hypotonic with a sodium content of 10 to 20
mmol/L (230 to 460 mg/L). Overconsumption of such fluids may decrease the rate of serum sodium decline but is unlikely to prevent EAH. Currently, the American College of Sports Medicine recommends an intake of 0.5 to 0.7 g sodium/L of water as the appropriate level of sodium intake to replace the sodium that is lost in sweat during endurance events [07260].

**Therapy of EAH**

Ideally, medical facilities at endurance events should be able to measure serum or plasma sodium concentrations in any athlete who manifests symptoms that are compatible with EAH or EAHE. However, this may not be universally feasible, and caregivers may have to act empirically on the suspicion of EAH or EAHE as the cause of symptoms. It is crucial for on-site caregivers to be vigilant for the possibility of EAH and not diagnose incorrectly volume depletion and implement a reflex therapy of normal saline infusion. In 2005, a consensus panel made specific recommendations for the treatment of EAH and EAHE. The specific treatment recommended depends on the level of symptoms that the athlete is exhibiting at the time of presentation. Most forms of mild hyponatremia (serum sodium 130 to 135 mmol/L) will be asymptomatic and found only by laboratory testing. Most athletes with mild, asymptomatic hyponatremia will require only fluid restriction and observation until spontaneous diuresis occurs. It is important that hydration with intravenous 0.9 percent sodium chloride be used with utmost caution because this therapy runs the potential risk for further decreasing the serum sodium if AVP levels remain elevated in some athletes. Furthermore, the absorption of large amounts of retained hypotonic fluids in the gastrointestinal tract may continue to lower the serum sodium for some time after the event is finished. Therefore, intravenous hydration with sodium should be reserved for athletes who manifest clear clinical signs of volume depletion and used cautiously with mandatory monitoring of serum sodium levels. Furthermore, cases of pulmonary edema have been described in individuals who received aggressive hydration with 0.9 percent sodium Monitoring of urinary sodium and potassium concentrations and calculation of the urinary free water excretion rate can be helpful in this situation. Athletes who are excreting free water can be monitored safely without need for intravenous fluids, whereas athletes with a negative free water clearance should not receive 0.9 percent sodium because this may worsen the hyponatremia. The treatment of severe (serum natrium <120 mmol/L) or symptomatic EAH requires the administration of hypertonic saline. There are some important considerations when deciding to treat EAH with hypertonic saline. First is the assumption that all EAH is acute (<48 h). This allows the correction of the hyponatremia to be done rapidly and safely. The second consideration is that no cases of osmotic demyelination syndrome have been reported with the treatment of EAH. However, there is no general consensus on the amount of hypertonic saline to be given in athletes with EAH. In the field, it has been suggested that experienced medical staff may give 100 ml of 3 percent saline over 10 min...m This has been suggested to be safe, raising the serum sodium concentration 2 to 3 mmol/L in a short period of time, and should be used in athletes who exhibit symptoms of severe hyponatremia (confusion, vomiting, respiratory insufficiency). The use of hypertonic saline has been shown to induce a greater-than-expected increase in the serum sodium, likely as a result of a decrease in AVP, and the restoration of a dilute urine; therefore, it is imperative that all athletes who receive therapy for EAH or EAHE be transported to a medical center where the serum sodium can be monitored closely. Use of hypertonic saline should be continued in the hospital to correct the hyponatremia using standard protocols. In general, 3 percent hypertonic saline can be given at 1 to 2 ml/kg per h with close monitoring of both serum electrolytes and urinary sodium and potassium excretion. In cases of severe antidiuresis, the rate of infusion may need to be increased to 3 to 4 ml/kg per h. Once significant water diuresis begins, the rate of infusion can be decreased or stopped. Special mention should be made of the patient who presents with severe EAHE and pulmonary edema. It is imperative that these patients receive emergent therapy with 3 percent
hypertonic saline despite evidence of volume overload. The addition of a loop diuretic can be considered in two circumstances: significant volume overload and significant antidiuresis with a very elevated urinary osmolality, sodium, and/or potassium level. Hypokalemia can develop during athletic events especially after the event is completed. It is important that the potential for hypokalemia be appreciated because it can have important implications for treatment. First, hypokalemia is a risk factor for the development of osmotic demyelination that is associated with correction of chronic hyponatremia. Whether hypokalemia is a risk factor for poor neurologic outcomes that are associated with therapy for acute hyponatremia is not known. Second, replacement of potassium deficits will increase the serum sodium as sodium shifts out of cells. With concomitant potassium repletion, the serum sodium may rise faster than anticipated, and correction of hyponatremia should be less aggressive [07260].

Exercise-associated hyponatremia (EAH), as defined by a blood sodium concentration less than 135 mmol/L, may lead to hypotonic encephalopathy with fatal cerebral edema. Understanding the pathogenetic role of antidiuresis may lead to improved strategies for prevention and treatment. Normonatremic marathon runners were tested pre- and post-race for creatine kinase, interleukin-6, cortisol, prolactin, and arginine vasopressin. Similar testing also was carried out in runners with encephalopathy caused by EAH, including 2 cases with fatal cerebral edema. Normonatremic runners (n=33; 2001) with a mean 3 percent decrease in body weight showed a 40-fold increase in interleukin-6, which was significantly correlated with increases in creatine kinase, cortisol, and prolactin but not arginine vasopressin. Collapsed runners with EAH (n=22; 2004) showed a mean blood urea nitrogen less than 15 mg/dL with measurable plasma levels of arginine vasopressin (>0.5 pg/mL) in 43 percent of cases. Two marathon runners with fatal cerebral edema additionally showed less than maximally dilute urines (>100 mmol/kg/H₂O) and urine sodium concentration greater than 25 mEq/L. Cases of EAH fulfill the essential diagnostic criteria for the syndrome of inappropriate antidiuretic hormone secretion (SIADH). Runners with hypotonic encephalopathy at subsequent races were treated with intravenous hypertonic (3 %) saline on the basis of this paradigm, which resulted in rapid clinical improvement without adverse effects. Release of muscle-derived interleukin-6 may play a role in the nonosmotic secretion of arginine vasopressin, thereby linking rhabdomyolysis to the pathogenesis of EAH [07261].

Exercise-associated hyponatraemia (EAH) is an acute-onset imbalance in the tonicity of extracellular fluids during or after endurance exercise which results in a blood sodium concentration <135 mmol/L. Both excessive fluid intake and a concurrent decrease in urine formation contribute to this rapid-onset, predominantly dilutional, decrease in serum sodium, which can result in life-threatening pulmonary and cerebral edema. Marathon runners with hypotonic encephalopathy related to EAH, including two cases with fatal cerebral oedema, demonstrated non-osmotic secretion of arginine vasopressin and fulfilled the essential diagnostic criteria for the syndrome of inappropriate antidiuretic hormone secretion (SIADH). The pathophysiology of SIADH as the proximate cause of EAH accounts for otherwise puzzling clinical observations such as cases occurring after only moderate fluid intake or presenting hours after races. This formulation provides a framework for enhancing prevention by monitoring weight changes during races to detect positive fluid balance before the onset of mental status changes. Most importantly, SIADH supports a strategy for use of oral and intravenous hypertonic solutions, including 3 percent sodium chloride, for the emergent treatment of moderate and life-threatening symptoms of hypotonic encephalopathy, respectively [07262].

Over the past 2 decades, exercise-associated hyponatraemia (EAH) has emerged as an important complication of prolonged endurance physical activities. Data collected since the first reports of EAH have strongly implicated a dilutional hyponatraemia from inappropriate retention of body water as the primary cause of EAH. Although high rates of fluid
consumption clearly contribute to the pathogenesis of EAH, a review of the available data does not support the view that EAH can be ascribed solely to excess drinking. Because the kidney is exquisitely sensitive to low plasma levels of the antidiuretic hormone arginine vasopressin (AVP) and because many non-osmotic stimuli to AVP secretion normally occur during prolonged endurance exercise activity, it is more likely that a combination of higher than normal fluid intakes in the setting of modest elevations of plasma AVP levels from a variety of potential stimuli during prolonged physical activity accounts for the majority of cases of EAH. In any individual, the degree to which AVP secretion is stimulated and whether it can be suppressed with sufficient fluid ingestion, will determine their susceptibility to EAH as a result of fluid ingestion both before and after physical activity, accounting for the high degree of individual variability in the occurrence of this potentially life-threatening metabolic disorder [07263].

**Bicarbonate**

Although a considerable amount of published literature exists on the ergogenic potential of ingesting sodium bicarbonate (NaHCO₃) prior to short-term, high intensity exercise, very little exists on optimal loading times prior to exercise. The purpose of this study was to determine the influence of NaHCO₃ supplementation timing on repeated sprint ability (RSA). Eight males completed three (randomized and counter balanced) trials of ten, 10 s sprints separated by 50 seconds of active recovery (1:5 work-to-rest) on a non-motorized treadmill (NMT). Prior to each trial, subjects ingested 0.3 g/kg BW of NaHCO₃ at 60 (H1), 120 (H2) or 180 (H3) min prior to exercise. Additionally, subjects assessed any side effects (gastrointestinal (GI) discomfort) from the NaHCO₃ ingestion via a visual analog scale (VAS). Blood buffering was assessed using a two-way ANOVA with repeated measures, while repeated sprint performance and GI discomfort was assessed via a one-way ANOVA with repeated measures. Blood-buffering capacity was not different at pre exercise times. Average speed (AS), average power (AP) and total distance (TD) covered progressively declined over the ten sprints, however there was no difference between conditions. Incidence of GI discomfort was significantly higher from pre ingestion at all time points with the exception of 180 min, while severity was only different between 90 and 180 min. Ingestion times (between 60 and 180 min) did not influence the blood buffering or ergogenic potential of NaHCO₃ as assessed by RSA. However, VAS scores indicated that at 180 min post ingestion an individual is less prone to experience significant GI discomfort [11408].

Lactate (La) and H⁺-ions are unequally distributed in the blood between plasma and red blood cells (RBCs). There is no data concerning the effects of an oral ingestion of bicarbonate (HCO₃⁻) on repeated high intensity sprint exercise and La and H⁺ distribution between plasma and RBCs. Since an oral ingestion of bicarbonate leads to a higher efflux of La from the working skeletal muscle to the plasma, as it was shown by previous studies, this would lead to a higher gradient of La between plasma and RBCs. Although a higher gradient leads to a higher uptake, it is even more difficult for the RBCs to take up La fast enough, due to the more stressed transport system. Since RBCs function to transport La from the working muscle and help to maintain a concentration difference between plasma and muscle, this potentially increases performance during repeated sprint exercise (e.g. 4 × 30 s). The major goal of one investigation was to test this hypothesis. Eleven male participants ingested either a solution of sodium bicarbonate (NaHCO₃) or placebo (CaCO₃). Thereafter all performed four maximal 30 s sprints with 5 min of passive rest. During the resting periods concentrations of bicarbonate La and H⁺ where measured in both blood compartments (plasma and RBCs). There were no significant differences in the La-ratios between plasma and RBCs between both interventions. These results indicate that the La/H⁺ co-transport is
Metabolic acidosis is associated with skeletal muscle proteolysis, and alkali supplementation has shown improvements in lean body mass and urinary nitrogen wasting in several studies. However, the association of acidosis with functional outcomes has not been examined on a population-based level. 2,675 nationally representative adults 50 years or older in the National Health and Nutrition Examination Survey 1999-2002. Serum bicarbonate level <23 mEq/L was present in 23 percent of the cohort. Compared with participants with bicarbonate levels ≥23 mEq/L, those with bicarbonate levels <23 mEq/L had higher body mass index and serum albumin levels; were more likely to have low socioeconomic status, a diagnosis of diabetes mellitus, or glomerular filtration rate <60 mL/min/1.73 m²; and were less likely to use diuretics. Serum bicarbonate level <23 mEq/L compared with ≥23 mEq/L was associated with low gait speed (odds ratio 1.43) and low peak torque (odds ratio 1.36) after multivariable adjustment. The association with low peak torque was modified by race/ethnicity in women, but not men. Lower serum bicarbonate levels are associated with slower gait speed and decreased quadriceps strength in older adults. Further studies should examine the effect of alkali therapy on functional outcomes [11410].

The aim of one study was to determine the effect and reliability of acute and chronic sodium bicarbonate ingestion for 2000-m rowing ergometer performance (watts) and blood bicarbonate concentration [HCO₃⁻]. In a crossover study, 7 well-trained rowers performed paired 2000-m rowing ergometer trials under 3 double-blinded conditions: (1) 0.3 grams per kilogram of body mass (g/kg BM) acute bicarbonate; (2) 0.5 g/kg BM daily chronic bicarbonate for 3 d; and (3) calcium carbonate placebo, in semi-counterbalanced order. For 2000-m performance and bicarbonate amount it was examined differences in effects between conditions via pairwise comparisons, with differences interpreted in relation to the likelihood of exceeding smallest worthwhile change thresholds for each variable. There were only trivial differences in 2000-m performance between placebo (277 ± 60 W), acute bicarbonate (280 ± 65 W) and chronic bicarbonate (282 ± 65 W); however, [HCO₃⁻] was substantially greater after acute bicarbonate, than with chronic loading and placebo. Typical error for 2000-m mean power was 2.1 percent for acute bicarbonate, 3.6 percent for chronic bicarbonate, and 1.6 percent for placebo. It was concluded that performance in 2000-m rowing ergometer trials may not substantially improve after acute or chronic bicarbonate loading. However, performances will be reliable with both acute and chronic bicarbonate loading protocols [12456].

The aim of one study was to examine the effects of sodium bicarbonate (NaHCO₃) administration on lower-body, hypertrophy-type resistance exercise (HRE). Using a double-blind randomized counterbalanced design, 12 resistance-trained male participants (mean age 20 years) ingested 0.3 g/kg of NaHCO₃ or placebo 60 min before initiation of an HRE regimen. The protocol employed multiple exercises: squat, leg press, and knee extension, utilizing four sets each, with 10-12 repetition-maximum loads and short rest periods between sets. Exercise performance was determined by total repetitions generated during each exercise, total accumulated repetitions, and a performance test involving a fifth set of knee extensions to failure. Arterialized capillary blood was collected via fingertip puncture at four time points and analyzed for pH, [HCO₃⁻], base excess (BE), and lactate [Lac(-)]. NaHCO₃ supplementation induced a significant alkaline state. NaHCO₃ administration resulted in significantly more total repetitions than placebo after the exercise protocol. These findings demonstrate ergogenic efficacy for NaHCO₃ during HRE and warrant further investigation into chronic training applications [13606].

At rest, administration of the short-chain fatty acid acetate suppresses fat oxidation without
affecting carbohydrate utilization. The combined effect of increased acetate availability and exercise on substrate utilization is, however, unclear. With local ethics approval, it was studied the effect of ingesting either sodium acetate (NaAc) or sodium bicarbonate (NaHCO$_3$) at a dose of 4 mmol/kg body mass 90 min before completing 120 min of exercise at 50 percent VO$_{2\text{peak}}$. Six healthy young men completed the trials after an overnight fast and ingested the sodium salts in randomized order. As expected NaAc ingestion decreased resting fat oxidation (mean ± SD; 0.09 ± 0.02 vs. 0.07 ± 0.02 g/min pre- and post-ingestion respectively) with no effect upon carbohydrate utilization. In contrast, NaHCO$_3$ ingestion had no effect on substrate utilization at rest. In response to exercise, fat and CHO oxidation increased in both trials, but fat oxidation was lower (0.16 ± 0.10 vs 0.29 ± 0.11 g/min) and carbohydrate oxidation higher (1.67 ± 0.35 vs 1.44 ± 0.22 g/min) in the NaAc trial compared with the NaHCO$_3$ trial during the first 15 min of exercise. Over the final 75 min of exercise an increase in fat oxidation and decrease in carbohydrate oxidation was observed only in the NaAc trial. These results demonstrate that increasing plasma acetate concentration suppresses fat oxidation both at rest and at the onset of moderate-intensity exercise [13607].

During intensive anaerobic exercise with a large glycolytic component, one major cause of fatigue is believed to be acidosis caused by high levels of hydrogen ions (H$^+$) in the muscle fibers. The increase in (H$^+$) corresponds to a decrease in muscle and blood pH, can slow glycolysis, interfere with calcium release from the endoplasmic reticulum and calcium ion binding, and increase the perception of fatigue after some types of exercises. A number of buffers can be used by the body, but the primary method for buffering the H$^+$ is thought to be either bicarbonate or hemoglobin. For the past 35 years, several studies have investigated the use of sodium bicarbonate (SB) as an ergogenic aid. The participants have typically been men, and efficacy (improved performance and a decrease in H$^+$ concentration after exercise) has generally been seen at doses of at least 0.3 g/kg body mass. A meta-analysis by suggests that ingestion of SB at 0.3-0.5 g/kg body mass improves mean power by 1.7 ± 2.0 percent during high-intensity races of short duration (1–10 min). Timing of ingestion ranging from 60 min - 180 min before exercise did not influence buffering capacity or the ergogenic potential of SB (0.3 g/kg body mass) as assessed by repeated sprint ability. However, visual analog scale scores indicated that at 180 minutes post-ingestion, an individual is less prone to experiencing significant gastrointestinal discomfort. It has also been demonstrated that swimmers ingesting 0.3 g/kg body mass of SB can enhance blood buffering potential and positively influence interval swim performance. One study examined the effect of simultaneous supplementation of extracellular buffer sodium bicarbonate (SB) and intracellular buffer beta-alanine (BA) on maximal sprint swimming. Thirteen competitive male swimmers completed 4 different treatments (placebo [PL], SB, BA + PL, and BA + SB) in a crossover procedure. PL or SB supplementation (0.3 g/kg body weight) was ingested 60 min before two maximal 100-m freestyle swims that were performed with a passive recovery of 12-min between each swim. Because of the known long washout period for carnosine, four weeks of BA supplementation (4.8 g per day) was started after the first week of PL or SB supplementation and performance testing. The first maximal swims were similar, but the increase in time of the second versus the first 100-m swimming time was 1.5 s more in PL than in SB. Blood pH values were significantly (p < 0.05) greater in the SB and in the BA + SB groups compared to the PL and BA + PL values. There were no differences in peak blood lactate between the treatments. It was concluded that supplementing with SB prior to performing maximal sprint swimming with repetitions under 60 s improves performance. However, co-supplementation with SB and BA did not confer any added benefit on maximal swim performance [13608].

One study examined the acute effects of NaHCO$_3$ ingestion on repetitions to failure and rating of perceived exertion (RPE) in the back squat and bench press in trained males. Eight resistance trained males took part in this double-blind, randomized cross-over experimental
study whereby they ingested NaHCO$_3$ (0.3 g/kg body mass) or placebo (sodium chloride NaCl: 0.045 g/kg body mass) solution 60 mins before completing a bout of resistance exercise (3 sets of bench press and back squat exercise to failure at an intensity of 80 percent 1 repetition maximum). Experimental conditions were separated by at least 48 hours. Participants completed more repetitions to failure in the back squat following NaHCO$_3$ ingestion but not for bench press. Mean ± SD of total repetitions was 31.3 ± 15.3 and 24.6 ± 16.2 for back squat and 28.7 ± 12.2 and 26.7 ± 10.2 for bench press in NaHCO$_3$ and placebo conditions respectively. Repetitions to failure decreased as set increased for the back squat and bench press. RPE significantly increased with set for the back squat and bench press. There was no significant change in blood lactate across time or between conditions. There were however treatment X time interactions for blood pH and blood HCO$_3$ concentration. Following ingestion blood pH and HCO$_3$ concentration were greater for the NaHCO$_3$ condition compared to the placebo condition. The results of this study suggest that sodium bicarbonate ingestion can enhance resistance exercise performance using a repetition to failure protocol in the first exercise in a resistance exercise session [13609].

**Physiology**

Defense of extracellular pH constancy against lactic acidosis can be estimated from changes (Delta) in lactic acid ([La]), HCO$_3^-$, pH and PCO$_2$ in blood plasma because it is equilibrated with the interstitial fluid. These quantities were measured in earlobe blood during and after incremental bicycle exercise in 13 untrained (UT) and 21 endurance-trained (TR) males to find out if acute and chronic exercise influence the defense. During exercise the capacity of non-bicarbonate buffers ($\beta$(nbi) = -Delta[La] . DeltapH(-1) - Delta[HCO$_3^-$] . DeltapH(-1)) available for the extracellular fluid (mainly hemoglobin, dissolved proteins and phosphates) amounted to 32 ± 2 and 20 ± 2 mmol/L in UT and TR, respectively, which was a significant difference. During recovery $\beta$(nbi) decreased to significantly in both groups corresponding to values previously found at rest by in vivo CO$_2$ titration. Bicarbonate buffering ($\beta$(bi)) amounted to 44-48 mmol/L during and after exercise. The large exercise $\beta$(nbi) seems to be mainly caused by an increasing concentration of all buffers due to shrinking of the extracellular volume, exchange of small amounts of HCO$_3^-$ or H$^+$ with cells and delayed HCO$_3^-$ equilibration between plasma and interstitial fluid. Increase of concentration of HCO$_3^-$ during titration by these mechanisms augments total $\beta$ and thus the calculated $\beta$(nbi) more than $\beta$(bi) because it reduces DeltapH and Delta[HCO$_3^-$] at constant Delta[La]. The smaller rise in exercise $\beta$(nbi) in TR than UT may be caused by an increased extracellular volume and an improved exchange of lactic acid, bicarbonate and H$^+$ between trained muscles and blood [07265].

**Effect on exercise**

Bicarbonate loading is a popular ergogenic aid used primarily by athletes in short-duration, high-intensity sporting events and competitions. Controlled experimental trials have shown that small (worthwhile) benefits can obtained from acute doses of bicarbonate taken before exercise. Gastrointestinal problems encountered by some athletes limit the widespread use of this practice, however. The transfer of positive research findings to the competitive environment has proved problematic for some individuals. More recent applications involve serial ingestion of bicarbonate over several days before competition or during high-intensity training sessions over a few weeks. A number of research questions need to be addressed to enhance applications of bicarbonate loading in the elite sport environment. One commentary examined some of research and practical issues of bicarbonate loading used to enhance both training and competitive performance [07266].
Sodium bicarbonate ingestion has been shown to improve performance in single-bout, high intensity events, probably due to an increase in buffering capacity, but its influence on single-bout swimming performance has not been investigated. The effects of sodium bicarbonate supplementation on 200 m freestyle swimming performance were investigated in elite male competitors. Following a randomised, double blind counterbalanced design, 9 swimmers completed maximal effort swims on 3 separate occasions: a control trial (C); after ingestion of sodium bicarbonate (SB: NaHCO₃ 300 mg/kg body mass); and after ingestion of a placebo (P: CaCO₃ 200 mg/kg body mass). The SB and P agents were packed in gelatine capsules and ingested 90-60 min prior to each 200 m swim. Mean 200 m performance times were significantly faster for SB than C or P. Base excess, pH and blood bicarbonate were all elevated pre-exercise in the SB compared to C and P trials. Post-200 m blood lactate concentrations were significantly higher following the SB trial compared with P and C. It was concluded that SB supplementation can improve 200 m freestyle performance time in elite male competitors, most likely by increasing buffering capacity.

The effects of exercise on energy substrate metabolism persist into the postexercise recovery period. It was sought to derive bicarbonate retention factors (k) to correct for carbon tracer oxidized, but retained from pulmonary excretion before, during, and after exercise. Ten men and nine women received a primed-continuous infusion of ¹³C-bicarbonate (sodium salt) under three different conditions: before, during, and 3 h after 90 min of exercise at 45 percent peak oxygen consumption (VO₂peak); before, during, and 3 h after 60 min of exercise at 65 percent VO₂peak and during a time-matched resting control trial, with breath samples collected for determination of ¹³CO₂ excretion rates. Throughout the resting control trial, k was stable and averaged 0.83 in men and women. During exercise, average k in men was 0.93 at 45 percent VO₂peak and 0.94 at 65 percent VO₂peak and in women k was 0.91 at 45 percent VO₂peak and 0.92 at 65 percent VO₂peak with no significant differences between intensities or sexes. After exercise at 45 percent VO₂peak k returned rapidly to control values in men and women, but following exercise at 65 percent VO₂peak k was significantly less than control at 30 and 60 min postexercise in men (0.74 and 0.72, respectively) and women (0.75 and 0.76, respectively) with no significant postexercise differences between men and women. We conclude that bicarbonate retention is transiently increased in men and women for the first hour of postexercise recovery following endurance exercise bouts of hard but not moderate intensity.

Ingestion of agents that modify blood buffering action may affect high-intensity performance. Here we present a meta-analysis of the effects of acute ingestion of three such agents – sodium bicarbonate, sodium citrate and ammonium chloride – on performance and related physiological variables (blood bicarbonate, pH and lactate). A literature search yielded 59 useable studies with 188 observations of performance effects. To perform the mixed-model meta-analysis, all performance effects were converted into a percentage change in mean power and were weighted using standard errors derived from exact p-values, confidence limits (CLs) or estimated errors of measurement. The fixed effects in the meta-analytic model included the number of performance-test bouts (linear), test duration (log linear), blinding (yes/no), competitive status (athlete/nonathlete) and sex (male/female). Dose expressed as buffering mmoL/kg/body mass (BM) was included as a strictly proportional linear effect interacted with all effects except blinding. Probabilistic inferences were derived with reference to thresholds for small and moderate effects on performance of 0.5 and 1.5 percent, respectively. Publication bias was reduced by excluding study estimates with a standard error >2.7 percent. The remaining 38 studies and 137 estimates for sodium bicarbonate produced a possibly moderate performance enhancement of 1.7 percent with a typical dose of 3.5 mmoL/kg/BM (about 0.3 g/kg/BM) in a single 1-minute sprint, following blinded consumption by male athletes. In the 16 studies and 45 estimates for sodium citrate, a typical dose of 1.5 mmoL/kg/BM (about 0.5 g/kg/BM) had an unclear effect on performance.
of 0.0 percent, while the five studies and six estimates for ammonium chloride produced a possibly moderate impairment of 1.6 percent with a typical dose of 5.5 mmol/kg/BM (about 0.3 g/kg/BM). Study and subject characteristics had the following modifying small effects on the enhancement of performance with sodium bicarbonate: an increase of 0.5 percent with a 1 mmol/kg/BM increase in dose; an increase of 0.6 percent with five extra sprint bouts; a reduction of 0.6 percent for each 10-fold increase in test duration (e.g. 1-10 minutes); reductions of 1.1 percent with nonathletes and 0.7 percent with females. Unexplained variation in effects between research settings was typically ±1.2 percent. The only noteworthy effects involving physiological variables were a small correlation between performance and pre-exercise increase in blood bicarbonate with sodium bicarbonate ingestion, and a very large correlation between the increase in blood bicarbonate and time between sodium citrate ingestion and exercise. The approximate equal and opposite effects of sodium bicarbonate and ammonium chloride are consistent with direct performance effects of pH, but sodium citrate appears to have some additional metabolic inhibitory effect. Important future research includes studies of sodium citrate ingestion several hours before exercise and quantification of gastrointestinal symptoms with sodium bicarbonate and citrate. Although individual responses may vary, it was recommended ingestion of 0.3-0.5 g/kg/BM sodium bicarbonate to improve mean power by 1.7 percent in high-intensity races of short duration [11586].

Decrease in peripheral oxygen delivery may impact exercise performance in athletes with exercise-related arterial oxygen desaturation (ERD). It was evaluated whether sodium bicarbonate ingestion would be effective to reduce ERD and what is the consequences upon exercise performance. Seventy highly trained athletes performed an incremental treadmill cardiopulmonary exercise test (incCPX) and a high intensity constant speed test (ctCPX) on separate days. Subjects who developed ERD by pulse oximetry were randomly allocated to oral sodium bicarbonate or placebo during 5 days. At the end of treatment subjects repeated both tests. ERD prevalence was 33 percent during the incCPX (17 % severe, 48% moderate and 35 % mild) and 34 percent (5 % severe, 37 % moderate and 58 % mild) in the ctCPX. Athletes who developed ERD by pulse oximetry were randomly allocated to oral sodium bicarbonate or placebo during 5 days. At the end of treatment subjects repeated both tests. ERD prevalence was 33 percent during the incCPX (17 % severe, 48% moderate and 35 % mild) and 34 percent (5 % severe, 37 % moderate and 58 % mild) in the ctCPX. Active treatment, but not placebo, reduced ERD during ctCPX (P<0.05). However, there were no significant positive effects on main parameters of aerobic function and endurance exercise capacity. It was concluded that sodium bicarbonate was effective in lessening ERD during ctCPX in athletes. However, this intervention failed to improve maximal and submaximal exercise capacity in these subjects [13610].

Effect on symptoms from the stomach

Sodium bicarbonate (NaHCO₃) is often ingested at a dose of 0.3 g/kg body mass (BM), but ingestion protocols are inconsistent in terms of using solution or capsules, ingestion period, combining NaHCO₃ with sodium citrate (Na₃C₆H₅O₇), and coingested food and fluid. To quantify the effect of ingesting 0.3 g/kg NaHCO₃ on blood pH, [HCO₃⁻], and gastrointestinal (GI) symptoms over the subsequent 3 hr using a range of ingestion protocols and, thus, to determine an optimal protocol in a crossover design, 13 physically active subjects undertook 8 NaHCO₃ experimental ingestion protocols and 1 placebo protocol. Capillary blood was taken every 30 min and analyzed for pH and [HCO₃⁻]. GI symptoms were quantified every 30 min via questionnaire. Statistics used were pairwise comparisons between protocols; differences were interpreted in relation to smallest worthwhile changes for each variable. A likelihood of >75% was a substantial change. [HCO₃⁻] and pH were substantially greater than in placebo for all other ingestion protocols at almost all time points. When NaHCO₃ was coingested with food, the greatest [HCO₃⁻] (30.9 mmol/kg) and pH (7.49) and lowest incidence of GI symptoms were observed. The greatest incidence of GI side effects was
observed 90 min after ingestion of 0.3 g/kg NaHCO₃ solution. It was concluded that the changes in pH and [HCO₃⁻] for the 8 NaHCO₃-ingestion protocols were similar, so an optimal protocol cannot be recommended. However, the results suggest that NaHCO₃ coingested with a high-carbohydrate meal should be taken 120-150 min before exercise to induce substantial blood alkalosis and reduce GI symptoms [11248].

**Sodium bicarbonate and sodium citrate**

Athletes utilise many different strategies to enhance performance. Among the more popular ergogenic aids are sodium bicarbonate (NaHCO₃) or sodium citrate, collectively recognised as “buffers.” These substances, permitted for use by the World Anti-Doping Agency code, potentially provide the body with improved resistance to the fatigue caused by changes in acid-base balance. Typically, resting human arterial blood pH is approximately pH 7.4, slightly alkalotic, but after strenuous exercise may fall to about 7.1, while muscle pH decreases to about 6.8. Buffers such as NaHCO₃ and sodium citrate will increase the buffering capacity by increasing, for example, the amount of bicarbonate that can be utilised, say, by increasing the pH to 7.5. Studies in the 1980s on intense running using NaHCO₃ as a buffer suggested that ingesting this substance could enhance performance in elite athletes running 400-800 m. An ergogenic benefit in 200 m freestyle swimming performance after NaHCO₃ ingestion has also been reported. Researchers found that, after ingesting an acute load of 0.3 g/kg bw of NaHCO₃ and undergoing a loading sequence of creatine, swimmers who undertook an interval protocol (2×100 m freestyle with 10 min passive rest between bouts) experienced an improvement in time to complete the second swim. A recent study has suggested that, in order to overcome the gastrointestinal upset often associated with buffering, a progressive intake of 600 mg/kg bodyweight, split into several doses over the day, can be used as an alternative to the acute dose protocol. More conclusive, perhaps, is the ergogenic potential observed in recreational team sport participants during repeated sprints (5×6 s) or multiple effort bouts. Similar to earlier studies, it was observed a lower blood [H⁺] and higher [HCO₃⁻] after supplementing with 0.3 g/kg bw NaHCO₃. No differences were reported in total work or percentage fatigue, but improvements in sprints were observed. NaHCO₃ provision also resulted in significantly higher post-test muscle lactate values, attributed by the authors to a high rate of glycolytic flux in the muscle. However, another study failed to find any evidence of improved repeated sprint ability in college wrestlers. A NaHCO₃ dose of 0.3 g/kg body weight was used in a randomised controlled trial on 10 well-trained male cyclists undertaking 1 h maximal effort cycle ergometry. The cyclists performed, on average, 13 and 14 percent greater total with NaHCO₃ than control and placebo, respectively. More recently, no difference in NaHCO₃ (0.3 g/kg body weight) and control trials lasting about 60 min in total was observed. It was concluded that both NaHCO₃ and sodium citrate are effective, the optimal amount being 0.3 g/kg body weight. Users should test the response to buffer ingestion to improve their own performance prior to any competitive event, as both buffers can contribute to GI upset. It appears that short- and long-term high-intensity exercise, and possibly high-intensity longer-duration performance, can benefit from the ergogenic effects of these buffers. Loading sequences and timing of pre-exercise doses tend to be different in most studies, leading to confusion regarding effectiveness of the various buffering substances [09343].

**Energy need**

It has been demonstrated that athletes' dietary intake was relatively well-balanced according to the recommended dietary allowances (RDAs). In contrast, other studies have shown that athletes may have low energy intake or imbalance of protein and fat and insufficient minerals
and vitamins. Nonetheless, it was hypothesized that practicing a sport may allow young adults to have a nutritional status closer to recommended values. The purpose of one experiment was to study the nutritional status of young French adults, particularly to compare the nutritional status of trained young male and female athletes to those of young sedentary control subjects, and to national RDAs. A total of 85 young adults were recruited and filled a 4-day food and physical activity record. Dietary intake, energy expenditure, energy balance, carbohydrate, protein, fat, water, vitamins, and minerals were recorded. Energy intake values were $9874 \pm 3050$ kJ for the athletes and $7506 \pm 1845$ kJ for control subjects. Athletes' nutritional status was closer to French RDAs than those of sedentary subjects who present a lower energy intake, a greater percentage of the energy intake from fat and lower values for minerals and vitamins. In conclusion, practicing a sport may allow athletes to balance their energy intake and expenditure and could be a good way to have a nutritional status closer to RDAs. Educational programs for students on proper food selection, eating habits and physical activity are needed to improve the nutritional status of these young French adults, particularly in sedentary students [09322].

**Meal frequency**

Admittedly, research to date examining the physiological effects of meal frequency in humans is somewhat limited. More specifically, data that has specifically examined the impact of meal frequency on body composition, training adaptations, and performance in physically active individuals and athletes is scant. Until more research is available in the physically active and athletic populations, definitive conclusions cannot be made. However, within the confines of the current scientific literature, it can be asserted that:

- increasing meal frequency does not appear to favorably change body composition in sedentary populations
- if protein levels are adequate, increasing meal frequency during periods of hypoenergetic dieting may preserve lean body mass in athletic populations
- increased meal frequency appears to have a positive effect on various blood markers of health, particularly LDL cholesterol, total cholesterol, and insulin
- increased meal frequency does not appear to significantly enhance diet induced thermogenesis, total energy expenditure or resting metabolic rate
- increasing meal frequency appears to help decrease hunger and improve appetite control

Although athletic and physically active populations have not been independently studied in this domain, given the beneficial outcomes that increasing meal frequency exerts on a variety of health markers in non-athletic populations, it appears as if increasing meal frequency in athletic populations is warranted in terms of improving blood markers of health. Nutrient timing research has demonstrated the importance of protein ingestion before, during, and following physical activity. Social and cultural definitions of an actual "meal" (vs snack) vary greatly and time between "meals" is arbitrary. In other words, if the "time-lag" is very short, it may increase the number of feedings as opposed to a study with a greater "time-lag". Taking all of this into account, it appears from the existing (albeit limited) body of research that increased meal frequency may not play a significant role in weight loss/gain when under-reporting, restrained eating, and exercise are accounted for in the statistical analyses. Furthermore, most, but not all of the existing research, fails to support the effectiveness of increased meal frequency on the thermic effect of food, resting metabolic rate, and total energy expenditure. However, when energy intake is limited, increased meal frequency may likely decrease hunger, decrease nitrogen loss, improve lipid oxidation, and improve blood markers such as total and LDL cholesterol, and insulin. Nonetheless, more well-designed research studies involving various meal frequencies, particularly in physically active/athletic populations are warranted [11246].
Carbohydrates

One review considered aspects of the optimal nutritional strategy for recovery from prolonged moderate to high intensity exercise. Dietary carbohydrate represents a central component of post-exercise nutrition. Therefore, carbohydrate should be ingested as early as possible in the post-exercise period and at frequent (i.e. 15- to 30-minute) intervals throughout recovery to maximize the rate of muscle glycogen resynthesis. Solid and liquid carbohydrate supplements or whole foods can achieve this aim with equal effect but should be of high glycaemic index and ingested following the feeding schedule described above at a rate of at least 1 g/kg/h in order to rapidly and sufficiently increase both blood glucose and insulin concentrations throughout recovery. Adding ≥0.3 g/kg/h of protein to a carbohydrate supplement results in a synergistic increase in insulin secretion that can, in some circumstances, accelerate muscle glycogen resynthesis. Specifically, if carbohydrate has not been ingested in quantities sufficient to maximize the rate of muscle glycogen resynthesis, the inclusion of protein may at least partially compensate for the limited availability of ingested carbohydrate. Some studies have reported improved physical performance with ingestion of carbohydrate-protein mixtures, both during exercise and during recovery prior to a subsequent exercise test. While not all of the evidence supports these ergogenic benefits, there is clearly the potential for improved performance under certain conditions, e.g. if the additional protein increases the energy content of a supplement and/or the carbohydrate fraction is ingested at below the recommended rate. The underlying mechanism for such effects may be partly due to increased muscle glycogen resynthesis during recovery, although there is varied support for other factors such as an increased central drive to exercise, a blunting of exercise-induced muscle damage, altered metabolism during exercise subsequent to recovery, or a combination of these mechanisms [10434].

During postexercise recovery, optimal nutritional intake is important to replenish endogenous substrate stores and to facilitate muscle-damage repair and reconditioning. After exhaustive endurance-type exercise, muscle glycogen repletion forms the most important factor determining the time needed to recover. Postexercise carbohydrate (CHO) ingestion has been well established as the most important determinant of muscle glycogen synthesis. Coingestion of protein and/or amino acids does not seem to further increase muscle glycogen synthesis rates when CHO intake exceeds 1.2 g/kg per hour. However, from a practical point of view it is not always feasible to ingest such large amounts of CHO. The combined ingestion of a small amount of protein (0.2-0.4 g/kg per hour with less CHO (0.8 g/kg per hour) stimulates endogenous insulin release and results in similar muscle glycogen-repletion rates as the ingestion of 1.2 g/kg per hour CHO. Furthermore, postexercise protein and/or amino acid administration is warranted to stimulate muscle protein synthesis, inhibit protein breakdown, and allow net muscle protein accretion. The consumption of about 20 g intact protein, or an equivalent of about 9 g essential amino acids, has been reported to maximize muscle protein-synthesis rates during the first hours of postexercise recovery. Ingestion of such small amounts of dietary protein 5 or 6 times daily might support maximal muscle protein-synthesis rates throughout the day. Consuming CHO and protein during the early phases of recovery has been shown to positively affect subsequent exercise performance and could be of specific benefit for athletes involved in multiple training or competition sessions on the same or consecutive days [10512].

Consuming carbohydrate-rich meals before continuous endurance exercise improves performance, yet few studies have evaluated the ideal preexercise meal for high-intensity intermittent exercise, which is characteristic of many team sports. Now it was the purpose to investigate the effects of low- and high-glycemic-index (GI) meals on metabolism and
performance during high-intensity, intermittent exercise. Sixteen male participants completed three 90-min high-intensity intermittent running trials in a single-blinded random order, separated by about 7 d, while fasted (control) and 2 hr after ingesting an isoenergetic low-GI (lentil), or high-GI (potato and egg white) preexercise meal. Serum free fatty acids were significantly higher and insulin lower throughout exercise in the fasted condition, but there were no differences in blood glucose during exercise between conditions. Distance covered on a repeated-sprint test at the end of exercise was significantly greater in the low-GI and high-GI conditions than in the control. Rating of perceived exertion was lower in the low-GI condition than in the control. In a subsample of 5 participants, muscle glycogen availability was greater in the low-and high-GI conditions versus fasted control before the repeated-sprint test, with no differences between low and high GI. When exogenous carbohydrates are not provided during exercise both low- and high-GI preexercise meals improve high-intensity, intermittent exercise performance, probably by increasing the availability of muscle glycogen. However, the GI does not influence markers of substrate oxidation during high-intensity, intermittent exercise [10513].

Ingesting carbohydrate plus protein during prolonged variable intensity exercise has demonstrated improved aerobic endurance performance beyond that of a carbohydrate supplement alone. The purpose of one study was to determine if a supplement containing a mixture of different carbohydrates (glucose, maltodextrin, and fructose) and a moderate amount of protein given during endurance exercise would increase time to exhaustion (TTE), despite containing 50 percent less total carbohydrate than a carbohydrate-only supplement. We also sought post priori to determine if there was a difference in effect based on percentage of ventilatory threshold (VT) at which the subjects cycled to exhaustion. Fifteen trained male and female cyclists exercised on 2 separate occasions at intensities alternating between 45 and 70 percent VO$_{2\text{max}}$ for 3 hours, after which the workload increased to 74-85 percent VO$_{2\text{max}}$ until exhaustion. Supplements (275 mL) were provided every 20 minutes during exercise, and these consisted of a 3 percent carbohydrate/1.2% protein supplement (MCP) and a 6 percent carbohydrate supplement (CHO). For the combined group (n=15), TTE in MCP did not differ from CHO. However, for subjects cycling at or below VT (n=8), TTE in MCP was significantly greater than for CHO. There were no significant differences in TTE for the above VT group (n=7). The results suggest that, compared to a traditional 6% percentCHO supplement, a mixture of carbohydrates plus a moderate amount of protein can improve aerobic endurance at exercise intensities near the ventilatory threshold, despite containing lower total carbohydrate and caloric content [10514].

Rowing is an endurance sport with an important skill component. One study evaluated the effects of carbohydrate (CHO) solution ingestion on rowing technique before and after 60 min of ergometer rowing. On two occasions, eight male rowers rowed as a group in an indoor rowing tank for 6 min at 18 strokes per minute before and after 60 min of ergometer rowing at 60 percent of VO$_{2\text{max}}$. Participants ingested either a 6 percent CHO solution or a flavoured placebo in a randomised, counterbalanced fashion. Physiological and technical measures were analysed using a repeated measures analysis of variance. There were no differences in heart rate or rating of perceived exertion between the treatments, indicating similar physiological strain. There were no changes in stroke length over the 60-min period with either treatment. Oar handle velocity decreased from 49.7 ± 3.6 to 47.4 ± 2.8 °/s over the 60-min period in placebo group but did not change in CHO (48.9 ± 2.2 to 48.2 ± 1.9 °/s). Oar handle curves did not differ between CHO and placebo. Maximum blade depth declined similarly by 20 ± 16 placebo in both groups. This change is likely to decrease the resistance of the water upon the oar blade during the drive phase of the rowing stroke. In conclusion, selected measures of rowing tank technique deteriorated in response to 60 min of ergometer rowing in both placebo and CHO. Ingestion of CHO may help to maintain velocity of movement as rowers fatigue [10509].
Health care professionals advocate that athletes who are susceptible to exercise-associated muscle cramps (EAMCs) should moderately increase their fluid and electrolyte intake by drinking sport drinks. Some clinicians have also claimed drinking small volumes of pickle juice effectively relieves acute EAMCs, often alleviating them within 35 seconds. Others fear ingesting pickle juice will enhance dehydration-induced hypertonicity, thereby prolonging dehydration. To determine if ingesting small quantities of pickle juice, a carbohydrate-electrolyte (CHO-e) drink, or water increases plasma electrolytes or other selected plasma variables a crossover study was performed. Nine euhydrated, healthy men completed the study. Mean fluid intake was 86 ± 17 mL. Plasma sodium concentration, plasma magnesium concentration, plasma calcium concentration, plasma osmolality, and plasma volume did not change during the 60 minutes after ingestion of each fluid. Water ingestion slightly decreased plasma potassium concentration at 60 minutes. At these volumes, ingestion of pickle juice and CHO-e drink did not cause substantial changes in plasma electrolyte concentrations, plasma osmolality, or plasma volume in rested, euhydrated men. Concern that ingesting these volumes of pickle juice might exacerbate an athlete’s risk of dehydration-induced hypertonicity may be unwarranted. If EAMCs are caused by large electrolyte loss due to sweating, these volumes of pickle juice or CHO-e drink are unlikely to restore any deficit incurred by exercise [09325].

The purpose of one study was to examine the influences of a carbohydrate (CHO) mouth rinse on self-selected running speeds during a 30-min treadmill run. Ten endurance-trained men performed 2 trials, each involving a 10-min warm-up at 60 percent VO2max followed by a 30-min run. The run was performed on an automated treadmill that allowed the spontaneous selection of speeds without manual input. Participants were asked to run at speeds that equated to a rating of perceived exertion of 15, mouth rinsing with either a 6 percent CHO or taste-matched placebo solution. In addition to recording self-selected speeds and total distance covered the authors assessed the runners’ subjective feelings. The total distance covered was significantly greater during the CHO than during the placebo trial. Faster speeds selected during the first 5 min of exercise corresponded with enhanced feelings of pleasure when mouth rinsing with the CHO solution. Mouth rinsing with a CHO solution increased total distance covered during a self-selected 30-min run in comparison with mouth rinsing with a color- and taste-matched placebo [09326].

The aim of one study was to investigate the pre- and during-race nutritional intake of cyclists competing in a 210-km 1-day ultraendurance cycle race. Forty-five endurance-trained male cyclists participated in this dietary survey and completed a 3-day dietary record. Mean reported carbohydrate (CHO) intake over the 3 days before the race (5.6 ± 1.7 g/kg) was below the recommended guidelines of 7-10 g/kg. Although 57 percent of participants indicated that they CHO loaded 1-3 days before the race, only 23 percent of these participants achieved CHO intakes of ≥ 7 g/kg over the 3-day period before the race, demonstrating a discrepancy between perceived and actual intakes of CHO. Most participants indicated the use of CHO supplements before (84 %) and during (98 %) the race and achieved a CHO intake of 63 ± 23 g/hr during the race. Although most cyclists failed to meet recommended prerace CHO intakes, most achieved the recommended CHO intakes during the race [09327].

One study investigated the effects of short-term dietary changes on metabolism and duathlon performance. Eleven men underwent a high-fat (HF; >65% fat from energy) or a high-carbohydrate (HC) diet (>60 % carbohydrate from energy). Energy intake was individualized, and commercially available foods were prepared and packaged for each participant 48 hr before they completed a laboratory-based duathlon (5-km run, 30 km cycling, and 10-km run). Blood samples were obtained before, immediately after, and 1 and 2 hr after the
duathlon for determination of glucose, insulin, and glucagon. Oxygen consumption, ratings of perceived exertion, and respiratory-exchange ratio were assessed, and fat and carbohydrate oxidation were estimated before, during, and after the duathlon. Dietary records indicated a significant difference in fat content ingested before the duathlons. Time to complete the duathlon did not differ between the HC- and the HF-diet trials. Carbohydrate-oxidation rate was significantly higher during the HC-diet trial than during the HF-diet trial. Fat-oxidation rates were also significantly higher in the HF-diet trial than in the HC-diet trial. No differences in of perceived exertion were found between dietary trials. Blood glucose concentration was significantly higher immediately after the duathlon in the HC-diet trial than in the HF-diet trial and remained higher 1 and 2 hr after the duathlon. It was concluded that Duathlon performance was not altered by short-term changes in dietary fat or carbohydrate composition despite higher blood glucose concentrations under the HC condition [09328].

The aim of one study was to determine if the carbohydrate (CHO) availability alters the rate of increase in the rating of perceived exertion (RPE) during high intensity exercise and whether this would be associated with physiological changes. Six males performed high intensity exercise after 48 h of controlled, high CHO (80 %) and low CHO (10 %) diets. Time to exhaustion was lower in the low compared to high CHO diet. The rate of increase in RPE was greater and the VO$_2$ slow component was lower in the low CHO diet than in the control. There was no significant condition effect for cortisol, insulin, pH, plasma glucose, potassium, or lactate concentrations. Multiple linear regression indicated that the total amplitude of VO$_2$ and perceived muscle strain accounted for the greatest variance in the rate of increase in RPE. These results suggest that cardiorespiratory variables and muscle strain are important afferent signals from the periphery for the RPE calculations [10396].

The aim of one study was to investigate the prerace and during-race carbohydrate intakes of elite-level triathletes contesting draft-legal Olympic-distance triathlon (ODT) events. Self-reported prerace and during-race nutrition data were collected at 3 separate ODT events from 51 elite senior and under-23 triathletes. One hundred twenty-nine observations of food and fluid intake representing actual prerace (n=62) and during-race (n=67) nutrition practices from 36 male and 15 female triathletes were used in the final analysis of this study. Female triathletes consumed significantly more carbohydrate on the morning before race start when corrected for body mass and race start time than their male counterparts. Male and female triathletes consumed 26 percent more energy (kJ/kg) and 24 percent more carbohydrate (g/kg) when commencing a race after midday (1:00-1:30 p.m.) than for a late morning (11:00-11:15 a.m.) race start. During the race, triathletes consumed less than 60 g of carbohydrate on 66 percent of occasions, with average total race intakes of 48 ± 25 and 49 ± 25 g carbohydrate for men and women, respectively. Given average race times of 1:57:07 hr and 2:08:12 hr, hourly carbohydrate intakes were about 25 g and 23 g for men and women, respectively. Although most elite ODT triathletes consume sufficient carbohydrate to meet recommended prerace carbohydrate intake guidelines, during-race carbohydrate intakes varied considerably, with many failing to meet recommended levels [10397].

The effect of moderate dehydration and consequent fluid replenishment on short-duration maximal treadmill performance was studied in eight healthy, fit, young males. The study involved a within subject, blinded, crossover, placebo design. Initially, all subjects performed a baseline exercise test using an individualized treadmill protocol structured to induce exhaustion in 7 to 10 min. On each of the three subsequent testing days, the subjects exercised at 70-75 percent VO$_{2\text{max}}$ for 60 min at 29-33 degrees C, resulting in a dehydration weight loss of 1.8-2.1 percent body weight. After 60 min of rest and recovery at 22 C, subjects performed the same treadmill test to voluntary exhaustion, which resulted in a small reduction in VO$_{2\text{max}}$ and a decline in treadmill performance by 3% relative to the baseline results. Following another 60 min rest and recovery, subjects ingested the same amount of
fluid lost in the form of one of three lemon-flavored, randomly assigned commercial drinks, namely Crystal Light (placebo control), Gatorade® and Rehydrate Electrolyte Replacement Drink, and then repeated the treadmill test to voluntary exhaustion. VO₂max returned to baseline levels with Rehydrate, while there was only a slight improvement with Gatorade and Crystal Light. There were no changes in heart rate or ventilation with all three different replacement drinks. Relative to the dehydrated state, a 6.5 percent decrease in treadmill performance time occurred with Crystal Light, while replenishment with Gatorade, which contains fructose, glucose, sodium and potassium, resulted in a 2.1 percent decrease. In contrast, treatment with Rehydrate, which comprises fructose, glucose polymer, calcium, magnesium, sodium, potassium, amino acids, thiols and vitamins, resulted in a 7.3 percent increase in treadmill time relative to that of the dehydrated state. The results indicate that constituents other than water, simple transportable monosaccharides and sodium are important for maximal exercise performance and effective recovery associated with endurance exercise-induced dehydration [10398].

Carbohydrate is an essential part of the human diet and is the macronutrient that supplies the greatest fraction of total energy intake for most people. Because of its central role in energy metabolism during exercise, it plays a vital role in the athlete's diet. Though well-chosen foods can meet carbohydrate needs in many situations, carbohydrate supplements may be useful before, during and after exercise to help athletes achieve their nutrition goals. These supplements may be in solid, liquid or gel format and may or may not contain other nutrients. Judicious selection of carbohydrate-containing foods and supplements in the overall diet and around exercise sessions can help athletes to optimise training and competition performance. In this review, the authors have included two tables giving current recommendations for carbohydrate intakes for sport, as well as a range of products that can help to meet these goals [10223].

The term “carbohydrate” refers to members of a large family of organic compounds composed of carbon, hydrogen and oxygen with the general formula Cₘ(H₂O)ₙ. Carbohydrates can exist as single molecules (monosaccharides) such as glucose (a six-carbon hexose with the formula C₆H₁₂O₆), but these can polymerise to form chains that vary in length from two (disaccharides) to tens of thousands of glucose units (polysaccharides). ‘Sugars’ is the term used to refer to monosaccharides such as glucose (dextrose) and fructose (fruit sugar), and the disaccharides sucrose (table sugar: one molecule of glucose and one of fructose) and lactose (milk sugar: glucose plus galactose). Five carbon pentoses include the sugar ribose, which forms an important part of several key molecules such as the adenine nucleotides (ATP, ADP and AMP) and RNA. Glycogen, the storage form of carbohydrate in liver and in muscle, has a complex glucose polymer structure and is in many ways similar to starch, which acts as a storage form of carbohydrate in plants. The polymerised form occupies much less space but also, being almost insoluble, can be stored without large amounts of extra water being retained by the cells. The total amount of carbohydrate stored in the body is small, with a maximum of about 100 g in the liver and 400–500 g in the muscles: these amounts depend on the preceding diet, and will be reduced by fasting and by exercise. Liver glycogen can be broken down to glucose and released into the bloodstream where it is available to all tissues to act as a fuel. This is especially important for the brain, which relies heavily on blood glucose as a fuel, and for other tissues such as the red blood cells which use blood glucose as their only substrate. The muscle store of glycogen is more immediately available when the muscles are called on to do work, but it is not so readily available to other tissues. Resting muscle can meet the majority of its energy demand by the oxidation of any available fuels, including fat as well as carbohydrate. During exercise, the rate of carbohydrate utilisation, and its contribution to the total fuel mix, varies according to a range of factors including the intensity and duration of exercise, the training state of the athlete and the effects of carbohydrate intake prior to and during the
session. There are several ways in which body carbohydrate stores are critical for sports performance. Carbohydrate is the primary fuel for high-intensity work when the metabolic demand requires the recruitment of high glycolytic muscle fibres; inadequate muscle glycogen stores will limit the performance of single or repeated high-intensity bouts. At moderate exercise intensities of long duration, the depletion of muscle glycogen is associated with fatigue and reduction in work capacity as the muscle becomes more reliant on fat as an energy substrate. Reductions in blood glucose concentration can also occur during exercise due to a mismatch between liver glucose release and muscle glucose uptake. In some athletes or events, this may progress to frank hypoglycaemia and obvious signs of fatigue, disorientation and impaired work capacity. However, central fatigue (or sub-optimal performance) may occur with more subtle changes in blood glucose concentrations or carbohydrate availability to the central nervous system. This can become manifest in terms of sub-optimal work capacity via reductions in pacing strategies or muscle fibre recruitment or as impairments of the skill and concentration which underpin the outcomes in many sports. Total body carbohydrate stores are limited and often substantially less than the fuel requirements of intensive training and competition sessions. Athletes are therefore guided to consume dietary sources of carbohydrates to avoid or delay the depletion of body carbohydrate stores during exercise. It should be noted that sports nutrition guidelines no longer promote a “high carbohydrate” diet for all athletes. Instead, general targets are provided to allow athletes to meet the carbohydrate fuel requirements for their specific training and competition schedules, with suggestions for total amounts of carbohydrate that might be consumed over a day, as well as goals for intake before and during exercise, or in the recovery period between one session and the next. There is good evidence that carbohydrate intake strategies which maintain high carbohydrate availability during exercise and prevent carbohydrate depletion are associated with enhanced endurance and performance. Such strategies include glycogen supercompensation prior to endurance and ultraendurance events; ingesting a carbohydrate-rich meal in the hours before events of prolonged (>90 min) sustained or intermittent exercise; carbohydrate intake during sustained high-intensity exercise lasting about 60 min or prolonged sustained/intermittent exercise, and in the recovery period between two bouts of carbohydrate-demanding exercise. Carbohydrate is an essential ingredient of effective sports drinks; water and carbohydrate have independent and additive performance-enhancing effects when ingested during endurance exercise. The primary source of carbohydrate comes from the diet, and sugar-rich and starch-rich foods can contribute to energy and fuel needs as well as providing other useful nutrients for health and performance. However, special sports products containing substantial amounts of carbohydrate provide a valuable nutrition aid in some situations. The advantages of these products include taste appeal, provision of a known amount of carbohydrate to meet a specific sports nutrition goal, simultaneous provision of other important nutrients for sports nutrition goals, and gastrointestinal characteristics promoting quick digestion and absorption. Other benefits relate to characteristics that make the products practical to consume around exercise sessions (low-bulk, conveniently packaged) or in the athlete’s lifestyle (portable, non-perishable, minimal preparation). When these sports products are used by an athlete to meet the sports nutrition goals or guidelines, they are likely to enhance performance. In fact, the performance benefits achieved by addressing a situation that would otherwise result in low carbohydrate availability are robust, ranking carbohydrate supplements among the performance enhancers with the strongest evidence base in sports nutrition. In the sports world, where millions of products with special ingredients are marketed with claims of enhanced performance, it is ironic that sports foods containing an everyday nutrient (carbohydrate) may be best able to deliver on these claims. Although some of these sports foods – for example, sports drinks – have crossed into the general food supply and are consumed outside the context of sport, when they are used appropriately to support the carbohydrate needs of exercise or recovery they are likely to produce detectable benefits [10223].
The objective was to determine the effects of carbohydrate (CHO) supplementation on exercise-induced hormone responses and post-training intramyocellular lipid stores (IMCL). Twenty-four elite male athletes (28 ± 1 years) were randomized to receive CHO (maltodextrin solution) or zero energy placebo solution (control group). The high-intensity running protocol consisted of 10×800 m at 100 percent of the best 3000-m speed and 2×1000 m maximal bouts in the morning and a submaximal 10-km continuous easy running in the afternoon of day 9. IMCL concentrations were assessed by 1H-MRS before (-day 9) and after training (day 9) in soleus (SO) and tibialis anterior (TA) muscles. Blood hormones were also measured before, during, and post-exercise. The percent change in TA-IMCL was higher in the CHO group than in the control group. Insulin concentrations were higher in the CHO group post-intermittent running compared to control. Circulating levels of free fatty acids and GH were lower in the CHO group. The decline in performance in the 2nd 1000-m bout was also attenuated in this group compared to control. The hormonal milieu (higher insulin and lower GH levels) in the CHO group, together with unchanged free fatty acid levels, probably contributed to the increased IMCL stores. This greater energy storage capacity may have improved post-exercise recovery and thus prevented performance deterioration [12381].

Carbohydrate and fat are the two primary fuel sources oxidized by skeletal muscle tissue during prolonged (endurance-type) exercise. The relative contribution of these fuel sources largely depends on the exercise intensity and duration, with a greater contribution from carbohydrate as exercise intensity is increased. Consequently, endurance performance and endurance capacity are largely dictated by endogenous carbohydrate availability. As such, improving carbohydrate availability during prolonged exercise through carbohydrate ingestion has dominated the field of sports nutrition research. As a result, it has been well-established that carbohydrate ingestion during prolonged (>2 h) moderate-to-high intensity exercise can significantly improve endurance performance. Although the precise mechanism(s) responsible for the ergogenic effects are still unclear, they are likely related to the sparing of skeletal muscle glycogen, prevention of liver glycogen depletion and subsequent development of hypoglycemia, and/or allowing high rates of carbohydrate oxidation. Currently, for prolonged exercise lasting 2-3 h, athletes are advised to ingest carbohydrates at a rate of 60 g/h (1.0-1.1 g/min) to allow for maximal exogenous glucose oxidation rates. However, well-trained endurance athletes competing longer than 2.5 h can metabolize carbohydrate up to 90 g/h (1.5-1.8 g/min) provided that multiple transportable carbohydrates are ingested (e.g. 1.2 g/min glucose plus 0.6 g/min of fructose). Surprisingly, small amounts of carbohydrate ingestion during exercise may also enhance the performance of shorter (45-60 min), more intense (>75 % peak oxygen uptake; VO₂peak) exercise bouts, despite the fact that endogenous carbohydrate stores are unlikely to be limiting. The mechanism(s) responsible for such ergogenic properties of carbohydrate ingestion during short, more intense exercise bouts has been suggested to reside in the central nervous system. Carbohydrate ingestion during exercise also benefits athletes involved in intermittent/team sports. These athletes are advised to follow similar carbohydrate feeding strategies as the endurance athletes, but need to modify exogenous carbohydrate intake based upon the intensity and duration of the game and the available endogenous carbohydrate stores. Ample carbohydrate intake is also important for those athletes who need to compete twice within 24 h, when rapid repletion of endogenous glycogen stores is required to prevent a decline in performance. To support rapid post-exercise glycogen repletion, large amounts of exogenous carbohydrate (1.2 g/kg/h) should be provided during the acute recovery phase after exhaustive exercise. For those athletes with a lower gastrointestinal threshold for carbohydrate ingestion immediately post-exercise, and/or to support muscle re-conditioning, co-ingesting a small amount of protein (0.2-0.4 g/kg/h) with less carbohydrate (0.8 g/kg/h) may provide a feasible option to achieve similar muscle glycogen repletion rates [13611].
It is now well established that protein supplementation after resistance exercise promotes increased muscle protein synthesis, which ultimately results in greater net muscle accretion, relative to exercise alone or exercise with supplementary carbohydrate ingestion. However, it is not known whether combining carbohydrate with protein produces a greater anabolic response than protein alone. Recent recommendations have been made that the composition of the ideal supplement post-exercise would be a combination of a protein source with a high glycemic index carbohydrate. This is based on the hypothesis that insulin promotes protein synthesis, thus maximising insulin secretion will maximally potentiate this action. However, it is still controversial as to whether raising insulin level, within the physiological range, has any effect to further stimulate muscle protein synthesis. One commentary reviewed the evidence underpinning the recommendation to consume carbohydrates in addition to a protein supplementation after resistance exercise for the specific purpose of increasing muscle mass. The paucity of data will be discussed, thus our conclusions are that further studies are necessary prior to any conclusions that enable evidence-based recommendations to be made [13612].

There is a consensus claiming an ergogenic effect of carbohydrates ingested in the proximity of or during a performance bout. However, in performance studies, the protocols that are used are often highly standardized (e.g. fasted subjects, constant exercise intensity with time-to-exhaustion tests), and do not necessarily reflect competitive real-life situations. Therefore, we aimed at systematically summarizing all studies with a setting mimicking the situation of a real-life competition (e.g. subjects exercising in the postprandial state and with time-trial-like performance tests such as fixed distance or fixed time tests). We performed a PubMed search by using a selection of search terms covering inclusion criteria for sport, athletes, carbohydrates, and fluids, and exclusion criteria for diseases and animals. This search yielded 16,658 articles and the abstract of 16,508 articles contained sufficient information to identify the study as non-eligible for this review. The screening of the full text of the remaining 150 articles yielded 17 articles that were included in this review. These articles described 22 carbohydrate interventions covering test durations from 26 to 241 min (mostly cycling). It was observed no performance improvement with half of the carbohydrate interventions, while the other half of the interventions had significant improvement between 1 and 13 percent (improvement with one of five interventions lasting up to 68 min and with 10 of 17 interventions lasting between 70 and 241 min). Thus, when considering only studies with a setting mimicking real-life competition, there is a mixed general picture about the ergogenic effect of carbohydrates ingested in the proximity of or during a performance bout with an unlikely effect with bouts up to perhaps 70 min and a possible but not compelling ergogenic effect with performance durations longer than about 70 min [13613].

Carbohydrates are one of the two main fuels for sport activities, and their relevance for optimal sport performance is undisputed among experts. In general, there is a consensus claiming an ergogenic effect of carbohydrates ingested just before or during a performance bout. However, in scientifically aimed studies the subjects are often fasted overnight even in performance studies. The reason for this is probably that the metabolism in fasted subjects is in a more balanced state, which might be more easily reproduced than a postprandial state. However, the recommendation to athletes is not to compete in a fasted state because of potentially reduced liver glycogen stores and a subsequent negative effect on performance. While this concern might be unsubstantiated (overnight-fasted well-trained subjects can have more than twofold higher liver glycogen levels compared to overnight-fasted non-athletes, about 130 g versus about 50 g, respectively), athletes almost intuitively do not compete in a fasted state. Further, a test mode assessing how long a subject can exercise at a given intensity is common in “performance” studies (e.g. time-to-exhaustion tests). This is also does not reflect the real-life situation as usually a sporting event, at least in elite sports,
requires performing either as fast as possible for a given distance (e.g. races) or as well as possible within a given time (e.g. team sports) [13613].

The current consensus indicates that carbohydrates ingested in the proximity of or during a performance bout are ergogenic. However, the application of rigorous criteria to a systematic review, such as excluding fasted subjects and time-to-exhaustion test modes, led to a less convincing picture. It was observed no significant performance improvement with most of the performance bouts lasting less than 70 min, and the results with longer performance bouts indicated a significant improvement with 10 of 17 interventions. The absence of clear evidence is, nevertheless, not clear evidence of an absent effect. This is particularly true for the present review as we discarded many studies because relevant information was missing in the articles. As mentioned above, we encountered studies among other with missing information on age, gender, prandial state, or \( \text{VO}_{2\text{max}} \) of the subjects, missing information on the blinding or randomization of the interventions, or missing information on the drink volume ingested during the intervention. Thus, it cannot be excluded that some or even all of these discarded studies would have met the inclusion criteria if only the description were appropriate, and that then the outcome would have been a different one. Being conservative, however, it can be stated that with shorter duration events up to perhaps 70 min an ergogenic effect of carbohydrates ingested in the proximity of or during a performance bout is unlikely with trained (but not elite) male athletes in a real-life competition. The picture for longer durations is slightly more in favor than against the current consensus, but it is too heterogeneous for a solid conclusion [13613].

**Effects of carbohydrate loading**

The purpose of the present study was to examine the effects of a high- or low-carbohydrate (CHO) diet on performance, aerobic and anaerobic contribution, and metabolic responses during supramaximal exercise. Six physically-active men first performed a cycling exercise bout at 115 percent maximal oxygen uptake to exhaustion after following their normal diet for 48 h (about 50 % of CHO, control test). Seventy-two hours after, participants performed a muscle glycogen depletion exercise protocol, followed by either a high- or low-CHO diet (about 70 and 25 % of CHO, respectively) for 48 h, in a random, counterbalanced order. After the assigned diet period (48 h), the supramaximal cycling exercise bout (115 % maximal oxygen consumption) to exhaustion was repeated. The low-CHO diet reduced time to exhaustion when compared with both the control and the high-CHO diet (-19 and -32 %, respectively). The reduced time to exhaustion following the low-CHO diet was accompanied by a lower total aerobic energy contribution (-39 %) compared with the high-CHO diet. However, the aerobic and anaerobic energy contribution at the shortest time to exhaustion (isotime) was similar among conditions. The low-CHO diet was associated with a lower blood lactate concentration, with no effect on the plasma concentration of insulin, glucose and \( \text{K}^+ \). In conclusion, a low-CHO diet reduces both performance and total aerobic energy provision during supramaximal exercise. As peak \( \text{K}^+ \) concentration was similar, but time to exhaustion shorter, the low-CHO diet was associated with an earlier attainment of peak plasma \( \text{K}^+ \) concentration [13614].

**Glucose homeostasis in athletes**

The maintenance of normal blood glucose levels at rest and during exercise is critical. The maintenance of blood glucose homeostasis depends on the coordination and integration of several physiological systems, including the sympathetic nervous system and the endocrine system. During prolonged exercise increased demand for glucose by contracting muscle causes to increase glucose uptake to working skeletal muscle. Increase in glucose uptake by working skeletal muscle during prolonged exercise is due to an increase in the translocation
of insulin and contraction sensitive glucose transporter-4 (GLUT4) proteins to the plasma membrane. However, normal blood glucose level can be maintained by the augmentation of glucose production and release through the stimulation of liver glycogen breakdown, and the stimulation of the synthesis of glucose from other substances, and by the mobilization of other fuels that may serve as alternatives. Both feedback and feedforward mechanisms allow glycemia to be controlled during exercise. One review focused on factors that control blood glucose homeostasis during prolonged exercise [07297].

Physical activity is essential for weight control, for limiting onset and complications of chronic disorders and for preventing impaired insulin sensitivity. Little is known about the glycaemic adaptations of physically active subjects, especially elite and professional athletes. Thus, it was evaluated the glycaemic control in elite and professional cyclists by assessing fasting plasma glucose (FPG) and glycated haemoglobin (HbA1c). The study population consisted of 47 male professional road cyclists, 72 male elite road cyclists and 58 male sedentary blood donors. A significant difference was observed for FPG between sedentary controls and either elite or professional cyclists. Athletes showed a consistent trend towards higher HbA1c values, reaching statistical significance between sedentary individuals and professional cyclists. In multiple linear regression analysis, the intensity of physical exercise is inversely correlated with FPG and directly correlated with HbA1c. These results demonstrate a significant association between intensity of the training regimen and both FPG and HbA1c, highlighting the need for establishing the appropriate critical difference for the measurement of FPG and HbA1c according to the training and exercise workload [07298].

**Effect on perceived exertion**

The aim of this study was to determine if the carbohydrate (CHO) availability alters the rate of increase in the rating of perceived exertion (RPE) during high intensity exercise and whether this would be associated with physiological changes. Six males performed high intensity exercise after 48 h of controlled, high CHO (80 %) and low CHO (10 %) diets. Time to exhaustion was lower in the low compared to high CHO diet. The rate of increase in RPE was greater and the VO₂ slow component was lower in the low CHO diet than in the control. There was no significant condition effect for cortisol, insulin, pH, plasma glucose, potassium, or lactate concentrations. Multiple linear regression indicated that the total amplitude of VO₂ and perceived muscle strain accounted for the greatest variance in the rate of increase in RPE. These results suggest that cardiorespiratory variables and muscle strain are important afferent signals from the periphery for the RPE calculations [10399].

One study was designed to determine the effects of ingesting a carbohydrate (CHO) solution on affective states and rating of perceived exertion (RPE) during prolonged intermittent high-intensity exercise. Seventeen male soccer players completed a prolonged intermittent high-intensity exercise protocol for 90 min on two occasions, separated by at least 7 days. Participants consumed either a 6.4 percent CHO (0.6 g/kg body mass (BM)/h) or an artificially sweetened placebo (PLA) solution immediately before (8 mL/kg BM) and every 15 min (3 mL/kg BM) during exercise in a double-blind, counterbalanced design. Pleasure-displeasure, perceived activation, RPE and plasma glucose concentration was assessed. The results showed that compared with the CHO trial, perceived activation were lower in the placebo trial during the last 30 min of exercise and this was accompanied by lowered plasma glucose concentrations. In the CHO trial, RPE was maintained in the last 30 min of exercise but carried on increasing in the PLA trial. Therefore, CHO ingestion during prolonged high-intensity exercise appears to elicit an enhanced perceived activation profile that may impact upon task persistence and performance. This finding is in addition to the physiological and metabolic benefits of the exogenous energy supply [07270].
The purpose of one study was to examine the effect of carbohydrate supplementation on differentiated and undifferentiated ratings of perceived exertion (RPE) during prolonged intermittent exercise and recovery. Twelve male subjects cycled for 2.0 h at 64 percent $W_{\text{max}}$ and 73 percent $V_{\text{O2peak}}$ with 3-min rest intervals interspersed every 10 min (2.6 h of total exercise time, including rest intervals) with placebo (P) or carbohydrate (C) beverages. RPE was assessed during the last minute of each 10-min exercise interval and then every 30 s during the 3-min recovery period. The pattern of change in RPE over time was significantly different between C and P ingestion, with attenuated RPE responses found for both overall body (O) and legs (L). A significant main effect was found for recovery RPE-O between C and P ingestion, with attenuated RPE responses found in the later part of the 2-h run. C relative to P ingestion was associated with higher respiratory exchange ratios and plasma levels of glucose and with lower levels of plasma cortisol. These data indicate that carbohydrate supplementation attenuates perceived exertion during prolonged intermittent exercise and recovery [07271].

Effect on endurance exercise

The main aim of one study was to investigate the influence of consuming a 6 percent carbohydrate-electrolyte (CHO-E) solution on the intermittent, high-intensity endurance performance and capacity of adolescent team games players. Fifteen participants (mean age 13 years) performed two trials separated by 3-7 days. In each trial, they completed 60 min of exercise composed of four 15-min periods of part A of the Loughborough Intermittent Shuttle Test, followed by an intermittent run to exhaustion (part B). In a double-blind, randomised, counterbalanced fashion participants consumed either the 6 percent CHO-E solution or a non-carbohydrate (CHO) placebo (5 ml/kg BM) during the 5 min pre-trial and after each 15-min period of part A (2 ml/kg BM). Time to fatigue was increased by 25 percent during part B when CHO was ingested, with distance covered in part B also significantly greater in the CHO trial. No significant between-trials differences were observed for mean 15-m sprint time, peak sprint time, or heart rate during part A. These results demonstrate, for the first time, that ingestion of a CHO-E solution significantly improves the intermittent, high-intensity endurance running capacity of adolescent team games players during an exercise protocol designed to simulate the physiological demands of team games [10400].

The purpose of one study was to determine if the type of diet can influence blood glucose levels during a regular swimming training session. Twelve elite male swimmers were enrolled for this study. Swimmers were put on two isocaloric three-day diets, containing either a high percentage of carbohydrate (HCD-65 %) or a low percentage of carbohydrate (LCD-35 %) before performing the two 110 minutes training sessions (inter-spaced by 6-8 days) at an intensity of 75 percent of peak heart rates. Blood glucose was collected and analyzed at 0, 20, 60 and 110 minutes of exercise. Blood glucose under both dietary conditions initially dropped and thereafter rose again by the end of the 110 minute training session. The data revealed that blood glucose never dropped to hypoglycemic levels, but showed a different pattern of changes throughout training. The carbohydrate content of the diet followed for a period of three days prior to testing, does not appear to directly affect blood glucose levels throughout a swimming training session lasting over 90 minutes, in elite national level skimmers [08389].

Rapid and complete rehydration, or restoration of fluid spaces, is important when acute illness or excessive sweating has compromised hydration status. Many studies have investigated the effects of graded concentrations of sodium and other electrolytes in rehydration solutions; however, no study to date has determined the effect of carbohydrate on fluid retention when electrolyte concentrations are held constant. The purpose of one
study was to determine the effect of graded levels of carbohydrate on fluid retention following exercise-induced dehydration. Fifteen heat-acclimatized men exercised in the heat for 90 min with no fluid to induce 2-3 percent dehydration. After a 30-min equilibration period, they received, over the course of 60 min, one of five test beverages equal to 100 percent of the acute change in body mass. The experimental beverages consisted of a flavored placebo with no electrolytes (P), placebo with electrolytes (P + E), 3 percent, 6 percent, and 12 percent carbohydrate solutions with electrolytes. All beverages contained the same type and concentration of electrolytes (18 meq/l Na⁺, 3 meq/l K⁺, 11 meq/l Cl⁻). Subjects voided their bladders at 60, 90, 120, 180, and 240 min, and urine specific gravity and urine volume were measured. Blood samples were taken before exercise and 30, 90, 180, and 240 min following exercise and were analyzed for glucose, sodium, hemoglobin, hematocrit, renin, aldosterone, and osmolality. Body mass was measured before and after exercise and a final body mass was taken at 240 min. There were no differences in percent dehydration, sweat loss, or fluid intake between trials. Fluid retention was significantly greater for all carbohydrate beverages compared with P. P + E was not different from water, 3 percent or 6 percent but was significantly less than 12 percentretention of the ingested fluid. No difference was found between the carbohydrate beverages. Carbohydrate at the levels measured exerts a mild influence on fluid retention in postexercise recovery [09323].

One study examined the effect of consuming carbohydrate-(CHO) electrolyte solution on running performance after different-glycemic-index (GI) meals. Nine men completed 3 trials in a randomized counterbalanced order, with trials separated by at least 7 days. Two hours before the run after an overnight fast, each participant consumed a high-GI (GI=83) or low-GI (GI=36) CHO meal or low-energy sugar-free Jell-O (GI=0, control). The two isocaloric GI meals provided 1.5 g available CHO/kg body mass. During each trial, 2 ml/kg body mass of a 6.6 percent CHO-electrolyte solution was provided immediately before exercise and every 2.5 km after the start of running. Each trial consisted of a 21-km performance run on a level treadmill. The participants were required to run at 70 percent VO₂max during the first 5 km of the run. They then completed the remaining 16 km as fast as possible. There was no difference in the time to complete the 21-km run and there were no differences in total CHO and fat oxidation throughout the trials, despite differences in preexercise blood glucose, serum insulin, and serum free-fatty-acid concentrations. It was concluded that when a CHO-electrolyte solution is consumed during a 21-km run, the GI of the preexercise CHO meal makes no difference in running performance [09324].

There is a growing body of research on the influence of ingesting carbohydrate-electrolyte solutions immediately prior to and during prolonged intermittent, high-intensity exercise (team games exercise) designed to replicate field-based team games. One review presents the current body of knowledge in this area, and identifies avenues of further research. Almost all early work supported the ingestion of carbohydrate-electrolyte solutions during prolonged intermittent exercise, but was subject to methodological limitations. A key concern was the use of exercise protocols characterized by prolonged periods at the same exercise intensity, the lack of maximal- or high-intensity work components and long periods of seated recovery, which failed to replicate the activity pattern or physiological demand of team games exercise. The advent of protocols specifically designed to replicate the demands of field-based team games enabled a more externally valid assessment of the influence of carbohydrate ingestion during this form of exercise. Once again, the research overwhelmingly supports carbohydrate ingestion immediately prior to and during team games exercise for improving time to exhaustion during intermittent running. While the external validity of exhaustive exercise at fixed prescribed intensities as an assessment of exercise capacity during team games may appear questionable, these assessments should perhaps not be viewed as exhaustive exercise tests per se, but as indicators of the ability to maintain high-intensity exercise, which is a recognized marker of performance and fatigue during field-based team
games. Possible mechanisms of exercise capacity enhancement include sparing of muscle glycogen, glycogen resynthesis during low-intensity exercise periods and attenuated effort perception during exercise. Most research fails to show improvements in sprint performance during team games exercise with carbohydrate ingestion, perhaps due to the lack of influence of carbohydrate on sprint performance when endogenous muscle glycogen concentration remains above a critical threshold of 200 mmol/kg dry weight. Despite the increasing number of publications in this area, few studies have attempted to drive the research base forward by investigating potential modulators of carbohydrate efficacy during team games exercise, preventing the formulation of optimal carbohydrate intake guidelines. Potential modulators may be different from those during prolonged steady-state exercise due to the constantly changing exercise intensity and frequency, duration and intensity of rest intervals, potential for team games exercise to slow the rate of gastric emptying and the restricted access to carbohydrate-electrolyte solutions during many team games. The review highlights fluid volume, carbohydrate concentration, carbohydrate composition and solution osmolality; the glycaemic index of pre-exercise meals; fluid and carbohydrate ingestion patterns; fluid temperature; carbohydrate mouthwashes; carbohydrate supplementation in different ambient temperatures; and investigation of all of these areas in different subject populations as important avenues for future research to enable a more comprehensive understanding of carbohydrate ingestion during team games exercise [11436].

Effects of a carbohydrate beverage on the physiological responses to load carriage were examined. Ten fit male participants (age: 28 ± 9 years, body mass: 82 ± 11 kg, O2max: 55 ± 6 mL/kg/min) completed two test conditions in random order, walking on a treadmill (6.5 km/h)) for 120 min, carrying a 25-kg backpack. At 0 and 60 min of exercise participants consumed 250 mL of a placebo (flavoured water) (PLA) or 6.4 percent carbohydrate (CHO) beverage. There were no differences in O2max respiratory exchange ratio (RER), heart rate or EMG activity of m. rectus femoris, m. vastus lateralis, m. semitendinosus and m. biceps femoris between conditions at minute 5 of exercise. The increase in O2max between minutes 5 and 120 was less during CHO than PLA (8 ± 5 vs. 14 ± 6 %). RER decreased during PLA, from 0.96 ± 0.05 at minute 5 to 0.87 ± 0.04 at minute 120, but not during CHO. Heart rate increased between minutes 5 and 120 during PLA (16 ± 10 %) and CHO (12 ± 6 %), with no difference between conditions. EMG peak RMS did not change between minutes 7 and 107 during PLA or CHO for the leg muscles. However, individual responses in EMG were highly variable (i.e. both increases and decreases in RMS). It was concluded that carbohydrate intake during load carriage reduced the O2max drift, which could be partially attributed to higher carbohydrate oxidation rates. Despite muscle fatigue/damage previously being identified as a cause of O2max drift, it appears that carbohydrate had no effect on neuromuscular responses during load carriage [11384].

One study was a systematic review with meta-analysis examining the efficacy of carbohydrate (CHO) ingestion compared with placebo (PLA) on endurance exercise performance in adults. Relevant databases were searched to January 2011. Included studies were PLA-controlled, randomized, crossover designs in which CHO ingestion not exceeding 8 percent and between 30 and 80 g/h during exercise of ≥1 h was evaluated via time trial (TT) or exercise time to exhaustion (TTE). The between-trial standardized mean differences (effect size, ES) and pooled estimates of the effect of CHO ingestion were calculated. Of the 41,175 studies from the initial search, 50 were included. The ES for submaximal exercise followed by TT was significant as was the effect size for TT. The weighted mean improvement in exercise performance favored CHO ingestion. TTE and submaximal exercise followed by TTE also showed significant effects, with weighted mean improvements of 15 and 54 percent, respectively, with CHO ingestion. Similar trends were evident for subanalyses of studies using only male or trained participants, for exercise of 1-3 h duration, and where CHO and PLA beverages were matched for electrolyte content. The data support
that ingestion of CHO between 30 and 80 g/h enhances endurance exercise performance in adults [11250].

Effects of a carbohydrate beverage on the physiological responses to load carriage were examined. Ten fit male participants completed two test conditions in random order, walking on a treadmill for 120 min, carrying a 25-kg backpack. At 0 and 60 min of exercise participants consumed 250 mL of a placebo (flavoured water) (PLA) or 6.4 percent carbohydrate (CHO) beverage. There were no differences in respiratory exchange ratio (RER), heart rate or EMG activity of m. rectus femoris, m. vastus lateralis, m. semitendinosus and m. biceps femoris between conditions at minute 5 of exercise. The increase between minutes 5 and 120 was less during CHO than PLA. RER decreased during PLA, from 0.96 ± 0.05 at minute 5 to 0.87 ± 0.04 at minute 120, but not during CHO. Heart rate increased between minutes 5 and 120 during PLA and CHO, with no difference between conditions. EMG peak RMS did not change between minutes 7 and 107 during PLA or CHO for the leg muscles. However, individual responses in EMG were highly variable (i.e. both increases and decreases in RMS). It was concluded that carbohydrate intake during load carriage reduced the O₂ drift, which could be partially attributed to higher carbohydrate oxidation rates. Despite muscle fatigue/damage previously being identified as a cause of O₂ drift, it appears that carbohydrate had no effect on neuromuscular responses during load carriage [11251].

An athlete’s carbohydrate intake can be judged by whether total daily intake and the timing of consumption in relation to exercise maintain adequate carbohydrate substrate for the muscle and central nervous system (“high carbohydrate availability”) or whether carbohydrate fuel sources are limiting for the daily exercise programme (“low carbohydrate availability”). Carbohydrate availability is increased by consuming carbohydrate in the hours or days prior to the session, intake during exercise, and refuelling during recovery between sessions. This is important for the competition setting or for high-intensity training where optimal performance is desired. Carbohydrate intake during exercise should be scaled according to the characteristics of the event. During sustained high-intensity sports lasting about 1 h, small amounts of carbohydrate, including even mouth-rinsing, enhance performance via central nervous system effects. While 30-60 g/h is an appropriate target for sports of longer duration, events >2.5 h may benefit from higher intakes of up to 90 g/h. Products containing special blends of different carbohydrates may maximize absorption of carbohydrate at such high rates. In real life, athletes undertake training sessions with varying carbohydrate availability. Whether implementing additional “train-low” strategies to increase the training adaptation leads to enhanced performance in well-trained individuals is unclear [11252].

One study investigated the effects of high and low glycemic index (GI) 24 h recovery meals on the physiological responses and subsequent athletic performance, following a glycogen depleting protocol. Ten well trained cyclists participated in two trials in a randomized cross-over design. On day 1, subjects performed a glycogen depleting protocol after which they then consumed either high or low GI recovery diets over the next 24 h, which provided 8 g/kg BW of carbohydrate. On day 2, the subjects returned to the laboratory, 2-3 h postprandial, to perform a 40 km time trial (TT) on the Velotron cyclePro© ergometer. No difference was observed in TT performance times between the high GI trial and the low GI trial. Additionally, no differences in carbohydrate, fat oxidation or blood glucose concentration was observed. The results of the study suggest that the ingestion of a high GI carbohydrate 24 h recovery diet following glycogen depleting exercise, has no greater effect on endurance performance than consuming a low GI carbohydrate 24 h recovery diet. It may be concluded from these results that, provided enough carbohydrate is consumed during a 24 h recovery period, there is no difference in subsequent endurance performance [11254].
One study determined if recreational type of endurance exercise is limited by a short-term fast, such as an overnight fast or benefited by a carbohydrate supplement prior to and during endurance exercise. Six individuals ran at 70 percent VO₂max for 90 min under three dietary conditions (fed, fasted for 16 to 18 h, fasted plus CHO). RPE, RER, BG (blood glucose), and La (lactate) were similar between conditions throughout 90 min of exercise. FFA was higher only in the fed and fasted groups after exercise. It was concluded that the psychosomatic sensation, physiologic, and metabolic data all indicated that endurance exercise for up to 90 min for fit individuals is not limited by a short-term fast or enhanced by carbohydrate supplementation. These findings are of interest to persons who exercise to maintain and enhance health and are not concerned with elite performance [07272].

The purpose of one investigation was to determine the effects of 2.5 hours of cycling with and without carbohydrate supplementation on gross efficiency. Trained cyclists (n=15) were tested for VO₂max and lactate threshold during incremental tests to exhaustion. On 2 separate visits, cyclists performed 2.5 hours of cycling on an indoor trainer. A carbohydrate (C) or placebo (P) beverage was randomly provided and counterbalanced for each of the trials. Gross efficiency, cycling economy, power output, VO₂, lactate, and blood glucose were measured every 20 minutes during the 2.5-hour ride. Muscle glycogen was measured immediately before and after the ride from the vastus lateralis. Results indicated that power output and VO₂ decreased over time but were not different between trials. Relative gross efficiency and cycling economy during C were greater than P at 40 and 150 minutes. Blood glucose significantly decreased in P and was lower than C at all time points. Respiratory exchange ratio decreased over time in both trials, with a significant treatment effect at 40 and 150 minutes. Muscle glycogen decreased by 65 percent during both conditions but demonstrated no treatment effect. It was concluded that carbohydrate supplementation during 2.5 hours of cycling attenuated the decrease in gross efficiency possibly by maintaining blood glucose levels. This suggests that the positive effect of carbohydrate supplementation on endurance performance may be through the maintenance of metabolic efficiency [07269].

Curvilinear dose-response relationship of carbohydrate (0-120 g/h) and performance
There is a lack of consensus regarding the optimal range of carbohydrate (CHO) ingestion rates recommended for endurance athletes. One study investigated the relationship between CHO dose and cycling time trial performance to identify an optimal range of CHO ingestion rates for endurance performance. Fifty-one cyclists and triathletes (28 ± 7 years) across four research sites completed four trials. Each trial consisted of a 2-h constant load ride at 95 percent of the workload that elicited a 4-mmol/L blood lactate concentration immediately followed by a computer-simulated 20-km time trial, which subjects were asked to complete as quickly as possible. Twelve CHO electrolyte (18 mmol/L Na, 3 mmol/L K, and 11 mmol/L Cl) beverages (three at each site) were tested in a double-blind manner, providing subjects 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 g CHO (1:1:1 glucose-fructose-maltodextrin) per hour during the 2-h constant load ride at a fluid intake rate of 1 L/h. All subjects also consumed a noncaloric placebo on one counterbalanced test occasion. Data were natural log transformed, subjected to a mixed-model analysis, and are reported as adjusted treatment means. It was estimate incremental performance improvements of 1.0, 2.0, 3.0, 4.0, and 4.7 percent at 9, 19, 31, 48, and 78 g·h, respectively, with diminishing performance enhancement seen at CHO levels >78 g/h. It was concluded that CHO beverage ingestion and endurance (about 160 min) performance appear to be related in a curvilinear dose-response manner, with the best performance occurring with a CHO (1:1:1 glucose-fructose-maltodextrin) ingestion rate of 78 g/h [13615].

During exercise
Research on the performance effects of acute carbohydrate supplementation is comprehensive. Eighty-eight randomized crossover studies in which carbohydrate supplements were consumed with or without protein before and/or during exercise provided 155 estimates for performance effects in time-to-exhaustion tests or in time trials with or without a preload. For the mixed-model meta-analysis, all effects were converted into percentage changes in mean power in a non-preloaded time trial and weighted using percentage standard errors derived from exact p-values (in a minority of studies) or from estimated errors of measurement (in all other studies). Publication bias was assessed with a plot of t-values for the random-effect solutions versus standard errors. Probabilistic inferences were derived with reference to thresholds for small, moderate and large effects on performance of 0.5, 1.5 and 2.7 percent. Publication bias was reduced by excluding studies with a standard error >1.25 percent. In the remaining 73 studies and 122 estimates, the meta-analysed performance effects of carbohydrate supplements ranged from clear large improvements of >6 percent to clear moderate impairments of >2 percent. The best supplement inferred from the analysis consisted of a 3-10 percent carbohydrate-plus-protein drink providing 0.7 g/kg/h glucose polymers, 0.2 g/kg/h fructose and 0.2 g/kg/h protein. Substantial increases in the benefit of a supplement were probably small with an additional 9-hour fast and with the inclusion of 0.2 g/kg/h of protein, probably small to moderate with ingesting the first bolus not at the start of exercise but 1-4 hours before exercise, and possibly small with increasing the frequency of ingestion by three boluses per hour. Substantial reductions in the benefit of a supplement were possibly moderate with a supplement providing >0.25 g/kg/h fructose, and possibly small with an increase in ambient temperature of 10°C. The effect in subjects with maximal oxygen consumption higher by 10 mL/kg/min was probably trivial, and the effects of exercise duration were dependent on the concentration of carbohydrate plus protein in the supplement. The effect of including salt was unexpectedly trivial, and the effect of gender was unclear. Carbohydrate supplements with an appropriate composition and administration regimen can have large benefits on endurance performance. More research and better reporting are required to investigate the moderating effects of gender and salt [11437].

The effect of carbohydrate (CHO) consumption during prolonged endurance running on post-exercise inflammation and hepcidin levels was investigated. Eleven well-trained male endurance athletes completed a graded exercise test, followed by two experimental running trials in a randomized order. The two experimental trials consisted of a 90 min run at 75 percent of the peak oxygen uptake velocity (vO2peak), while consuming a solution with either 6 percent CHO or a placebo (PLA) equivalent at 3 ml/kg every 20 min. Serum interleukin-6 (IL-6), free hemoglobin (Hb), haptoglobin (Hp), hepcidin and iron parameters were assessed throughout the post-run recovery period. Serum iron and IL-6 were significantly elevated immediately post-run in both CHO and PLA, with no differences between trials. Serum-free Hb increased and Hp decreased significantly immediately post-run in both conditions. Serum soluble transferrin receptor levels were significantly below the baseline at 3 and 24 h post-run in both conditions. Serum hepcidin concentration recorded 3 h post-run in both conditions was significantly elevated, and had returned to the baseline by 24 h post-run. Thus, the use of a 6 percent CHO solution at 3 ml/kg/20 min during endurance running did not attenuate the inflammatory response and subsequent increase in serum hepcidin levels during the post-run recovery period [11413].

One study was designed to determine the effects of ingesting a carbohydrate (CHO) solution on affective states and rating of perceived exertion (RPE) during prolonged intermittent high-intensity exercise. Seventeen male soccer players completed a prolonged intermittent high-intensity exercise protocol for 90 min on two occasions, separated by at least 7 days. Participants consumed either a 6.4% CHO (0.6 g/kg body mass (BM)/h) or an artificially sweetened placebo (PLA) solution immediately before (8 mL/kg BM) and every 15 min (3
mL/kg BM) during exercise in a double-blind, counterbalanced design. Pleasure-displeasure, perceived activation, RPE and plasma glucose concentration was assessed. The results showed that compared with the CHO trial, perceived activation were lower in the placebo trial during the last 30 min of exercise and this was accompanied by lowered plasma glucose concentrations. In the CHO trial, RPE was maintained in the last 30 min of exercise but carried on increasing in the PLA trial. Therefore, CHO ingestion during prolonged high-intensity exercise appears to elicit an enhanced perceived activation profile that may impact upon task persistence and performance. This finding is in addition to the physiological and metabolic benefits of the exogenous energy supply [07282].

During and after sessions of high-intensity intermittent running exercise

One study evaluated the acute effects of carbohydrate supplementation on heart rate (HR), rate of perceived exertion (RPE), metabolic and hormonal responses during and after sessions of high-intensity intermittent running exercise. Fifteen endurance runners (26 years) performed two sessions of intermittent exercise under carbohydrate (CHO) and placebo (PLA) ingestion. The sessions consisted of 12 x 800 m separated by intervals of 1 min 30 s at a mean velocity corresponding to the previously performed 3-km time trial. Both the CHO and PLA sessions were concluded within approximately 28 min. Blood glucose was significantly elevated in both sessions and mean blood lactate was significantly higher in the CHO. The metabolic stress induced by the exercise model used was confirmed by the elevated HR (approximately 182 bpm) and RPE (approximately 18 on the 15-point Borg scale) for both conditions. No significant differences in plasma insulin, cortisol or free fatty acids were observed during exercise between the two trials. During the recovery period, free fatty acid and insulin concentrations were significantly lower in the CHO trial. Supplementation with CHO resulted in higher lactate associated with lipolytic suppression, but did not attenuate the cortisol, RPE or HR responses [06253].

Effect of pre-exercise carbohydrate-loading on endurance performance

One study examined the influence of 3-day isoenergetic carbohydrate (CHO) loading with different glycemic index (GI) and glycemic load (GL) meals on running performance and metabolic responses. With a randomized crossover design, nine male runners performed a 1-h run at 70 percent VO$_{2\text{max}}$ followed by a 10-km performance run after a 3-day diet adaptation, which involved different GI and GL meals (CHO intake (%), GI, and GL per day were 73%, 80, and 553 percent for the high GI and high GL (HH); 73%, 36, and 249 for the low GI and low GL (LL); and 31%, 79, and 227 for the high GI and low GL (HL), respectively). There were no differences in the time to complete the 10-km run between the two high-CHO trials; however, the performance in the LL trial was improved as compared to that in the HL trial. It appears that the amount, rather than the nature, of the CHO consumed during the 3-day isoenergetic CHO loading may be the most overriding factor on subsequent metabolism and endurance run performance [07274].
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The purpose of one study was to investigate the effect of pre-test carbohydrate (CHO) ingestion on anaerobic-threshold assessment using the lactate-minimum test (LMT). Fifteen competitive male distance runners capable of running 10 km in 33.5-43 min were used as subjects. LMT was performed following CHO (2x300 mL, 7 % solution) or comparable placebo (Pl) ingestion, in a double-blind, randomized order. The LMT consisted of two high-intensity 1 min treadmill runs (17-21 km/h), followed by an 8 min recovery period. Subsequently, subjects performed 5 min running stages, incremented by 0.6 km/h and separated by 1 min blood-sampling intervals. Tests were terminated after 3 consecutive increases in blood-lactate concentration (La) had been observed. Finger-tip capillary blood was sampled for lactate concentration and blood-glucose determination 30 min before the test's onset, during the recovery phase following the 2 high-intensity runs, and following each of the subsequent 5 min stages. Heart rate (HR) and rating of perceived exertion (RPE) were recorded after each stage. The lactate-minimum speed (LMS) was determined from the individual [La]-velocity plots and was considered reflective of the anaerobic threshold. Pre-test CHO ingestion had no effect on LMS, nor on concentration of lactate and glucose concentration at that speed, or on HR and RPE responses. Pre-test CHO ingestion therefore does not affect LMS or the LMT-estimated anaerobic threshold [07276].

One study compared effects of carbohydrate (CHO) and rest on oxidative stress during exercise. Cyclists (n=12) completed 4 randomized trials at 64 percent Wattsmax under 2 conditions (continuous cycling for 2 h [C] and cycling with 3-min rest every 10 min for 2.6 h [R]). Subjects cycled under each condition while receiving 6 percent CHO and placebo (PLA). CHO and PLA were given preexercise (12 mL/kg) and during exercise (4 mL/kg for 15 minutes). Blood was collected preexercise, postexercise, and 1 h postexercise and assayed for F2-isoprostanes, hydroperoxides (LH), nitrite, antioxidant capacity, glucose, insulin, cortisol, and epinephrine. F2-isoprostanes and LH were lower in CHO. Glucose, cortisol, and epinephrine exhibited significant effects, with postexercise levels of glucose higher and cortisol and epinephrine lower in CHO during the R condition. This pattern was identical in the C condition. Oxidative stress during cycling was unaffected by use of short rest intervals but was diminished by CHO [07277].

The purpose of this investigation was to determine the effects of 2.5 hours of cycling with and without carbohydrate supplementation on gross efficiency (GE). Trained cyclists (n=15) were tested for VO2max and lactate threshold during incremental tests to exhaustion. On 2 separate visits, cyclists performed 2.5 hours of cycling on an indoor trainer. A carbohydrate (C) or placebo (P) beverage was randomly provided and counterbalanced for each of the trials. Gross efficiency, cycling economy, power output, VO2, lactate, and blood glucose were measured every 20 minutes during the 2.5-hour ride. Muscle glycogen was measured immediately before and after the ride from the vastus lateralis. Results indicated that power output and VO2 decreased over time but were not different between trials. Relative GE and cycling economy during C were greater than P at 40 and 150 minutes. Blood glucose significantly decreased in P and was lower than C at all time points. Respiratory exchange ratio decreased over time in both trials, with a significant treatment effect at 40 and 150 minutes. Muscle glycogen decreased by 65 percent during both conditions but demonstrated no treatment effect. It was concluded that carbohydrate supplementation during 2.5 hours of cycling attenuated the decrease in GE possibly by maintaining blood glucose levels. This suggests that the positive effect of carbohydrate supplementation on endurance performance may be through the maintenance of metabolic efficiency [07278].
The purpose of one study was to investigate the effect of pre-test carbohydrate (CHO) ingestion on anaerobic-threshold assessment using the lactate-minimum test (LMT). Fifteen competitive male distance runners capable of running 10 km in 33.5-43 min were used as subjects. LMT was performed following CHO (2x300 mL, 7 % solution) or comparable placebo (Pl) ingestion, in a double-blind, randomized order. The LMT consisted of two high-intensity 1 min treadmill runs (17-21 km/h), followed by an 8 min recovery period. Subsequently, subjects performed 5 min running stages, incremented by 0.6 km/h and separated by 1 min blood-sampling intervals. Tests were terminated after 3 consecutive increases in blood-lactate concentration ([La]) had been observed. Finger-tip capillary blood was sampled for [La] and blood-glucose determination 30 min before the test's onset, during the recovery phase following the 2 high-intensity runs, and following each of the subsequent 5 min stages. Heart rate (HR) and rating of perceived exertion (RPE) were recorded after each stage. The lactate-minimum speed (LMS) was determined from the individual [La]-velocity plots and was considered reflective of the anaerobic threshold. Pre-test CHO ingestion had no effect on LMS, nor on [La] and glucose concentration at that speed, or on HR and RPE responses. Pre-test CHO ingestion therefore does not affect LMS or the LMT-estimated anaerobic threshold [07279].

Carbohydrate ingested 30-60 min before exercise may result in hypoglycaemia during exercise, a phenomenon often called rebound or reactive hypoglycaemia. There is considerable confusion regarding pre-exercise carbohydrate feeding with advice that ranges from “consume carbohydrate in the hour before exercise” to “avoid carbohydrate in the 60 min prior to exercise.” It was analysed the studies available in the literature to draw conclusions about the use of carbohydrate in the pre-exercise period. Without performing a meta-analysis, it is clear that the risk of reduced performance is minimal as almost all studies point towards unaltered or even improved performance. This is despite the rather large metabolic changes that occur in response to pre-exercise carbohydrate feeding. It can be concluded that advice to avoid carbohydrate feeding in the hour before exercise is unfounded. Nevertheless athletes may develop symptoms similar to those of hypoglycaemia, even though they are rarely linked to actual low glucose concentrations. An individual approach may therefore be necessary to minimize these symptoms even though they do not appear to be related to exercise performance [11249].

One study examined the effects of pre-exercise food on different glycemic indexes (GI) on exercise metabolism and endurance running capacity. Nine subjects performed 3 exercise trials on different days 15 min after ingesting: lentils (LGI), potatoes (HGI), and placebo. Each subject ingested an equal amount of each food (1 g/kg body mass) and ran on a level treadmill for 5 min at 60 percent, 45 min at 70 percent and then at 80 percent of VO\textsubscript{2max} until exhaustion. Serum glucose concentrations were higher 15 min after the HGI trial compared to the LGI and placebo trials. In addition, serum glucose levels were higher during the LGI trial at the time of exhaustion compared to the HGI and placebo trials. Plasma insulin levels, 15 min after ingestion, were higher in the HGI trial as compared to the LGI and placebo trials. Exercise time was longer during the LGI trial compared to the placebo, but the time to exhaustion in the HGI condition did not differ from the placebo. These results suggest that lentils, the LGI food, ingested 15 min before prolonged exercise maintained euglycemia during exercise and enhanced endurance running capacity [11253].

The effects of dietary factors such as carbohydrate (CHO) on endurance-running performance have been extensively studied under laboratory-based and simulated field conditions. Evidence from "real-life" events, however, is poorly characterized. The purpose of this observational study was to examine the associations between prerace and in-race nutrition tendencies and performance in a sample of novice marathoners. Forty-six college students (36 women and 10 men) age 21.3 ± 3.3 years recorded diet for 3 d before, the
morning of, and during a 26.2-mile marathon. Anthropometric, physiological, and performance measurements were assessed before the marathon so the associations between diet and marathon time could be included as part of a stepwise-regression model. Mean marathon time was 266 ± 42 min. A pre-marathon 2-mile time trial explained 73% of the variability in marathon time. Day-before + morning-of CHO was the only other significant predictor of marathon time, explaining an additional 4 percent of the variability in marathon time. Other factors such as age, body-mass index, gender, day-before + morning-of energy, and in-race CHO were not significant independent predictors of marathon time. In this sample of primarily novice marathoners, Day-before + morning-of CHO intake was associated with faster marathon time, independent of other known predictors. These results suggest that novice and recreational marathoners should consider consuming a moderate to high amount of CHO in the 24-36 hr before a marathon [13617].

Meal frequency of pre-exercise carbohydrate feedings
One study compared the effect of single and multiple carbohydrate feedings before exercise on biochemical and physiological responses during exercise. Eight males performed 3 runs for 1 h at 70 percent VO$_{2\text{max}}$ after consuming a meal containing 2.5 g carbohydrate per kg body mass in a single dose 3 h before exercise (SF), the same meal in 5 equal doses at 3, 2.5, 2, 1.5, and 1 h before exercise (MF), or a liquid placebo 3 h before exercise (P). RER and carbohydrate oxidation rates were higher in SF and MF compared to P trials, but there was no difference between SF and MF trials. Pre-exercise insulin was 2.0- and 3.4-fold higher in SF and MF, respectively, compared to P, and 1.7-fold higher in MF compared to SF. Glycerol and NEFA were higher in P compared to SF and MF trials before and at the end of exercise. In conclusion, a carbohydrate meal containing 2.5 g/kg ingested in doses over 3 h before running produced higher hyperinsulinemia pre-exercise than that produced when the meal was consumed in a single dose. Nevertheless, estimated carbohydrate utilization and adipose tissue lipolysis during exercise after multiple feedings seemed to be as high as after a single feeding [07284].

The day before
To investigate whether the “overnight second-meal effect” results in altered substrate oxidation during the postprandial period following breakfast and subsequent sub-maximal exercise in women. Seven recreationally active women were recruited for the study. In each trial, participants were provided with their evening meal on day 1, which was composed of either high glycaemic index (HGI) or low glycaemic index (LGI) carbohydrates (CHO). On day 2, participants were provided with a standard HGI breakfast and then performed a 60 min run at 65 percent VO$_{2\text{max}}$ 3 h later. The incremental area under the curve (IAUC) for plasma glucose concentrations during the postprandial period following breakfast was greater in the HGI trial compared to the LGI trial (P<0.01). Similarly, the IAUC for serum insulin concentrations was greater in the HGI trial than the LGI trial (P<0.05). No differences in plasma free-fatty acids (FFA) or plasma glycerol concentrations were found between trials during the postprandial period. During subsequent exercise, there were no significant differences in substrate metabolism. The glycaemic index of an evening meal does not alter substrate oxidation at rest following breakfast or during subsequent submaximal exercise in women. This study provides further evidence for the overnight second-meal effect on glycaemic responses following a LGI mixed evening meal [07280].

In women
The effect of different quantities of carbohydrate (CHO) intake on CHO metabolism during prolonged exercise was examined in endurance-trained females. On four occasions, eight females performed 2 h of cycling at approximately 60 percent VO$_{2\text{max}}$ with ingestion of beverages containing low (LOW, 0.5 g/min), moderate (MOD, 1.0 g/min), or high (HIGH, 1.5 g/min) amounts of CHO, or water only (WAT). Test solutions contained trace amounts of [U-
\[^{13}\text{C}]\) glucose. Indirect calorimetry combined with measurement of expired \[^{13}\text{C}]\text{CO}_2 \text{ and plasma }^{13}\text{C} \text{ enrichment enabled calculation of exogenous CHO, liver-derived glucose, and muscle glycogen oxidation during the last 30 min of exercise. The highest rates of exogenous CHO oxidation were observed in MOD, with no further increases in HIGH. Endogenous CHO oxidation was lowest in MOD. Compared with WAT, CHO ingestion reduced liver glucose oxidation during exercise by approximately 30 percent. Differential rates of muscle glycogen oxidation were observed with different CHO doses. It was concluded that in endurance-trained women, the highest rates of exogenous CHO oxidation and greatest endogenous CHO sparing was observed when CHO was ingested at moderate during exercise [07281].

**Effect on combat sports**

The purpose of the current study was to investigate elite female (n=21) and male (n=47) combat sports athletes' (n=68; mean age (± SD) 21 ± 4 years; mean height 177 ± 10 cm) dietary intake between weigh-in and the first bout in Olympic combat sports. The data were collected at 6 separate tournaments and measurements included estimated food records, time for recovery, and body weight (BW) at weigh-in and first match. In total, 33 athletes participated in wrestling and taekwondo, sports with extended recovery times, and 35 athletes in judo and boxing, sports with limited recovery time. The results displayed that despite a mean consumption of food and drinks corresponding to 4.2 kg, the athletes only regained an average of 1.9 kg BW during recovery. Water accounted for 86 percent of the total intake. For each litre of water consumed, athletes gained 0.57 kg BW, when excluding heavy weight athletes (n=5). Carbohydrate consumption was 5.5 g/kg BW, compared to the recommended 8-10 g/kg BW. In total, one-quarter of the consumed water originated from carbohydrate-rich drinks. Given the average recovery time of 18 (wrestling, taekwondo) versus 8 hours (judo, boxing), the former group consumed twice the amount of water, carbohydrates, protein, and fat as the latter group. In conclusion, a large proportion of the participants did not meet the recovery nutrition guidelines for carbohydrates. Also, the discrepancy between nutrient intake and weight gain points to the physiological barriers to retaining fluids during a limited recovery time after engaging in weight making practices [13618].

**Effect on tennis**

Carbohydrate supplementation is a popular nutritional practice used in tennis to enhance physical capacities, motor-skill performance, and delay fatigue. However, the effects of carbohydrate supplementation on physiological and perceptual responses during tennis match play are not established. A double blind, randomized, placebo-controlled crossover study was designed to determine the influence of carbohydrate supplementation (0.5g/kg/h) on glycemia, salivary hormones (cortisol and testosterone) concentration, salivary IgA concentration and ratings of perceived exertion (RPE) during 3 h of tennis match play in 12 well trained tennis players. The only significant difference between the two conditions was a lower salivary cortisol concentration post-match in the carbohydrate trial; however, there was a trend for higher glucose concentration and lower session-RPE following tennis match play in the carbohydrate condition, which may have some practical implications. There was no change in salivary testosterone, salivary IgA and RPE responses during tennis match play between conditions. These data indicate that carbohydrate ingestion during 3 h of competitive tennis match play helps to maintain glycemina and attenuates the increase in salivary cortisol concentration compared to placebo [0619].

**Effect on football**

1674
One study investigated how performance was affected after soccer players, in a postprandial state, ingested a 7 percent carbohydrate (CHO) solution compared to a placebo (0 % CHO) during a simulated soccer match. Using a double-blind placebo-controlled design, 22 trained male league soccer players (age: 24 ± 7 years, wt: 73 ± 12 kg, VO\textsubscript{2}max: 52 ± 4 mL O\textsubscript{2}/kg/min) completed two trials, separated by 7 days, during which they ingested, in random order, 700 mL of either a 7 percent CHO or placebo drink during a simulated soccer match. Ratings of perceived exertion (RPE), agility, timed and run to fatigue were measured during the trials. Change in agility times was not altered by CHO versus placebo ingestion. Timed runs to fatigue were 381 ± 267 s versus 294 ± 159 s for the CHO and placebo drinks, respectively. Body mass modified the relationship between time to fatigue and drink ingestion, such that lower body mass was associated with increased time to fatigue when the players ingested CHO, but not placebo. RPE values for the final stage of the simulated soccer match were 8.5 ± 1.7 and 8.6 ± 1.5 for the CHO and placebo drinks respectively. The group data showed that the 7 percent CHO solution (49 g CHO) did not significantly improve performance during a simulated soccer match in league soccer players who had normal pre-match nutrition. However, when adjusting for body mass, increasing CHO intake was associated with improved time to fatigue during the simulated soccer match [13620].

**Effect on sleep onset**

Dietary carbohydrate intake has been shown to increase the plasma concentration of tryptophan, a precursor of serotonin and sleep-inducing agent. To investigate the role of carbohydrate in sleep induction, it was explored the effect of glycemic index (GI) and meal time on sleep in healthy volunteers. It was compared the effect of high- and low-GI carbohydrate-based meals ingested 4 h before bedtime on sleep quality. It was also evaluated the effect of the timing of high-GI meals (4 h compared with 1 h) on sleep quality. Twelve healthy men (aged 18-35 year) were administered standard, isocaloric (3212 kJ; 8 % of energy as protein, 1.6 % of energy as fat, and 90.4 % of energy as carbohydrate) meals of either Mahatma (low GI = 50) or Jasmine (high GI = 109) rice 4 h before their usual bedtime. On another occasion, the high-GI meal was given 1 h before bedtime. The participants underwent a familiarization night followed by 3 test nights in random order 1 week apart. A significant reduction in the mean sleep onset latency was observed with a high-GI compared with a low-GI meal consumed 4 h before bedtime. The high-GI meal given 4 h before bedtime showed a significantly shortened sleep onset latency compared with the same meal given 1 h before bedtime. No effects on other sleep variables were observed. It was shown that a carbohydrate-based high-GI meal resulted in a significant shortening of sleep onset latency in healthy sleepers compared with a low-GI meal and was most effective when consumed 4 h before bedtime. The relevance of these findings to persons with sleep disturbance should be determined in future trials [07283].

**Mouth rinse with carbohydrate solutions**

Ingesting carbohydrate-electrolyte solutions during exercise has been reported to benefit self-paced time-trial performance. The mechanism responsible for this ergogenic effect is unclear. For example, during short duration (≤1 hour), intense (>70 % maximal oxygen consumption) exercise, euglycaemia is rarely challenged and adequate muscle glycogen remains at the cessation of exercise. The absence of a clear metabolic explanation has led authors to speculate that ingesting carbohydrate solutions during exercise may have a 'non-metabolic' or 'central effect' on endurance performance. This hypothesis has been explored by studies investigating the performance responses of subjects when carbohydrate solutions are mouth rinsed during exercise. The solution is expectorated before ingestion, thus removing the provision of carbohydrate to the peripheral circulation. Studies using this
method have reported that simply having carbohydrate in the mouth is associated with improvements in endurance performance. However, the performance response appears to be dependent upon the pre-exercise nutritional status of the subject. Furthermore, the ability to identify a central effect of a carbohydrate mouth rinse may be affected by the protocol used to assess its impact on performance. Studies using functional MRI and transcranial stimulation have provided evidence that carbohydrate in the mouth stimulates reward centres in the brain and increases corticomotor excitability, respectively. However, further research is needed to determine whether the central effects of mouth-rinsing carbohydrates, which have been seen at rest and during fatiguing exercise, are responsible for improved endurance performance [11256].

The aim of one study was to determine the effect of a carbohydrate mouthwash on running time-trial performance. On two separate occasions, seven recreationally active males completed a preloaded (15 min at 65 %VO$_{2\text{max}}$) time-trial of 45 min in duration on a motorized treadmill. At 6-min intervals during the preload and time-trial, participants were given either a 6 percent maltodextrin, 3 percent lemon juice solution (carbohydrate trial) or a 3 percent lemon juice placebo mouthwash (placebo trial) in a double-blind, randomized crossover design. Heart rate, oxygen consumption (VO$_2$), respiratory exchange ratio (RER), and ratings of perceived exertion (RPE) were measured during the preload, and blood glucose and lactate were measured before and after the preload and time-trial. There were no significant differences in distance covered between trials. Furthermore, there were no significant between-trial differences in heart rate and running speed during the time-trial, or VO$_2$, RER or RPE during the preload. Blood lactate and glucose increased as a result of the exercise protocol, with no between-trial differences. In conclusion, there was no positive effect of a carbohydrate mouthwash on running performance of approximately 1 h duration [07305].

It has been previously reported that carbohydrate (CHO) mouth rinse can improve exercise performance. The proposed mechanism involves increased activation of brain regions believed to be responsible for reward/motivation and motor control. Since strength-related performance is affected by central drive to the muscles, it seems reasonable to hypothesize that the positive CNS response to oral CHO sensing may counteract the inhibitory input from the muscle afferent pathways minimizing the drop in the central drive. The purpose of one study was to test if CHO mouth rinse affects maximum strength and strength endurance performance. Twelve recreationally strength-trained healthy males took part in the study. All of the tests were performed in the morning, after an 8 h overnight fasting. Subjects were submitted to a maximum strength test (1-RM) and a strength endurance test (six sets until failure at 70 % of 1-RM), in separate days under three different experimental conditions (CHO mouth rinse, placebo-PLA mouth rinse and control-CON) in a randomized crossover design. The CHO mouth rinse (25 mL) occurred before every attempt in the 1-RM test, and before every set in the endurance strength test. Blood glucose and lactate were measured immediately before and 5 min post-tests. There were no significant differences in 1-RM between experimental conditions. Furthermore, there were no significant between-trial differences in heart rate and running speed during the time-trial, or VO$_2$, RER or RPE during the preload. Blood lactate and glucose increased as a result of the exercise protocol, with no between-trial differences. In conclusion, there was no positive effect of a carbohydrate mouthwash on running performance of approximately 1 h duration [11257].

To investigate the influence of ingesting versus mouth rinsing a carbohydrate-electrolyte solution on 1-h running performance, after a 14- to 15-h fast, 10 endurance-trained male runners completed three 1-h performance runs separated by 1 week. In random order, runners ingested either a 8 mL/kg body mass of either a 6.4 percent carbohydrate-electrolyte solution (CHO) or a placebo solution (P) 30 min before or a 2 mL/kg body mass at 15 min
intervals throughout the 1 h run. On a separate occasion, runners mouth rinsed (R) a 6.4 percent CHO, i.e., without ingestion, at the same times as in the ingestion trials. A greater distance was covered after the mouth rinse and ingestion of a 6.4 percent CHO during a 1 h performance run than when mouth rinsing the same solution or mouth rinsing followed by the ingestion of the same volume of a placebo solution [11258].

Carbohydrate during exercise has been demonstrated to improve exercise performance even when the exercise is of high intensity (>75 % VO_{2max}) and relatively short duration (approximately 1 h). It has become clear that the underlying mechanisms for the ergogenic effect during this type of activity are not metabolic but may reside in the central nervous system. Carbohydrate mouth rinses have been shown to result in similar performance improvements. This would suggest that the beneficial effects of carbohydrate feeding during exercise are not confined to its conventional metabolic advantage but may also serve as a positive afferent signal capable of modifying motor output. These effects are specific to carbohydrate and are independent of taste. The receptors in the oral cavity have not (yet) been identified and the exact role of various brain areas is not clearly understood. Further research is warranted to fully understand the separate taste transduction pathways for simple and complex carbohydrates and how these differ between mammalian species, particularly in humans [10401].

It is well known that carbohydrate (CHO) supplementation can improve performance in endurance exercises through several mechanisms such as maintenance of glycemia and sparing endogenous glycogen as well as the possibility of a central nervous-system action. Some studies have emerged in recent years in order to test the hypothesis of ergogenic action via central nervous system. Recent studies have demonstrated that CHO mouth rinse can lead to improved performance of cyclists, and this may be associated with the activation of brain areas linked to motivation and reward. These findings have already been replicated in other endurance modalities, such as running. This alternative seems to be an attractive nutritional tool to improve endurance exercise performance [10402].

It was investigated the influence of ingesting versus mouth-rinsing a carbohydrate-electrolyte solution on 1 h running performance. Following a 14-15 h fast, ten endurance-trained male runners completed three 1 h performance runs separated by 1 week. In random order runners either ingested 8 ml/kg body mass of either a 6.4 percent carbohydrate-electrolyte solution (CHO) or placebo solution (P) 30 min prior to and 2 ml per kg body mass at 15 min intervals throughout the 1 h run. On a separate occasion runners mouth-rinsed (R) a 6.4 percent carbohydrate-electrolyte solution i.e. without ingestion, at the same times as in the ingestion trials. Total distances covered in the CHO, P and R trials were 14515 ± 756 m; 14190 ± 800 m and 14283 ± 758 m respectively. Runners covered 320 m more (during the CHO trial compared to the placebo trial and 230 m more in comparison to the mouth rinse trial. Thus, a greater distance was covered following the mouth-rinse and ingestion of a 6.4 percent carbohydrate-electrolyte solution during a 1 h performance run than when mouth-rinsing the same solution or mouth-rinsing followed by the ingestion of the same volume of a placebo solution [10403].

As the primary fuel for abiding sports, carbohydrate intake before, during, and after exercise, has a positive effect on endurance performance. Apart from being an energy substrate, the sight, smell, and taste of food may act as positive reinforcements. By generating promises for food intake, these senses play a role in reward prediction. As a result, the body starts to function as if it is going to receive food. Recent studies suggest that simple mouth rinses with a carbohydrate solution can improve endurance performance even for performances that last about an hour, that is, in conditions where glycogen stores should not be limiting. Both complex and simple sugars can elicit a mouth rinse effect. Intravenous infusion of glucose
with a similar effect on blood sugar regulation parameters, as compared to a swallowed glucose solution, did not affect performance in a about 1-hour-time trial. It seems that specific oropharyngeal receptors, linked to brain centers that are involved in motivation and reward, play a role in the ergogenic effect of carbohydrate mouth rinsing. Oropharyngeal receptors thus signal presence of carbohydrate to the brain. Mouth rinses with a carbohydrate solution facilitate corticomotor output and improve time-trial performance in well-trained subjects in a fasted state. It was tested for this effect in nonathletic subjects in fasted and nonfasted state. Thirteen healthy non-athletic males performed 5 tests on a cycle ergometer. After measuring maximum power output ($W_{\text{max}}$), the subjects cycled four times at 60 percent $W_{\text{max}}$ until exhaustion while rinsing their mouth every 5 minutes with either a 6.4 percent maltodextrin solution or water, one time after an overnight fast and another after a carbohydrate rich breakfast. Mouth rinsing with maltodextrin improved time-to-exhaustion in pre- and postprandial states. This was accompanied by reductions in the average and maximal rates of perceived exertion but no change in average or maximal heart rate was observed. It was concluded that carbohydrate mouth rinsing improves endurance capacity in both fed and fasted states in non-athletic subjects [11386].

The purpose of one study was to examine the effectiveness of carbohydrate and caffeine mouth rinses in enhancing repeated sprint ability. Previously, studies have shown that a carbohydrate mouth rinse (without ingestion) has beneficial effects on endurance performance that are related to changes in brain activity. Caffeine ingestion has also demonstrated positive effects on sprint performance. However, the effects of carbohydrate or caffeine mouth rinses on intermittent sprints have not been examined previously. Twelve males performed 5 × 6 s sprints interspersed with 24 s of active recovery on a cycle ergometer. Twenty-five milliliters of either a noncaloric placebo, a 6 percent glucose, or a 1.2 percent caffeine solution was rinsed in the mouth for 5 s prior to each sprint in a double-blinded and balanced cross-over design. Postexercise maximal heart rate and perceived exertion were recorded, along with power measures. A second experiment compared a combined caffeine-carbohydrate rinse with carbohydrate only. Compared with the placebo mouth rinse, carbohydrate substantially increased peak power in sprint 1, and both caffeine and carbohydrate improved mean power in sprint 1. Experiment 2 demonstrated that a combination of caffeine and carbohydrate improved sprint 1 power production compared with carbohydrate alone. It was concluded that carbohydrate and (or) caffeine mouth rinses may rapidly enhance power production, which could have benefits for specific short sprint exercise performance. The ability of a mouth-rinse intervention to rapidly improve maximal exercise performance in the absence of fatigue suggests a central mechanism [13621].

**Influence on testosterone levels**

One study examined the effect of dietary carbohydrate (CHO) consumption on the free testosterone to cortisol (fT/C) ratio during a short-term intense micro-cycle of exercise training. The fT/C ratio is a proposed biomarker for overreaching-overtraining (i.e. training stress or imbalance) in athletes. The ratio was studied in two groups, control-CHO (approximately 60 % of daily intake, n=12) and low-CHO (approximately 30 % of daily intake, n=8), of male subjects who performed three consecutive days of intensive training (approximately 70-75 % maximal oxygen consumption, 60 min per day) with a dietary intervention (on the day before and during training). Resting, pre-exercise blood samples were collected under standardized-controlled conditions before each day of training (pre 1, 2, 3) and on a fourth day after the micro-cycle (rest). Bloods were analyzed for free testosterone and cortisol via radioimmunoassay procedures. Subjects performed no additional physical activity other than prescribed training. Statistical analysis (ANCOVA) revealed the fT/C ratio decreased significantly from pre-study resting measurement (pre 1) to
the final post-study resting measurement (rest) in the low-CHO group (-43 %), but no change occurred in the control-CHO group (-3 %). Findings suggest if the fTC ratio is utilized as a marker of training stress or imbalance it is necessary for a moderately high diet of CHO to be consumed to maintain validity of any observed changes in the ratio value [10224].

Endurance performance and fuel selection while ingesting glucose (15, 30, and 60 g/h) was studied in 12 cyclists during a 2-h constant-load ride (approximately 77 % peak $O_2$ uptake) followed by a 20-km time trial. Total fat and carbohydrate (CHO) oxidation and oxidation of exogenous glucose, plasma glucose, glucose released from the liver, and muscle glycogen were computed using indirect respiratory calorimetry and tracer techniques. Relative to placebo, glucose ingestion increased the time trial mean power output. With 60 g/h glucose, mean power was 2.3, 0.4 to 4.2 percent higher, and 3.1, 0.5 to 5.7 percent higher than with 30 and 15 g/h, respectively, suggesting a relationship between the dose of glucose ingested and improvements in endurance performance. Exogenous glucose oxidation increased with ingestion rate, but endogenous CHO oxidation was reduced only with 30 and 60 g/h due to the progressive inhibition of glucose released from the liver (probably related to higher plasma insulin concentration) with increasing ingestion rate without evidence for muscle glycogen sparing. Thus ingestion of glucose at low rates improved cycling time trial performance in a dose-dependent manner. This was associated with a small increase in CHO oxidation without any reduction in muscle glycogen utilization [10225].

**Intake recommendations**

Carbohydrate intake during prolonged exercise has been shown to increase endurance capacity and improve performance. Until recently, the advice was to ingest 30-60 g of carbohydrate per hour. The upper limit was based on studies that demonstrated that intakes greater than 60-70 g/h would not result in greater exogenous carbohydrate oxidation rates. The lower limit was an estimated guess of the minimum amount of carbohydrate required for ergogenic effects. In addition, the advice was independent of the type, the duration or the intensity of the activity as well as the level of athlete. Since 2004, significant advances in the understanding of the effects of carbohydrate intake during exercise have made it possible to be much more prescriptive and individual with the advice. Studies revealed that oxidation rates can reach much higher values (up to 105 g/h) when multiple transportable carbohydrates are ingested (i.e. glucose:fructose). It has also been observed that carbohydrate ingested during shorter higher intensity exercise (1 h, 80 % $VO_2_{max}$) can improve performance, although mechanisms are distinctly different. These findings resulted in new recommendations that are dependent on the duration and intensity of exercise and not only specify the quantity of carbohydrate to be ingested but also the type [13616].

**Can low carbohydrate high fat provide fuel for sport?**

As for athletic performance, athletes have long been encouraged to load up with carbohydrates prior to and during endurance exercise because glycogen, the storage form of carbohydrate, was thought to be a more efficient fuel than fat. This has also been challenged of late by scientists who argue that fat provides more calories per gram and also has much larger body stores. After a week or two of carbohydrate deprivation, our bodies change from a carbohydrate metabolism to a fat metabolism with health and performance improvements. It would seem that fat is just as effective a fuel for endurance events. What is unclear is whether a high fat low carbohydrate diet is appropriate for athletes in intermittent high-intensity sports such as football or road cycling [13596].

**Carbohydrates and protein**
The incidence of heat illness and heat stroke is greater in older than younger people. In this context, exercise training regimens to increase heat tolerance in older people may provide protection against heat illness. Acute increases in plasma volume (PV) improve thermoregulation during exercise in young subjects, but there is some evidence that changes in PV in response to acute exercise are blunted in older humans. It was recently demonstrated that protein-carbohydrate (Pro-CHO) supplementation immediately after a bout of exercise increased PV and plasma albumin content (Albcont) after 23 h in both young and older subjects. It was also examined whether Pro-CHO supplementation during aerobic training enhanced thermoregulation by increasing PV and Albcont in older subjects. Older men aged 68 years exercised at moderate intensity, 60 min/day, 3 days/week, for 8 weeks, at 19 degrees C, and took either placebo (CNT; 0.5 kcal, 0 g protein/kg) or Pro-CHO supplement (Pro-CHO; 3.2 kcal, 0.18 g protein/kg) immediately after exercise. After training, it was found during exercise at 30 degrees C that increases in oesophageal temperature were attenuated more in Pro-CHO than CNT and associated with enhanced cutaneous vasodilatation and sweating. It was also confirmed similar results in young subjects after 5 days of training. These results demonstrate that post-exercise protein and CHO consumption enhance thermoregulatory adaptations especially in older subjects and provide insight into potential strategies to improve cardiovascular and thermoregulatory adaptations to exercise in both older and younger subjects [09329].

The purpose of one study was to determine whether an isocaloric beverage with added protein and vitamins would influence oxidative stress and inflammation after cycling to exhaustion as indicated by plasma protein carbonyls (PC), lipid hydroperoxides (LOOH), and interleukin-6 (IL-6). Twelve trained men (18-33 yr) volunteered and performed this randomized crossover study. Participants cycled at 70 percent of VO2peak until fatigue and at 80 percent VO2peak 22-24 hr later to fatigue with either carbohydrate or the special drink. Blood collected before the cycling at rest and 24, 48, and 72 hr after the exercise was analyzed for PC and LOOH spectrophotometrically and for IL-6 via an enzyme-linked immunosorbent assay. Protein carbonyls demonstrated significant treatment and time effects with no Treatment x Time interaction. PC was higher in the special drink treatment than with carbohydrate independent of time and increased at 24 (48 %), 48 (59 %), and 72 (67 %) hr after exercise compared with preexercise values. Resting LOOH and IL-6 did not have any significant changes with time or treatment. These data indicate that an isocaloric beverage with added protein and vitamins would drink after 2 cycling bouts to exhaustion will exacerbate the resting PC level compared with an isocaloric drink [09330].

It was examined whether protein-carbohydrate (CHO) supplementation immediately after exercise each day during aerobic training facilitated plasma volume (PV) expansion and thermoregulatory and cardiovascular adaptations in older men. Fourteen moderately active older men (68 ± 5 yr) were divided into two groups so as to have no significant differences in anthropometric measures, PV, and peak oxygen consumption rate (VO2peak)). Each group was provided with a mixture of protein and CHO (3.2 kcal, 0.18 g protein/kg body wt, Pro-CHO, n=7) or a non-protein and low-calorie placebo (0.5 kcal, 0 g protein/kg body wt, CNT, n=7) immediately after cycling exercise (60-75 % VO2peak), 60 min/day, 3 days/wk) each day for 8 wk at approximately 19 degrees C ambient temperature and approximately 43 percent relative humidity. Before and after training, we measured PV, cardiac stroke volume (SV), and esophageal temperature during 20-min exercise at 60 percent of pretraining VO2peak at 30 degrees C and 50 percent relative humidity. After training, PV significantly increased by approximately 6 percent in Pro-CHO, with an approximately 10 percent increase in SV during exercise. Thus postexercise protein-CHO supplementation during training caused PV expansion and facilitated thermoregulatory and cardiovascular adaptations, possibly providing a new training regimen for older men [09331].
This study examined whether increased plasma volume (PV) and albumin content in plasma for 23 h after exercise were attenuated in older subjects compared with in young adult subjects, and if this attenuation abated by supplementation with protein and carbohydrate (CHO) immediately after exercise. Eight moderately active older (approximately 68 yr) and 8 young (approximately 21 yr) men performed two trials: control and Pro-CHO in which subjects consumed placebo (0.5 kcal, 0 g protein, 0.5 mg Na⁺ in 3.2 ml total fluid volume/kg body wt) or protein and CHO mixture (3.2 kcal, 0.18 g protein, 0.5 mg Na⁺ in 3.2 ml total fluid volume/kg body wt) suppletions, respectively, immediately after high-intensity interval exercise for 72 min (8 sets of 4 min at 70-80 % peak oxygen consumption rate, VO₂peak, intemitted by 5 min at 20 percent VO₂peak). PV, albumin content, and plasma globulin content were measured before exercise, at the end of exercise, every hour from the 1st to the 5th hour after exercise, and at the 23rd hour after exercise. From 12 h before the start to the end of experiment, food intake was controlled to the age-matched recommended dietary allowances. It was found that during the first 4 h after exercise in controls, albumin content recovered less in the older than the young group by approximately 0.04 g/kg, which was a significant difference, while it generally recovered more with Pro-CHO than controls by approximately 0.09 and approximately 0.04 g/kg in the young and older group, respectively, accompanied by a greater increase in PV by approximately 1 and approximately 2 ml/kg, respectively, during the 23 h after exercise. Globulin content remained constant throughout the experiment in both trials for both age groups. Thus the attenuated responses of albumin content and plasma volume after exercise in older subjects were restored by protein and CHO supplementation immediately after exercise, similarly to young subjects [09332].

One study examined whether a carbohydrate (CHO) + casein hydrolysate beverage improved time-trial performance versus a CHO beverage delivering approximately 60 g CHO/hr. Markers of muscle disruption and recovery were also assessed. Thirteen male cyclists completed 2 computer-simulated 60-km time trials consisting of 3 laps of a 20-km course concluding with a 5-km climb (approximately 5 % grade). Participants consumed 200 ml of CHO (6 %) or carbohydrate + casein hydrolysate beverage (6 % + 1.8 % protein hydrolysate) every 5 km and 500 ml of beverage immediately postexercise. Beverage treatments were administered using a randomly counterbalanced, double-blind design. Plasma creatine phosphokinase (CK) and muscle-soreness ratings were assessed immediately before and 24 hr after cycling. All time differences between treatments occurred during the final lap, with protein hydrolysate ingestion explaining a significant proportion of between-trials differences over the final 20 km and final 5 km. Plasma CK levels and muscle-soreness ratings increased significantly after the CHO trial but not the CHO + casein hydrolysate trial. Late-exercise time-trial performance was enhanced with CHO + casein hydrolysate beverage ingestion compared with a beverage containing CHO provided at maximal exogenous oxidation rates during exercise. CHO casein hydrolysate ingestion also prevented increases in plasma CK and muscle soreness after exercise [09333].

Exercise-induced muscle damage (EIMD) leads to decrements in muscle performance and increases in intramuscular enzymes measured in the plasma, and to delayed onset of muscle soreness (DOMS), partly due to the activation of degradative pathways. It has been shown that milk-based carbohydrate-protein (CHO-P) can limit changes in markers of EIMD, possibly by attenuating protein degradation and (or) increasing protein synthesis. However, the timing of supplementation has received limited attention, and this may alter the response. One study examined the effects of acute milk-based CHO-P supplementation timing on the attenuation of EIMD. Four independent matched groups of 8 healthy males consumed milk-based CHO-P before (PRE), immediately after (POST), or 24 h after (TWENTY-FOUR) muscle-damaging exercise. Active DOMS, isokinetic muscle performance, reactive strength index (RSI), and creatine kinase (CK) were assessed immediately before and 24, 48, and 72
h after EIMD. POST and TWENTY-FOUR demonstrated a benefit in limiting changes in active DOMS, peak torque, and RSI over 48 h, compared with PRE. PRE showed a possible benefit in reducing increases in CK over 48 h and limiting changes in other variables over 72 h. Consuming milk-based CHO-P after muscle-damaging exercise is more beneficial in attenuating decreases in muscle performance and increases in active DOMS at 48 h than ingestion prior to exercise [10226].

The aim of one study was to determine whether adding protein to a CHO beverage would improve late-exercise cycle time-trial performance over CHO alone. Furthermore, it was examined the effects of coingesting protein with CHO during exercise on postexercise markers of sarcolemmal disruption and the recovery of muscle function. In a double-blind, crossover design, 12 trained male cyclists performed 120 min of steady-state (SS) cycling at approximately 55 percent VO_{2max} followed by a time trial lasting approximately 1 h. At 15-min intervals during SS exercise, participants consumed either a CHO or a CHO + protein (CHO + Pro) beverage (providing 65 g/h CHO or 65 g/h CHO plus 19 g/h protein). Twenty-four hours after the onset of the SS cycle, participants completed a maximum isometric strength test. At rest and 24 h postexercise, a visual analog scale was used to determine lower-limb muscle soreness, and blood samples were obtained for plasma creatine kinase concentration. Dietary control was implemented 24 h before and during the time course of each trial. Average power output sustained during time trial was similar for CHO and CHO + Pro, with no effect of treatment on the time to complete the time trial. Postexercise isometric strength significantly declined for CHO (15 %) and CHO + Pro (11 %) compared with baseline. Plasma creatine kinase concentrations, and visual analog scale soreness significantly increased at 24 h postexercise, with no difference between treatments. The present findings suggest that carbohydrate plus protein coingestion during exercise does not improve late-exercise time-trial performance, ameliorate markers of sarcolemmal disruption, or enhance the recovery of muscle function at 24 h postexercise over carbohydrates alone [10227].

One study examined the effect of amino acids in a carbohydrate beverage on cycling performance. Twelve male athletes cycled at 75 percent VO_{2peak} for 90 min followed by a ride to exhaustion at 85 percent VO_{2peak}, before (T1) and on 2 consecutive days (T2 and T3) after 2 weeks of supplementation with 3.6 percent carbohydrate plus 1 percent amino acids (AA) or 4.6 percent carbohydrate-only (CHO) isocaloric beverages. Muscle damage was assessed by plasma creatine kinase (CK), and muscle fatigue by changes in vertical jump pre- to postexercise. Muscle soreness, overall fatigue, and changes in mood state were assessed using questionnaires. Plasma CK was lower for amino acids in T3. Time to exhaustion decreased from T2 to T3 only in CHO. Vertical-jump change from pre- to postexercise was greater in T3 for the carbohydrate treatment. Total fatigue score and mood disturbance decreased significantly only with amino acids in T3. The addition of AA to a carbohydrate beverage after consecutive-day exercise bouts reduced muscle damage as indicated by CK levels, decreased fatigue, and maintained exercise performance compared with consuming carbohydrate alone [08390].

Endurance athletes commonly consume carbohydrate-electrolyte sports beverages during prolonged events. The benefits of this strategy are numerous--sports-beverage consumption during exercise can delay dehydration, maintain blood glucose levels, and potentially attenuate muscle glycogen depletion and central fatigue. Thus, it is generally agreed that carbohydrate-electrolyte beverages can improve endurance performance. A controversy has recently emerged regarding the potential role of protein in sports beverages. At least 3 recent studies have reported that carbohydrate-protein ingestion improves endurance performance to a greater extent than carbohydrate alone. In addition, carbohydrate-protein ingestion has been associated with reductions in markers of muscle damage and improved postexercise...
recovery. Although many of these muscle damage and recovery studies examined postexercise nutritional intake, recent evidence suggests that these benefits may be elicited with carbohydrate-protein consumption during exercise. These findings are intriguing and suggest that the importance of protein for endurance athletes has been underappreciated. However, 2 studies recently reported no differences in endurance performance between carbohydrate and carbohydrate-protein beverages. The varied outcomes may have been influenced by a number of methodological differences, including the amounts and types of carbohydrate or protein in the beverages, the exercise protocols, and the relative statistical power of the studies. In addition, although there are plausible mechanisms that could explain the ergogenic effects of carbohydrate-protein beverages, they remain relatively untested. One review examined the existing research regarding the efficacy of carbohydrate-protein consumption during endurance exercise [07291].

The majority of football players succumb to fatigue towards the end of the game. This study was designed to examine the influence of protein coingestion with carbohydrate (CHO) versus an isocaloric CHO supplement on subsequent running capacity towards the end of a simulated football match. Six male amateur football players participated in 3 trials applied in a randomized cross-over experimental design. A laboratory-based, football-specific intermittent exercise was allocated for 75 min interspersed with a 15-min recovery, immediately followed by run time to fatigue (RTF) at 80 percent peak oxygen consumption. In each trial, prior to exercise and during half-time, participants randomly ingested a placebo (PLC), 6.9 percent CHO, or 4.8 percent CHO plus 2.1 percent protein (CHO-P) supplements matched for color and taste. CHO-P resulted in longer RTF (23 ± 5 min) than did CHO (16 ± 3 min) and PLC (11 ± 2 min). Blood glucose was higher in CHO-P at the point of fatigue compared with CHO and PLC. Ratings of perceived exertion were lower in the CHO-P subjects at the onset of exercise and towards the end of intermittent exercise when compared with the PLC and CHO subjects. When protein was added to a CHO supplement, subsequent running capacity following limited recovery from intermittent exercise was enhanced. This improvement suggests that protein coingestion may exert an ergogenic benefit upon endurance capacity during intermittent activity [11522].

Influence on post-exercise glycogen levels of carbohydrates and protein
One study assessed whether liquid carbohydrate-protein (C+P) supplements, ingested early during recovery, enhance muscle glycogen resynthesis versus isoenergetic liquid carbohydrate (CHO) supplements, given early or an isoenergetic solid meal given later during recovery (PLB). Two hours after breakfast (7.0 kcal/kg; 0.3 g/kg P, 1.2 g/kg C, 0.1 g/kg F), six male cyclists performed a 60-min time trial (AMex). Pre- and postexercise, vastus lateralis glycogen concentrations were determined using nMRS. Immediately, 1 h, and 2 h postexercise, participants ingested C+P (4.8 kcal/kg; 0.8 g/kg C, 0.4 g/kg P), CHO (4.8 kcal/kg; 1.2 g/kg C), or PLB (no energy). Four hours postexercise, a solid meal was ingested. At that time, C+P and CHO received a meal identical to breakfast, whereas PLB received 21 kcal/kg (1 g/kg P, 3.6 g/kg C, 0.3 g/kg F); energy intake during 6 h of recovery was identical among treatments. After 6 h of recovery, measurement and cycling protocols (PMex) were repeated. Absolute muscle glycogen utilization was 18 percent greater during AMex relative to PMex but there were no differences between groups. During 6 h of recovery, muscle glycogen resynthesis was greater in C+P versus CHO or PLB. Cycling performance was similar among treatments during both AMex and PMex. It was concluded that C+P supplements, given early after exercise, enhance glycogen resynthesis relative to CHO and PLB. However, this does not influence performance in this type of exercise bout [06256].

Acute carbohydrate-protein ingestion has been shown to improve steady-state endurance performance. This study compared the effects of carbohydrate and carbohydrate-protein ingestion on self-regulated simulated multiple-sprint sport performance. Nine participants
completed two trials of a modified Loughborough Intermittent Shuttle Test involving 4 x 15 min blocks of regulated exercise followed by 2 x 15 min blocks of self-regulated exercise. Participants consumed 2.5 ml/kg of an 8 percent carbohydrate (CHO trial) or 6 percent carbohydrate plus 2 percent whey protein beverage (CHO-P trial) every 15 minutes. Distance covered (4 %) and maximal speed (6 %) decreased in the final 15 min of exercise, and whilst not significant, carbohydrate-protein elicited a very likely moderate and possibly small improvement in each variable, respectively. Average running speed declined in the final 15 min of the CHO trial only with protein providing a likely small improvement (2.7 %: ± 2.5 %). No differences between beverages were observed in body mass or plasma volume change, urine volume, heart rate, gut fullness, rating of perceived exertion (RPE), blood glucose or serum insulin. Blood urea concentration increased in the CHO-P trial only. These findings show carbohydrate-protein ingestion is likely to enhance multiple-sprint sport exercise performance above carbohydrate, potentially through altered central fatigue or increased protein oxidation [12383].

Protein supplements are consumed frequently by athletes and recreationally active adults for various reasons, including improved exercise performance and recovery after exercise. Yet, far too often, the decision to purchase and consume protein supplements is based on marketing claims rather than available evidence-based research. The purpose of one review was to provide a systematic and comprehensive analysis of the literature that tested the hypothesis that protein supplements, when combined with carbohydrate, directly enhance endurance performance by sparing muscle glycogen during exercise and increasing the rate of glycogen restoration during recovery. The analysis was used to create evidence statements based on an accepted strength of recommendation taxonomy. English language articles were searched with PubMed and Google Scholar using protein and supplements together with performance, exercise, competition, and muscle, alone or in combination as keywords. Additional articles were retrieved from reference lists found in these papers. Inclusion criteria specified recruiting healthy active adults less than 50 years of age and evaluating the effects of protein supplements in combination with carbohydrate on endurance performance metrics such as time-to-exhaustion, time-trial, or total power output during sprint intervals. The literature search identified 28 articles, of which 26 incorporated test metrics that permitted exclusive categorization into one of the following sections: ingestion during an acute bout of exercise (n=11) and ingestion during and after exercise to affect subsequent endurance performance (n=15). The remaining two articles contained performance metrics that spanned both categories. All papers were read in detail and searched for experimental design confounders such as energy content of the supplements, dietary control, use of trained or untrained participants, number of subjects recruited, direct measures of muscle glycogen utilization and restoration, and the sensitivity of the test metrics to explain the discrepant findings. The evidence statements assert that when carbohydrate supplementation was delivered at optimal rates during or after exercise, protein supplements provided no further ergogenic effect, regardless of the performance metric used. In addition, the limited data available suggested recovery of muscle glycogen stores together with subsequent rate of utilization during exercise is not related to the potential ergogenic effect of protein supplements. Many studies lacked ability to measure direct effects of protein supplementation on muscle metabolism through determination of muscle glycogen, kinetic assessments of protein turnover, or changes in key signaling proteins, and therefore could not substantiate changes in rates of synthesis or degradation of protein. As a result, the interpretation of their data was often biased and inconclusive since they lacked ability to test the proposed underlying mechanism of action. It was concluded that when carbohydrate is delivered at optimal rates during or after endurance exercise, protein supplements appear to have no direct endurance performance enhancing effect [13638].

Acute carbohydrate-protein ingestion has been shown to improve steady-state endurance
performance. This study compared the effects of carbohydrate and carbohydrate-protein ingestion on self-regulated simulated multiple-sprint sport performance. Nine participants completed two trials of a modified Loughborough Intermittent Shuttle Test involving 4 x 15 min blocks of regulated exercise followed by 2 x 15 min blocks of self-regulated exercise. Participants consumed 2.5 mL/kg of an 8 percent carbohydrate (CHO trial) or 6 percent carbohydrate plus 2 percent whey protein beverage (CHO-P trial) every 15 minutes. Distance covered (4.2 %) and maximal speed (6.1 %) decreased in the final 15 min of exercise, and whilst not significant, carbohydrate-protein elicited a very likely moderate and possibly small improvement in each variable, respectively. Average running speed declined in the final 15 min of the CHO trial only with protein providing a likely small improvement. No differences between beverages were observed in body mass or plasma volume change, urine volume, heart rate, gut fullness, rating of perceived exertion (RPE), blood glucose or serum insulin. Blood urea concentration increased in the CHO-P trial only. These findings show carbohydrate-protein ingestion is likely to enhance multiple-sprint sport exercise performance above carbohydrate, potentially through altered central fatigue or increased protein oxidation.

It is recommended that endurance athletes consume carbohydrate (CHO) supplements, providing 6-8 percent CHO concentration, during exercise >60 minutes to improve athletic performance. Recently research has compared carbohydrate-protein (CHO-P) supplementation to the traditionally used CHO supplementation during endurance exercise, following these supplementation recommendations, in controlled settings, but not under simulated applied conditions such as a field trial. Therefore, the purpose of one investigation was to test CHO and CHO-P supplementation under applied conditions such that commercially-available isocaloric (CHO-P & double-carbohydrate [CHO-CHO]) and isocarbohydrate (CHO-P & CHO) supplements were compared to a placebo (PLA), within an outdoor running field trial >60 minutes in order to assess their influence on endurance performance. Twelve male recreational runners completed four, 19.2 km runs, where they were instructed to run at a pace similar to race pace including a final sprint to the finish, which in this case was the final two laps of the course (1.92 km). Supplementation was provided before the start and in 4 km increments. Performance was measured by time to complete the 19.2 km run and last 1.92 km sprint. Analyses found no difference between supplements in time to complete the 19.2 km run or last 1.92 km sprint to the finish. Thus, when following recommendation for supplementation within a field trial, commercially available CHO and CHO-P supplements do not appear to enhance performance in male recreational runners.

In football
One study investigated the influence of carbohydrate supplementation on skill performance throughout exercise that replicates soccer match-play. Experimentation was conducted in a randomised, double-blind and cross-over study design. After familiarization, 15 professional academy soccer players completed a soccer match simulation incorporating passing, dribbling and shooting on two separate occasions. Participants received a 6 percent carbohydrate-electrolyte solution (CHO) or electrolyte solution (PL). Precision, success rate, ball speed and an overall index (speed-precision-success; SPS) were determined for all skills. Blood samples were taken at rest, immediately before exercise, every 15 min during exercise (first half: 15, 30 and 45 min; second half: 60, 75 and 90 min), and 10 min into the half time (half-time). Carbohydrate supplementation influenced shooting (time×treatment interaction), where CHO attenuated the decline in shot speed and SPS index. Supplementation did not affect passing or dribbling. Blood glucose responses to exercise were influenced by supplementation (time×treatment interaction), where concentrations were higher at 45 min and during half-time in CHO compared with PL. Blood glucose concentrations reduced by 30 ± 1 percent between half-time and 60 min in CHO. It was
concluded that carbohydrate supplementation attenuated decrements in shooting performance during simulated soccer match-play; however, further research is warranted to optimise carbohydrate supplementation regimes for high-intensity intermittent sports [12384].

In tennis
Twenty-two tennis players were individually studied on 2 occasions. They performed a prematch skill test, a 2-hr tennis match against an equally ranked opponent, and a postmatch skill test. A carbohydrate-electrolyte (CHO-E; Lucozade Sport) or flavor-matched placebo-electrolyte (PL) beverage was administered in a double-blind fashion. During the trials, heart-rate and movement intensity were monitored, and the match was recorded for performance analysis. There were no differences in skill-test scores pre- to postmatch or between trials (154 ± 38 pre- and 160 ± 35 postmatch on PL, 155 ± 36 pre- and 165 ± 33 postmatch on CHO-E). CHO-E ingestion elevated blood glucose concentration throughout the match, and participants reported feeling more energetic (general activation) and more tense (high activation) 1 hr into the match than at baseline. Participants in the CHO-E trial spent more time in moderate-intensity activity and less time in low-intensity activity than on PL. Performance analysis revealed that CHO-E ingestion increased overall serve success, success of first serves, and serves to the advantage side. Return success was greater during the second set of the match in the CHO-E trial. Differences in serve and return success were not associated with blood glucose response to CHO or player ability [12385].

Influence on DNA injuries
The aim of one study was to evaluate the effect of carbohydrate supplementation on free plasma DNA and conventional markers of training and tissue damage in long-distance runners undergoing an overload training program. Twenty-four male runners were randomly assigned to two groups (CHO group and control group). The participants were submitted to an overload training program (days 1-8), followed by a high-intensity intermittent running protocol (10 × 800 m) on day 9. The runners received maltodextrin solution (CHO group) or zero energy placebo solution as the control equivalent before, during, and after this protocol. After 8 days of intensive training, baseline LDH levels remained constant in the CHO group and increased in the control group. On day 9, LDH concentrations were lower in the CHO group than in the control group post-intermittent running. Carbohydrate ingestion attenuated the increase of free plasma DNA post-intermittent running when compared to the control group. Leukocyte counts were lower in the CHO group than in the control group post-intermittent running and at 80 min of recovery. Cortisol levels were positively correlated with free plasma DNA, leukocytes, and LDH. The results showed that ingestion of a carbohydrate beverage resulted in less DNA damage and attenuated the acute post-exercise inflammation response, providing better recovery during intense training [12386].

Influence on fat oxidation
The enhancement of fat oxidation during exercise is an aim for both recreational exercising individuals and endurance athletes. Nutritional status may explain a large part of the variation in maximal rates of fat oxidation during exercise. This review reveals novel insights into nutritional manipulation of substrate selection during exercise, explaining putative mechanisms of action and evaluating the current evidence. Lowering the glycaemic index of the pre-exercise meal can enhance lipid utilisation by up to 100 percent through reduced insulin concentrations, although its application may be restricted to specific training sessions rather than competition. Chronic effects of dietary glycaemic index are less clear and warrant future study before firm recommendations can be made. A flurry of recent advances has overturned the conventional view of l-carnitine supplementation, with skeletal muscle uptake possible under certain dietary conditions and providing a strategy to influence energy metabolism in an exercise intensity-dependent manner. Use of non-carbohydrate nutrients to stimulate muscle l-carnitine uptake may prove more beneficial for optimising lipid utilisation,
but this requires more research. Studies investigating fish oil supplementation on fat oxidation during exercise are conflicting. In spite of some strong putative mechanisms, the only crossover trial showed no significant effect on lipid use during exercise. Carbohydrates may increase NEFA availability although it is not clear whether these effects occur. Carbohydrates and caffeine can increase NEFA availability under certain circumstances which could theoretically enhance fat oxidation, yet strong experimental evidence for this effect during exercise is lacking. Co-administration of nutrients to maximise their effectiveness needs further investigation [12387].

Heavy aerobic exercise, particularly with short time periods between sessions, can result in incomplete muscle glycogen replenishment, increased indices of muscle disruption and soreness, and impaired performance in subsequent exercise. Recent studies have suggested that these aspects of muscle recovery may be influenced by the consumption of carbohydrate-protein (CHO + Pro) recovery beverages. For example, CHO + Pro intake following exercise may accelerate muscle glycogen replenishment rates compared to isocaloric CHO beverages consumed at sub-optimal intake rates (<1 g/kg BW/h); and glycogen repletion with CHO + Pro appears similar to optimal carbohydrate doses. Numerous studies have reported that post-exercise CHO + Pro ingestion attenuates changes in markers of post-exercise muscle disruption following exercise, such as creatine kinase (CK), myoglobin and lactate dehydrogenase. Others have reported that CHO + Pro intake is associated with reduced muscle soreness and enhanced post-exercise muscle function compared to when carbohydrate alone is consumed. However, the effects of CHO + Pro on these markers of recovery remain controversial, as other studies have reported no differences between CHO and CHO + Pro treatments on markers of muscle disruption, muscle soreness, or muscle function. Perhaps due to the varying effects of CHO + Pro on these factors, consumption of CHO + Pro supplements during recovery has been reported to augment performance during subsequent whole-body exercise in some but not all studies. The effects of different carbohydrate-protein (CHO + Pro) beverages were compared during recovery from cycling exercise. Twelve male cyclists (VO\textsubscript{2peak}: 65 ± 7 mL/kg/min) completed 1 h of high-intensity intervals (EX1). Immediately and 120 min following EX1, subjects consumed one of three calorically-similar beverages (285-300 kcal) in a cross-over design: carbohydrate-only (CHO; 75 g per beverage), high-carbohydrate/low-protein (HCLP; 45 g CHO, 25 g Pro, 0.5 g fat), or low-carbohydrate/high-protein (LCHP; 8 g CHO, 55 g Pro, 4 g fat). After 4 h of recovery, subjects performed subsequent exercise (EX2; 20 min at 70 % VO\textsubscript{2peak} + 20 km time-trial). Beverages were also consumed following EX2. Blood glucose levels (30 min after beverage ingestion) differed across all treatments (CHO > HCLP > LCHP), and serum insulin was higher following CHO and HCLP ingestion versus LCHP. Peak quadriceps force, serum creatine kinase, muscle soreness, and fatigue/energy ratings measured pre- and post-exercise were not different between treatments. EX2 performance was not significantly different between CHO (48.5 ± 1.5 min), HCLP (48.8 ± 2.1 min) and LCHP (50.3 ± 2.7 min). Beverages containing similar caloric content but different proportions of carbohydrate/protein provided similar effects on muscle recovery and subsequent exercise performance in well-trained cyclists [12395].

Previous studies have indicated that exercise-induced muscle damage might be attenuated by coingestion of protein and carbohydrate supplement. The purpose of one study was to compare the effect of three various ratios of carbohydrate-protein (CHO+PRO) supplements on resistance exercise-induced muscle damage indices. Twenty-eight untrained male students voluntarily participated in this study and were randomly assigned to one of the four groups: 1) CHO+PRO 2:1 ratio, n=7; 2) CHO+PRO 3:1 ratio, n=8; 3) CHO+PRO 4:1 ratio, n=7; 4) placebo group, n=6. They performed a single bout of resistance exercise (whole body: 3 set×8-10 reps with 70-75 % 1RM), with eccentric concentration. Every group consumed prepared CHO/PRO beverages (9 % concentration, 10 mL/kg/bw at different
ratios) or the same amount of placebo beverage before and in 15 min intervals during exercise. Blood samples were taken before the exercise bout and also at 1 and 24 h post-exercise. In addition, muscle soreness scores were recorded before and 1, 24, and 48 h postexercise. Serum creatine kinase (CK) and myoglobin (Mb) increased in all groups compared with pre-exercise but the significant difference among groups was observed in 24 h postexercise, in a way that both CK and Mb levels were higher in placebo group. Muscle soreness increased for all groups from pre to postexercise, but there was not any significant difference among groups at any time point. Findings of this study showed that CHO+PRO decreased serum CK and Mb at 24 h post exercise, but did not affect muscle soreness at any time points after exercise. Moreover, there were no significant differences between various ratios of CHO-PRO supplementation.

Carbohydrates and amino acids

This investigation examined chronic alteration of the acute hormonal response associated with liquid carbohydrate (CHO) and/or essential amino acid (EAA) ingestion on hormonal and muscular adaptations following resistance training. Thirty-two untrained young men performed 12 weeks of resistance training twice a week, consuming 675 ml of either, a 6 percent CHO solution, 6 g EAA mixture, combined CHO + EAA supplement or placebo (PLA). Blood samples were obtained pre- and post-exercise (week 0, 4, 8, and 12), for determination of glucose, insulin, and cortisol. 3-Methylhistidine excretion and muscle fibre cross-sectional area (fCSA) were determined pre- and post-training. Post-exercise cortisol increased during each training phase for PLA. No change was displayed by EAA; CHO and CHO + EAA demonstrated post-exercise decreases. All groups displayed reduced pre-exercise cortisol at week 12 compared to week 0. Post-exercise insulin concentrations showed no change for PLA; increases were observed for the treatment groups, which remained greater for CHO and CHO + EAA than PLA. EAA and CHO ingestion attenuated 3-methylhistidine excretion 48 h following the exercise bout. CHO + EAA resulted in a 26% decrease, while PLA displayed a 52 percent increase. fCSA increased across groups for type I, IIA, and IIB fibres, with CHO + EAA displaying the greatest gains in fCSA relative to PLA. These data indicate that CHO + EAA ingestion enhances muscle anabolism following resistance training to a greater extent than either CHO or EAA consumed independently. The synergistic effect of CHO + EAA ingestion maximises the anabolic response presumably by attenuating the post-exercise rise in protein degradation.

Carbohydrate versus carbohydrate plus protein

The purpose of this study was to investigate the effects of isocaloric carbohydrate (CHO) and carbohydrate-protein (CHO-Pro) supplements on time to exhaustion. Eleven moderately aerobically fit adults performed a maximal cycle ergometer test for the determination of VO\textsubscript{2max}. At least 72 hours later, the participants performed a time-to-exhaustion test at a power output equivalent to the power output when subjects were at 75 percent of their VO\textsubscript{2max}. Either the CHO or the CHO-Pro supplement was administered at 0, 30, 60, 90, and 120 minutes after this test. After 3 hours of recovery and supplement ingestion, a second time-to-exhaustion test was performed. This testing protocol was repeated for the third visit, but the supplement not given during the second visit was administered. The results indicated that there was no significant difference in time to exhaustion after isocaloric CHO (pretest 22.4 ± 2.84 minutes, posttest 25.4 ± 4.45 minutes) and CHO-Pro (pretest 22.3 ± 3.46 minutes, posttest 24.0 ± 5.08 minutes) supplementation. Carbohydrate and CHO-Pro ingestion after exercise appear to have similar effects on short-term recovery.

Carbohydrates plus whey
Whey protein is a supplemental protein source often used by athletes, particularly those aiming to gain muscle mass; however, direct evidence for its efficacy in stimulating muscle protein synthesis (MPS) is lacking. It was aimed to determine the impact of consuming whey protein on skeletal muscle protein turnover in the post-exercise period. Eight healthy resistance-trained young men (age=21 years; BMI=27) participated in a double-blind randomized crossover trial in which they performed a unilateral leg resistance exercise workout (4 sets of knee extensions and 4 sets of leg press; 8-10 repetitions/set; 80% of maximal), such that one leg was not exercised and acted as a rested (RE) comparator. After exercise, subjects consumed either an isoenergetic whey protein plus carbohydrate beverage (WHEY: 10 g protein and 21 g fructose) or a carbohydrate-only beverage (CHO: 21 g fructose and 10 g maltodextran). Subjects received pulse-tracer injections of L-[ring-2H5]phenylalanine and L-[15N]phenylalanine to measure MPS. Exercise stimulated a rise in MPS in the WHEY-EX and CHO-EX legs, which were greater than MPS in the WHEY-RE leg and the CHO-RE leg, respectively. The rate of MPS in the WHEY-EX leg was greater than in the CHO-EX leg. It was concluded that a small dose (10 g) of whey protein with carbohydrate (21 g) can stimulate a rise in MPS after resistance exercise in trained young men that would be supportive of a positive net protein balance, which, over time, would lead to hypertrophy [07292].

Ingesting carbohydrate plus protein following prolonged exercise may restore exercise capacity more effectively than ingestion of carbohydrate alone. The objective of the present study was to determine whether this potential benefit is a consequence of the protein fraction per se or simply due to the additional energy it provides. Six active males participated in three trials, each involving a 90-min treadmill run at 70 percent maximal oxygen uptake (run 1) followed by a 4-h recovery. At 30-min intervals during recovery, participants ingested solutions containing: 0.8 g carbohydrate/kg body mass/h plus 0.3 g/kg/h of whey protein isolate (CHO-PRO); 0.8 g carbohydrate/kg BM/h (CHO); or 1.1 g carbohydrate/kg BM/h (CHO-CHO). The latter two solutions matched the CHO-PRO solution for carbohydrate and for energy, respectively. Following recovery, participants ran to exhaustion at 70 percent maximal oxygen uptake (run 2). Exercise capacity during run 2 was greater following ingestion of CHO-PRO and CHO-CHO than following ingestion of CHO with no significant difference between the CHO-PRO and CHO-CHO treatments. In conclusion, increasing the energy content of these recovery solutions extended run time to exhaustion, irrespective of whether the additional energy originated from sucrose or whey protein isolate [07293].

**Carbohydrates with fat**

Pre-exercise meals containing carbohydrates (CHO) are recommended to athletes, although there is evidence to suggest that a high fat meal prior to exercise increases utilisation of fats yet may not adversely affect performance. This study investigated the effect of a high fat and high CHO pre-exercise meal prior to high intensity intermittent exercise. Ten male recreational soccer players performed a soccer specific protocol followed by a 1 km time trial 3½ h after ingesting one of 2 test meals, high fat meal (HFM) or a high CHO meal (HCM). Blood glucose, fatty acids (FA), glycerol, beta-hydroxybutyrate, lactate and insulin were assessed prior to the meal, pre-exercise, half-time, and post-exercise, whilst rates of CHO and fat oxidation were determined at 4 time points during the exercise as well as heart rate (HR) and rating of perceived exertion (RPE). Significant increases in FA, glycerol, β-hydroxybutyrate and fat oxidation after the HFM were observed, while CHO oxidation was significantly higher following the HCM. No performance effect was found for the 1 km time trial. These findings suggest that the type of meal ingested prior to soccer simulated exercise has an impact on metabolism, but not on the subsequent performance as determined in the present study [13641].
Milk carbohydrate and protein

Exercise-induced muscle damage leads to the degradation of protein structures within the muscle. This may subsequently lead to decrements in muscle performance and increases in intramuscular enzymes and delayed-onset muscle soreness. Milk, which provides protein and carbohydrate (CHO), may lead to the attenuation of protein degradation and an increase in protein synthesis that would limit the consequential effects of exercise-induced muscle damage. One study examined the effects of acute milk and milk-based protein-CHO supplementation on attenuating exercise-induced muscle damage. Delayed-onset muscle soreness was not significantly different between groups at any time point [08394].

Carbohydrates and caffeine

A round of golf lasts approximately 4 hours, during which time homeostasis could be challenged through either hypoglycemia or hypohydration. This might result in impaired motor skill or cognitive performance. Given the high cognitive demand of putting and the potential fatiguing effects from prolonged walking, the combination of a caffeine and carbohydrate drink could be beneficial in offsetting hypoglycemia and hypohydration. One study used a laboratory-simulated round of golf to examine the effect of an isotonic carbohydrate and caffeine sports drink on putting performance during a round of golf. After institutional ethics approval, 20 male golfers consumed either an isotonic sports drink containing caffeine (6.4 g carbohydrate and 16 mg caffeine per 100 mL) or a no-energy, flavour-matched placebo drink in a double-blind, randomized, counter-balanced crossover design. Drinks were consumed preround (5 mL/kg body mass) and at holes 6 and 12. Participants therefore consumed 1.6 mg/kg body mass of caffeine and 0.64 g/kg body mass of carbohydrate throughout the trial. Five and 2 m putting performance were assessed at each hole. Self-rated mood assessments were carried out every third hole. Putting performance over 5 m and 2 m and self-rated scores for alertness and relaxation showed a significant main effect for drink. Ratings of mental fatigue and tiredness significantly increased during the round. In experienced golfers, the consumption of an isotonic carbohydrate sports drink containing caffeine prior to and during a round of golf improved putting performance and increased feelings of alertness [09334].

Not all athletic competitions lend themselves to supplementation during the actual event, underscoring the importance of preexercise supplementation to extend endurance and improve exercise performance. Energy drinks are composed of ingredients that have been found to increase endurance and improve physical performance. The purpose of one study was to investigate the effects of a commercially available energy drink, ingested before exercise, on endurance performance. The study was a double-blind, randomized, crossover design. After a 12-hr fast, 6 male and 6 female trained cyclists consumed 500 ml of either flavored placebo or Red Bull Energy Drink (2.0 g taurine, 1.2 g glucuronolactone, 160 mg caffeine, 54 g carbohydrate, 40 mg niacin, 10 mg pantothenic acid, 10 mg vitamin B6, and 10 microg vitamin B12) 40 min before a simulated cycling time trial. Performance was measured as time to complete a standardized amount of work equal to 1 hr of cycling at 70 percent Wmax. Performance improved with energy drink compared with placebo, but there was no difference in rating of perceived exertion between treatments. Endorphin levels increased during exercise, with the increase for energy drink approaching significance over placebo. Substrate utilization, as measured by open-circuit spirometry, did not differ between treatments. These results demonstrate that consuming a commercially available energy drink before exercise can improve endurance performance and that this improvement might be in part the result of increased effort without a concomitant increase in perceived exertion.
Carbohydrate and caffeine are known to independently improve certain aspects of athletic performance. However, less is understood about physiological and performance outcomes when these compounds are coingested in a rehydration and carbohydrate-replacement strategy. The aim of one study was to examine the influence of adding a moderate dose of caffeine to a carbohydrate solution during prolonged soccer activity. Fifteen male soccer players performed two 90-min intermittent shuttle-running trials. They ingested a carbohydrate-electrolyte solution (CON) providing a total of 1.8 g/kg body mass (BM) of carbohydrate or a similar solution with added caffeine (CAF; 3.7 mg/kg BM). Solutions were ingested 1 hr before exercise and every 15 min during the protocol. Soccer passing skill and countermovement-jump height (CMJ) were quantified before exercise and regularly during exercise. Sprinting performance, heart rate, blood lactate concentration (La) and the subjective experiences of participants were measured routinely. Mean 15-m sprint time was significantly faster during CAF; over the final 15 min of exercise mean sprint times were CAF 2.48 ± 0.15 s versus CON 2.59 ± 0.2 s. Explosive leg power (CMJ) was improved during CAF. Heart rate was elevated throughout CAF, and ratings of pleasure were significantly enhanced. There were no significant differences in passing skill, rating of perceived exertion, La, or body-mass losses between trials. The addition of caffeine to the carbohydrate-electrolyte solution improved sprinting performance, countermovement jumping, and the subjective experiences of players. Caffeine appeared to offset the fatigue-induced decline in self-selected components of performance [10228].

Carbohydrates and electrolytes

To investigate the effect of ingesting a carbohydrate-electrolyte solution (CHO-E), in subjects with reduced carbohydrate stores, during an intermittent shuttle running test (LIST) on soccer passing (LSPT) and shooting (LSST) performance. Sixteen healthy male university soccer players ingested either a 6.4 percent CHO-E or placebo (PLA) solution during 90 min of the LIST (5 mL/kg BM before and 2 mL/kg BM every 15 min of exercise), in a double-blind, randomized, crossover design, with each trial separated by at least 7 d. On the evening before the main trial (17:00 h), subjects performed the glycogen-reducing cycling exercise (approximately 80 min at 70 % VO2max). They were then fed a low-carbohydrate evening meal and reported to the laboratory the following morning after a 10-h fast. Blood was collected at rest and after every 30 min of exercise; skill tests were performed before and after the LIST. The change in mean shooting performance from pre- to post-LIST was better in the CHO-E trial but not significantly different for the passing performance. Sprint performance during the LIST was quicker in the CHO-E trial. Plasma glucose was higher in the CHO-E trial after 90 min of exercise. It was concluded that ingestion of a carbohydrate-electrolyte solution improved sprinting performance, countermovement jumping, and the subjective experiences of players. Caffeine appeared to offset the fatigue-induced decline in self-selected components of performance [10228].

To examine the effect of the glycemic index (GI) of a pre-exercise (PRE-ex) meal on plasma cytokine responses and endurance performance when carbohydrate-electrolyte (CHO-E) drink was consumed during exercise. Eight endurance-trained male runners completed three trials in a randomized order. The pre-exercise meal consisted of either high-GI (HGI) (GI=83), low-GI (LGI) foods (GI=36) or control (CON) (low energy sugar-free jelly) was given to the participant 2h before a 21-km performance run on a level treadmill. During each trial, 2 ml/kg BM of 6.6 percent CHO-E solution was consumed immediately before exercise and every 2.5-km afterward. Blood samples were collected before (pre-meal), and 120 min after ingestion the meal (120 min), immediately (POST), and 60 min (POST-60 min) after exercise. No difference was found in time to complete the 21-km run between LGI and HGI. The interleukin-6 (IL-6) level increased by more than 100 times immediately after exercise in
the three trials and returned to the basal level only on LGI at POST-60 min. In contrast, interleukin-2 (IL-2) level showed a transitory but significant decrease at POST on CON. Glucose concentrations did not recover to the pre-meal level by POST-60 min on HGI only. Cortisol concentrations increased throughout the exercise and were significantly lower on LGI when compared with CON at POST-60 min. HGI and LGI demonstrated similar performance when CHO-E solution was consumed during a 21-km run. However, pre-exercise LGI meal attenuated the increases in cortisol and quickened the recovery of the increased IL-6 value [09336].

One study investigated the influence of consuming a 2, 6, and 10 percent carbohydrate-electrolyte (CHO-E) solution on the intermittent endurance capacity and sprint performance of adolescent team games players. Seven participants (five males and two females; mean age 13 ± 1 years, height 171 ± 5 cm, body mass 62.0 ± 6.3 kg) performed three trials separated by 3-7 days. In each trial, they completed four 15-min periods of part A of the Loughborough Intermittent Shuttle Test followed by an intermittent run to exhaustion (part B). Participants consumed 5 ml kg(-1) BM of the solution during the 5-min pre-exercise period, and a further 2 ml/kg BM every 15 min during part A. Intermittent endurance capacity increased by 34 percent with ingestion of the 6 percent CHO-E solution compared with the 10 percent solution (6 ± 1 vs 4 ± 2 min), equating to a distance of 931 ± 172 versus 706 ± 272 m). There was no significant difference between the 2 percent and 6 percent or the 2 and 10 percent solutions. Carbohydrate concentration did not significantly influence mean 15-m sprint time. These results suggest that the carbohydrate concentration of an ingested solution influences the intermittent endurance capacity of adolescent team games players with a 6 percent solution significantly more effective than a 10 percent solution [11382].

One study investigated the effects of drink composition on voluntary intake, hydration status, selected physiological responses and affective states during simulated gymnasium-based exercise. In a randomised counterbalanced design, 12 physically active adults performed three 20-min intervals of cardiovascular exercise at 75 percent heart rate maximum, one 20-min period of resistance exercise and 20min of recovery with ad libitum access to water (W), a carbohydrate-electrolyte solution (CES) or with no access to fluids (NF). Fluid intake was greater with CES than W and more adequate hydration was achieved in CES trials. Plasma glucose concentrations were highest with CES. Pleasure ratings were better maintained with ad libitum intake of CES. Under conditions of voluntary drinking, CES resulted in more adequate hydration and a better maintenance of affective states than W or NF during gymnasium-based exercise [11383].

**Influence of age and pubertal status on substrate utilization**

Substrate utilization during exercise is known to differ between children and adults, but whether these differences are related to pubertal status is unclear. The objective of this study was to investigate the effects of pubertal status on endogenous (CHOendo) and orally ingested exogenous (CHOexo) carbohydrate and fat oxidation rates during exercise. Twenty boys at the same chronological age (12 years) were divided into three pubertal groups (pre-pubertal, PP: n=7; early-pubertal, EP: n=7; mid- to late-pubertal, M-LP: n=6) and consumed either a placebo or 13C-enriched 6 percent CHO drink while cycling for 60 min at approximately 70 percent of their maximal aerobic power (VO2max). Another group of 14-year-old boys (pubertal, n=9) completed all procedures. Substrate utilization was calculated for the final 15 min of exercise using indirect calorimetry and stable isotope methodology. CHOexo significantly decreased fat and increased total CHO oxidation, irrespective of group. Fat oxidation was significantly higher in younger boys than in older boys, but similar among PP,
EP, and M-LP boys. CHOexo contributed to approximately 30 percent of energy expenditure (EE) in PP and EP, but to only 24 percent in M-LP, which was identical to the older boys (24%). CHOexo oxidation rate as a percentage of EE was inversely related to testosterone levels. It was concluded that reliance on CHOexo during exercise is particularly sensitive to pubertal status, with the highest oxidation rates observed in pre- and early-pubertal boys, independent of chronological age [07273].

**Carbohydrate gel**

The aim of one study was to investigate the effects of a carbohydrate (CHO) gel on performance after prolonged intermittent high-intensity shuttle running. Seven male soccer players performed 2 exercise trials, 7 d apart. On each occasion, participants completed five 15-min periods of intermittent variable-speed running, interspersed with periods of walking (Part A), followed by an intermittent run to exhaustion (Part B). Participants consumed either a CHO gel or placebo (PLA) immediately before exercise (0.89 mL/kg body mass, BM) and every 15 min thereafter (0.35 mL/kg BM). In addition, water was consumed at a rate of 5 mL/kg BM before and 2 mL/kg BM every 15 min during exercise. Blood glucose levels were significantly higher at 15, 30, and 60 min of exercise and at exhaustion in CHO than in PLA. During Part B, run time to exhaustion was significantly longer in the CHO trial. These results indicate that ingesting a CHO gel, along with water, improves performance after prolonged intermittent running in healthy male subjects, possibly by maintaining blood glucose levels during exercise [07285].

The aim of one study was to investigate the influence of ingesting a carbohydrate (CHO) gel on the intermittent endurance capacity and sprint performance of adolescent team games players. Eleven participants (mean age 13.5 ± 0.7 years, height 1.72 ± 0.08 m, body mass 62.1 ± 9.4 kg) performed two trials separated by 3-7 days. In each trial, they completed four 15 min periods of part A of the Loughborough Intermittent Shuttle Test, followed by an intermittent run to exhaustion (part B). In the 5 min pre-exercise, participants consumed 0.818 mL/kg BM of a CHO or a non-CHO placebo gel, and a further 0.327 mL/kg BM every 15 min during part A (38.0 ± 5.5 g CHO per h in the CHO trial). Intermittent endurance capacity was increased by 21 percent during part B when the CHO gel was ingested (4.6 ± 2.0 vs 3.8 ± 2.4 min), with distance covered in part B significantly greater in the CHO trial (787 ± 319 vs 669 ± 424 m). Gel ingestion did not significantly influence mean 15 m sprint time, peak sprint time, or heart rate. Ingestion of a CHO gel significantly increases the intermittent endurance capacity of adolescent team games players during a simulated team games protocol [11387].

The aim of one study was to investigate the effects of a carbohydrate (CHO) gel on performance after prolonged intermittent high-intensity shuttle running. Seven male soccer players performed 2 exercise trials, 7 days apart. On each occasion, participants completed five 15-min periods of intermittent variable-speed running, interspersed with periods of walking (Part A), followed by an intermittent run to exhaustion (Part B). Participants consumed either a CHO gel or placebo (PLA) immediately before exercise (0.89 mL/kg body mass, BM) and every 15 min thereafter (0.35 mL/kg BM). In addition, water was consumed at a rate of 5 mL/kg BM before and 2 mL/kg BM every 15 min during exercise. Blood glucose levels were higher at 15, 30, and 60 min of exercise and at exhaustion in CHO than in PLA. During Part B, run time to exhaustion was longer in the CHO trial. These results indicate that ingesting a CHO gel, along with water, improves performance after prolonged intermittent running in healthy male subjects, possibly by maintaining blood glucose levels during exercise [07286].

**Oral carbohydrate-protein gel**
Investigators have reported improved endurance performance and attenuated post-exercise muscle damage with carbohydrate-protein beverages (CHO+P) versus carbohydrate-only beverages (CHO). However, these benefits have been demonstrated only when CHO+P was administered in beverage-form, and exclusively in male subjects. Thus, the purposes of one study were to determine if an oral CHO+P gel improved endurance performance and post-exercise muscle damage compared to a CHO gel, and determine if responses were similar between genders. Thirteen cyclists (8 men, 5 women) completed two timed cycle-trials to volitional exhaustion at 75 percent of VO2peak. At 15-minute intervals throughout these rides, subjects received CHO or CHO+P gels, which were matched for carbohydrate content (CHO = 0.15 g CHO/kg bodyweight); CHO+P = 0.15 g CHO + 0.038 g protein/kg bodyweight). Trials were performed using a randomly counterbalanced, double-blind design. Subjects rode 13 percent longer when utilizing the CHO+P gel versus the CHO gel. In addition, men and women responded similarly to the CHO and CHO+P trials, with no significant treatment-by-gender effect. Postexercise creatine kinase (CK) was not significantly different between treatments. However, CK increased significantly following exercise in the CHO trial but not the CHO+P trial. Therefore, to prolong endurance performance and prevent increases in muscle damage, it is recommended that male and female cyclists consume CHO+P gels rather than CHO gels during and immediately following exercise [07287].

*Milk*

Resistance exercise (RE) preceding the provision of high-quality dairy protein supports muscle anabolism. Milk contains bioactive components, including two high-quality protein fractions, calcium and vitamin D, each of which has been shown modulate body composition (increasing lean mass and decreasing fat mass) under energy balance and hypoenergetic conditions. These dairy nutrients are also essential for skeletal health. Acutely, no study of RE and milk/whey consumption has been undertaken exclusively in female athletes, let alone women, nevertheless, studies with both men and women show increased lean mass accretion following milk/whey compared to soy/placebo. Currently, no longer-term RE studies with milk supplementation have been done in female athletes. However, trials in young recreationally active women demonstrated augmented increases in lean mass and decreases in fat mass with RE and milk or whey protein consumption. The amount of protein consumed post-exercise is also important; two trials using yogurt (5 g protein/6 oz) failed to demonstrate a positive change in body composition compared to placebo. For bone health, RE plus dairy improved bone mineral density at clinically important sites and reduced bone resorption. With energy restriction, in one study, higher dairy plus higher protein resulted in greater fat loss, lean mass gain and improved bone health in overweight women. In another study, milk and calcium supplementation showed no greater benefit. Neither trial exclusively utilized RE. Overall, RE and milk/dairy consumption positively impact body composition in women by promoting losses in fat, gains or maintenance of lean mass and preservation of bone [12388].

Sweat is produced during exercise to help dissipate some of the extra heat produced due to an increase in metabolic rate. Inadequate drink ingestion during exercise means athletes finish exercise hypohydrated and when the time between exercise bouts is short, effective rehydration strategies will be necessary to prevent subsequent performance impairment. For complete rehydration, drink volume must be sufficient to replace sweat losses as well as the additional water losses during recovery. Once a sufficient volume of drink is ingested it is the drink composition that dictates the rehydration success of the drink. It is well known that addition of sodium and some other nutrients to rehydration drinks enhances fluid balance restoration after exercise, but the effects of milk proteins have been less well documented. Skimmed milk is an effective post-exercise rehydration solution and enhances the restoration of fluid balance after exercise-induced dehydration to a greater extent than a carbohydrate-
electrolyte sports drink. Whilst there are a number of factors in skimmed milk that might be responsible for this enhancement of rehydration, it appears that some of the effect is due to the milk protein, as milk protein has been shown to be more effective for post-exercise rehydration than an isoenergetic amount of carbohydrate. Whilst the effects of whey protein on post-exercise rehydration are equivocal, whey protein addition to a carbohydrate-electrolyte rehydration solution certainly does not impair rehydration. Therefore, in situations where protein ingestion after exercise might be advantageous for the athlete, this protein might also enhance restoration of fluid balance [12389].

It has been found that ingestion of intrinsically labeled soy and milk proteins resulted in greater incorporation of nitrogen into serum proteins and urea when soy was ingested. The suggestion from these results, and from a modeling approach, was that proteins from soy are directed toward splanchnic metabolism, whereas milk proteins are directed to peripheral sites. It has also been shown that when milk and soy proteins are ingested after resistance exercise, milk protein resulted in a more positive net amino acid balance and a greater postexercise stimulation of protein synthesis. The acute findings observed should result, over the longer term, in a greater increase in muscle protein accrual with regular milk than with soy protein consumption. It was thus aimed to determine the long-term consequences of milk or soy protein or equivalent energy consumption on training-induced lean mass accretion. It was recruited 56 healthy young men who trained 5 d/week for 12 weeks on a rotating split-body resistance exercise program in a parallel 3-group longitudinal design. Subjects were randomly assigned to consume drinks immediately and again 1 h after exercise: fat-free milk (Milk; n=18); fat-free soy protein (Soy; n=19) that was isoenergetic, isonitrogenous, and macronutrient ratio matched to Milk; or maltodextrin that was isoenergetic with Milk and Soy (control group; n=19). Muscle fiber size, maximal strength, and body composition by dual-energy X-ray absorptiometry (DXA) were measured before and after training. No between-group differences were seen in strength. Type II muscle fiber area increased in all groups with training, but with greater increases in the Milk group than in both the Soy and control groups. Type I muscle fiber area increased after training only in the Milk and Soy groups, with the increase in the Milk group being greater than that in the control group. DXA-measured fat- and bone-free mass increased in all groups, with a greater increase in the Milk group than in both the Soy and control groups. The data show that the chronic consumption of fluid skim milk (500 mL) immediately and 1 h after resistance exercise promoted greater gains in FBFM (i.e. lean mass) than consumption of either an isonitrogenous, isoenergetic, and macronutrient ratio-matched soy protein-containing beverage or an isoenergetic carbohydrate drink. It was also observed a greater reduction in body fat mass associated with chronic postexercise milk consumption. On the basis of these results, extrapolations would predict that someone would gain twice as much lean mass with milk consumption as opposed to soy consumption, if only leg exercises were performed during a training program. Thus, it would appear that the acute findings may overestimate the magnitude of the effect in terms of differences in muscle protein accretion seen with soy and milk ingestion after exercise. Work comparing milk proteins with isolated whey and casein proteins indicates that postprandial nitrogen utilization with milk proteins may support greater anabolism than either isolated protein alone. The reason for this difference was not readily apparent, but in light of other data showing the superiority of milk proteins, including isolated casein. However, supplementation of soy protein with BCAAs can alter interorgan amino acid fluxes to favor muscle protein anabolism in aged and patient populations, whether this would be true in young persons under conditions of exercise-induced anabolism is not known. Hence, the data presented here, combined with previous data from chronic studies manipulating postexercise protein consumption, support the general thesis that immediate consumption of protein, particularly milk protein, after resistance exercise serves to maximize exercise-induced increases in muscle mass. In conclusion, immediate and 1-h postexercise milk consumption, as opposed to soy or isoenergetic carbohydrate, resulted in greater gains
in FBFM and type II muscle fiber area. Increases in type I muscle fiber area were greater in the Milk and the Soy groups than in the control group. All groups showed increased strength as a result of the training program; however, there were no between-group effects. A greater fat mass loss was seen in subjects who consumed the postexercise milk supplement than in both the Soy and control groups, which may be related to dietary calcium intake or an endogenous property of the milk proteins themselves [07309].

The effectiveness of low-fat milk, alone and with an additional 20 mmol/l NaCl, at restoring fluid balance after exercise-induced hypohydration was compared to a sports drink and water. After losing 1.8 percent of their body mass during intermittent exercise in a warm environment, eleven subjects consumed a drink volume equivalent to 150 percent of their sweat loss. Urine samples were collected before and for 5 h after exercise to assess fluid balance. Urine excretion over the recovery period did not change during the milk trials whereas there was a marked increase in output between 1 and 2 h after drinking water and the sports drink. Cumulative urine output was less after the milk drinks were consumed (611 and 550 mL for milk and milk with added sodium, respectively, compared to 1184 and 1205 mL for the water and sports drink). Subjects remained in net positive fluid balance or euhydrated throughout the recovery period after drinking the milk drinks but returned to net negative fluid balance 1 h after drinking the other drinks. The results of the present study suggest that milk can be an effective post-exercise rehydration drink and can be considered for use after exercise by everyone except those individuals who have lactose intolerance [07310].

Exercise-induced muscle damage (EIMD) leads to increases in intramuscular proteins observed in the blood stream and delayed onset of muscle soreness, but crucial for athletes are the decrements in muscle performance observed. Previous research has demonstrated that carbohydrate-protein supplements limit these decrements; however, they have primarily used isokinetic dynamometry, which has limited applicability to dynamic sport settings. Therefore, the aim of this study was to investigate the effects of a carbohydrate-protein milk supplement consumed after muscle-damaging exercise on performance tests specific to field-based team sports. Two independent groups of seven males consumed either 500 mL of milk or a control immediately after muscle-damaging exercise. Passive and active delayed onset of muscle soreness, creatine kinase, myoglobin, countermovement jump height, reactive strength index, 15 m sprint, and agility time were assessed before and 24, 48, and 72 h after EIMD. The Loughborough Intermittent Shuttle Test was also performed before and 48 h after EIMD. At 48 h, milk had a possible benefit for limiting increases in 10m sprint time and a likely benefit of attenuating increases in mean 15-m sprint time during the Loughborough Intermittent Shuttle Test. At 72 h, milk had a possible benefit for limiting increases in 15-m sprint time and a likely benefit for the attenuation of increases in agility time. All other effects for measured variables were unclear. It was concluded that the consumption of milk limits decrements in one-off sprinting and agility performance and the ability to perform repeated sprints during the physiological simulation of field-based team sports [13626].

One study investigated the relationship between the milk protein content of a rehydration solution and fluid balance after exercise-induced dehydration. On three occasions, eight healthy males were dehydrated to an identical degree of body mass loss (BML, approximately 1.8 %) by intermittent cycling in the heat, rehydrating with 150 percent of their BML over 1 h with either a 60 g/L carbohydrate solution (C), a 40 g/L carbohydrate, 20 g/L milk protein solution (CP20) or a 20 g/L carbohydrate, 40 g/L milk protein solution (CP40). Urine samples were collected pre-exercise, post-exercise, post-rehydration and for a further 4 h. Subjects produced less urine after ingesting the CP20 or CP40 drink compared with the C drink, and at the end of the study, more of the CP20 (59 %) and CP40 (64 %) drinks had been retained compared with the C drink (46 %). At the end of the study, whole-body net
fluid balance was more negative for trial C (-470 mL) compared with both trials CP20 (-181 mL) and CP40 (2107 mL). At 2 and 3 h after drink ingestion, urine osmolality was greater for trials CP20 and CP40 compared with trial C. The present study further demonstrates that after exercise-induced dehydration, a carbohydrate--milk protein solution is better retained than a carbohydrate solution. The results also suggest that high concentrations of milk protein are not more beneficial in terms of fluid retention than low concentrations of milk protein following exercise-induced dehydration [13627].

**Milk minerals**

Despite a high content of saturated fat, evidence from observational studies indicates that the consumption of dairy products may have a neutral effect or may be inversely associated with the risk of CVD. We aimed to examine whether milk minerals modify the effect of saturated fat on serum lipid profile. It was presented data from two studies. Study I had a randomised, blinded, parallel design (n 24 pigs) with a 10 d adaptation period during which a high-fat diet was fed to the pigs and a 14 d intervention period during which the same diet either enriched with milk minerals (MM group) or placebo (control group) was fed to the pigs. Study II had a randomised cross-over design (n 9 men) where the subjects were fed either a high-fat diet enriched with milk minerals (MM period) or a regular diet (control period). In both the studies, blood variables were measured before and after the intervention and faecal and urine samples were collected at the end of the dietary periods. The increase in plasma total cholesterol and LDL-cholesterol concentrations but not in HDL-cholesterol concentration was markedly lowered by milk minerals in both the studies. In the animal study, baseline adjusted total cholesterol and LDL-cholesterol concentrations in the MM group were 11 percent and 13 percent) lower compared with those in the control group after the intervention. Similarly in the human study, baseline adjusted total cholesterol and LDL-cholesterol concentrations were 6 percent and 9 percent lower after the MM period compared with those in the control period. HDL-cholesterol concentration was not lowered by milk minerals. These short-term studies indicate that the addition of milk minerals to a high-fat diet to some extent attenuates the increase in total cholesterol and LDL-cholesterol concentrations, without affecting HDL-cholesterol concentration [13630].

**Fermented milk**

One study investigated the effect of fermented milk supplementation on glucose metabolism associated with muscle damage after acute exercise in humans. Eighteen healthy young men participated in each of the three trials of the study: rest, exercise with placebo, and exercise with fermented milk. In the exercise trials, subjects carried out resistance exercise consisting of five sets of leg and bench presses at 70-100 percent 12 repetition maximum. Examination beverage (fermented milk or placebo) was taken before and after exercise in double-blind method. On the following day, we conducted an analysis of respiratory metabolic performance, blood collection, and evaluation of muscle soreness. Muscle soreness was significantly suppressed by the consumption of fermented milk compared with placebo. Serum creatine phosphokinase was significantly increased by exercise, but this increase showed a tendency of suppression after the consumption of fermented milk. Exercise significantly decreased the respiratory quotient, although this decrease was negated by the consumption of fermented milk. Furthermore, exercise significantly reduced the absorption capacity of serum oxygen radical although this reduction was not observed with the consumption of fermented milk. The results suggest that fermented milk supplementation improves glucose metabolism and alleviates the effects of muscle soreness after high-intensity exercise, possibly associated with the regulation of antioxidant capacity [13631].

**Effects depending on exercise status**

It has been suggested that milk protein intake for the improvement of metabolic health. 1697
However, the authors missed important insights into branched-chain amino acid (BCAA) metabolism under conditions of obesity and insulin resistance. They emphasized beneficial effects of milk protein ingestion for skeletal muscle but ignored adverse effects of BCAAs on adipose tissue and long-term beta-cell homeostasis. Obviously, milk’s physiological function promoting neonatal growth is not restricted to the musculoskeletal system. It is the intention of this perspective article to demonstrate that the evaluation of metabolic effects of milk protein consumption has to consider the nutritional and endocrine status and the level of physical activity of the milk protein consumer. Plasma BCAAs (leucine, isoleucine, valine) and glutamine/glutamate are increased in obesity, insulin resistance and type 2-diabetes (T2D). An extra daily intake of 53 g milk protein but not 53 g meat increased serum insulin and insulin resistance in 8-year-old boys. Impaired BCAA catabolism of adipocytes is a crucial metabolic deviation of obesity. As BCAA plasma levels in obesity are already elevated an additional BCAA influx may further deteriorate the pre-existing metabolic imbalance. In fact, the marked decrease in BCAA plasma levels resulting from gastric bypass surgery is associated with weight loss and improved insulin sensitivity. Palaeolithic, physically active hunter-gatherers consumed structural proteins like fish and meat. In contrast, modern Neolithic humans have “mutated” into physically inactive individuals, who particularly consume signalling proteins from milk providing abundant “fast dietary proteins” leading to high plasma BCAA and glutamine levels. Palaeolithic dairy-free diets exhibit lower insulin levels with improved insulin sensitivity protecting against the development of diseases of civilization. Milk protein consumption results in postprandial hyperinsulinemia in obese subjects, increases body weight of overweight adolescents and may thus deteriorate pre-existing metabolic disturbances of obese, insulin resistant individuals [13628].

Chocolate milk

An optimal post-exercise nutrition regimen is fundamental for ensuring recovery. Therefore, research has aimed to examine post-exercise nutritional strategies for enhanced training stimuli. Chocolate milk has become an affordable recovery beverage for many athletes, taking the place of more expensive commercially available recovery beverages. Low-fat chocolate milk consists of a 4:1 carbohydrate:protein ratio (similar to many commercial recovery beverages) and provides fluids and sodium to aid in post-workout recovery. Consuming chocolate milk (1.0-1.5 g/kg/h) immediately after exercise and again at 2 h post-exercise appears to be optimal for exercise recovery and may attenuate indices of muscle damage. Future research should examine the optimal amount, timing, and frequency of ingestion of chocolate milk on post-exercise recovery measures including performance, indices of muscle damage, and muscle glycogen resynthesis [12390].

Exercise-induced muscle damage (EIMD) leads to decrements in muscle performance, increases in intramuscular proteins and delayed-onset of muscle soreness (DOMS). Previous research demonstrated that one litre of milk-based protein-carbohydrate (CHO) consumed immediately following muscle damaging exercise can limit changes in markers of EIMD possibly due to attenuating protein degradation and/or increasing protein synthesis. If the attenuation of EIMD is derived from changes in protein metabolism then it can be hypothesised that consuming a smaller volume of CHO and protein will elicit similar effects. Three independent matched groups of 8 males consumed 500 mL of milk, 1,000 mL of milk or a placebo immediately following muscle damaging exercise. Passive and active DOMS, isokinetic muscle performance, creatine kinase (CK), myoglobin and interleukin-6 were assessed immediately before and 24, 48 and 72 h after EIMD. After 72 h 1,000 mL of milk had a likely benefit for limiting decrements in peak torque compared to the placebo. After 48 h, 1,000 mL of milk had a very likely benefit of limiting increases in CK in comparison to the placebo. There were no differences between consuming 500 or 1,000 mL of milk for changes in peak torque and CK. In conclusion, decrements in isokinetic muscle performance and
increases in CK can be limited with the consumption of 500 mL of milk [12086].

To maximize training quality, athletes have sought nutritional supplements that optimize recovery. This study compared chocolate milk (CHOC) with a carbohydrate replacement beverage (CRB) as a recovery aid after intense exercise, regarding performance and muscle damage markers in trained cyclists. Ten regional-level cyclists and triathletes completed a high-intensity intermittent exercise protocol, then 15-18 h later performed a performance trial at 85 percent of maximal oxygen uptake to exhaustion. Participants consumed 1.0 g carbohydrate/kg/h of a randomly assigned isocaloric beverage (CHOC or CRB) after the first high-intensity intermittent exercise session. The same protocol was repeated 1 week later with the other beverage. A 1-way repeated measures analysis of variance revealed no significant difference between trials for time to exhaustion at 85 percent of maximal oxygen uptake. The change in creatine kinase (CK) was significantly greater in the CRB trial than in the CHOC trial with differences not significant for CK levels before the second exercise session between the two trials. These findings indicate no difference between CHOC and this commercial beverage as potential recovery aids for cyclists between intense workouts [09337].

One study examined the effects of 3 recovery drinks on endurance performance following glycogen-depleting exercise. Nine trained male cyclists performed 3 experimental trials, in a randomized counter-balanced order, consisting of a glycogen-depleting trial, a 4-h recovery period, and a cycle to exhaustion at 70 percent power at maximal oxygen uptake. At 0 and 2 h into the recovery period, participants consumed chocolate milk, a carbohydrate replacement drink, or a fluid replacement drink. Participants cycled 51 percent and 43 percent longer after ingesting chocolate milk (32 ± 11 min) than after ingesting carbohydrate replacement drink (21 ± 8 min) or fluid replacement drink (23 ± 8 min). Chocolate milk is an effective recovery aid after prolonged endurance exercise for subsequent exercise at low-moderate intensities [09338].

Flavanol content
Dietary flavanols have been associated with reduced oxidative stress, however their efficacy in promoting recovery after exercise induced muscle damage is unclear. This study examined the effectiveness of acute consumption of cocoa-flavanols on indices of muscle recovery including: subsequent exercise performance, creatine kinase, muscle tenderness, force, and self-perceived muscle soreness. Eight endurance-trained athletes (VO2max 64 ± 8 mL/kg/min) completed a downhill running protocol to induce muscle soreness, and 48 h later completed a 5-K (kilometer) time trial. Muscle recovery measurements were taken at PRE, 24 h-POST, 48 h-POST, and POST-5K. Participants consumed 1.0 g of carbohydrate per kilogram of body weight of a randomly assigned beverage (CHOC: 0 mg flavanols vs CocoaCHOC: 350 mg flavanols per serving) immediately after the downhill run and again 2 h later. The same protocol was repeated three weeks later with the other beverage. An ANOVA revealed no significant difference between trials for 5 K completion time. No significant difference was found for creatine kinase (CK) levels, or muscle soreness between groups over time. These findings suggest that the acute addition of cocoa flavanols to low-fat chocolate milk offer no additional recovery benefits [13629].

Raisens
It was examined the metabolic, performance and gastrointestinal (GI) effects of supplementation with a natural food product (raisins) compared to a commercial product (sport chews). Eleven male (29 years) runners completed three randomized trials (raisins, chews and water only) separated by seven days. Each trial consisted of 80-min (75 %
VO$_2_{\text{max}}$ treadmill running followed by a 5-km time trial (TT). Heart rate (HR), respiratory exchange ratio (RER), blood lactate, serum free fatty acids (FFA), glycerol and insulin, plasma glucose and creatine kinase, GI symptoms and rating of perceived exertion (RPE) were recorded every 20-min. VO$_2$, HR, lactate, glycerol and RPE did not differ due to treatment. Average plasma glucose was maintained at resting levels during the sub-maximal exercise bout, and was significantly higher with chews than water only. RER and percent of non-protein macronutrient oxidation derived from carbohydrate was highest with chews, followed by raisins and water was the lowest during the sub-maximal exercise period. Serum FFA was higher in the water treatment versus both raisins and chews at 80 min of sub-maximal exercise. Serum insulin was higher with the chews than both raisins and water. Plasma creatine kinase, corrected for baseline values, for the last 40 min of the sub-maximal exercise bout, was higher with raisins compared to other treatments. The TT was faster for both carbohydrate supplements. GI disturbance was mild for all treatments. It was concluded that raisins and chews promoted higher carbohydrate oxidation and improved running performance compared to water only. Running performance was similar between the raisins and chews, with no significant GI differences [12397].

The purpose of one study was to examine the effects of a natural carbohydrate (CHO) source in the form of sun-dried raisins versus Sports Jelly Beans™ on endurance performance in trained cyclists and triathletes. Ten healthy men (18-33 years) completed 1 water-only acclimatization exercise trial and 2 randomized exercise trials administered in a crossover fashion. Each trial consisted of a 120-minute constant-intensity glycogen depletion period followed by a 10-km time trial (TT). During each experimental trial, participants consumed isocaloric amounts of sun-dried raisins or Sports Jelly Beans in 20-minute intervals. Measurements included time to complete 10-km TT, power output during 10-km TT, blood glucose levels and respiratory exchange ratio during glycogen depletion period, rate of perceived exertion (RPE), flow questionnaire responses, and a hedonic (i.e. pleasantness) sensory acceptance test. There were no significant differences in endurance performance for TT time or power, resting blood glucose levels, RPE, or flow experiences between sun-dried raisins and Sports Jelly Beans trials. However, the mean sensory acceptance scores were significantly higher for the sun-dried raisins compared to the beans. Consuming sun-dried raisins or Sports Jelly Beans during 120 minutes of intense cycling results in similar subsequent TT performances and are equally effective in maintaining blood glucose levels during exercise. Therefore, raisins are a natural, pleasant, cost-effective CHO alternative to commercial SJBs that can be used during moderate- to high-intensity endurance exercise [11527].

Research suggests that pre-exercise sources of dietary carbohydrate with varying glycemic indexes may differentially affect metabolism and endurance. One study was designed to examine potential differences in metabolism and cycling performance after consumption of moderate glycemic raisins vs. a high glycemic commercial sports gel. Eight endurance-trained male (n=4) and female (n=4) cyclists 30 ± 5 years of age completed two trials in random order. Subjects were fed 1 g carbohydrate per kilogram body weight from either raisins or sports gel 45 minutes prior to exercise on a cycle ergometer at 70 percent VO$_2_{\text{max}}$. After 45 minutes of submaximal exercise, subjects completed a 15-minute performance trial. Blood was collected prior to the exercise bout, as well as after the 45th minute of exercise, to determine serum concentrations of glucose, insulin, lactate, free fatty acids (FFAs), triglycerides, and beta-hydroxybutyrate. Performance was not different between the raisin and gel trials. Prior to exercise, serum concentrations of glucose and other fuel substrates did not differ between trials; however, insulin was higher for the gel versus raisin trial. After 45 minutes of exercise, insulin decreased to 14.2 ± 6.2 microU/mL and 13.3 ± 18.9 microU/mL for gel and raisin trials, respectively. The FFA concentration increased significantly during the raisin trial. Overall, minor differences in metabolism and no difference
in performance were detected between the trials. Raisins appear to be a cost-effective source of carbohydrate for pre-exercise feeding in comparison to sports gel for short-term exercise bouts [07288].

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Fruits and vegetables

Regular consumption of fruits and vegetables (FV) is widely regarded as an important contributor to a healthy diet. Inadequate consumption of plant foods is associated with an inadequate supply of important micronutrients like vitamins, phytochemicals and minerals. In athletes a deficit of these micronutrients can lead to excessive production of reactive oxygen and nitrogen species that induce tissue damage, a higher frequency of inflammatory processes, decreased immunity, increased susceptibility to injury, and prolonged recovery. But many athletes rarely achieve the recommended intake of FV due to difficult coordination of training activities and food intake, or to due to problems with digestion of FV. Therefore, in recent years more and more sports people have adopted supplemental FV concentrates to work around timing problems with uptake and the detrimental digestive effects during training of high FV intake. It is thought that supplementation of an athlete's basic diet with mixed FV concentrates can promote stable health and immunity, in order to provide a basis for optimal adaptation and performance. The intention of one article was to build a bridge between the science behind FV supplementation in exercise on the one hand and the practical relevance of its application on the other. For that purpose this paper addresses three questions: Is supplementation with a mixed FV concentrate to the athlete's diet appropriate to ensure stable health and immunity? Can supplementation with a mixed FV concentrate improve performance? Counseling guidance: how can sport nutrition advisors decide whether or not to supplement with mixed FV concentrates? [12393].

Cornstarch

The purpose of one study was to investigate differences in substrate oxidation between dextrose (DEX) and unmodified (UAMS) and acid/alcohol-modified (MAMS) cornstarches. Seven endurance-trained men participated in 2 h of exercise (66 % VO2peak) 30 min after ingesting 1 g/kg body weight of the experimental carbohydrate or placebo (PLA). Plasma glucose and insulin were elevated after DEX compared with UAMS, MAMS, and PLA.
Although MAMS and DEX significantly raised carbohydrate oxidation rate through 90 min of exercise, only MAMS persisted throughout 120 min. Exogenous-carbohydrate oxidation rate was higher in DEX than in MAMS and UAMS until 90 min of exercise. Acid/alcohol modification resulted in augmented carbohydrate oxidation with a small, sustained increase in exogenous-carbohydrate oxidation rate. MAMS appears to be metabolizable and available for oxidation during exercise [07290].

**Hydroxypropyl-distarch**

The aim of one study was to investigate the effects of hydroxypropyl-distarch phosphate (HDP) supplementation on postprandial energy metabolism and glucose-dependent insulinoetric polyepetide (GIP) in human subjects. A total of ten healthy male subjects, with a mean BMI of 24 kg/m², age 35 years and body weight 71 kg, participated in a randomised, cross-over, intervention study with two different test meals (1674 kJ) containing either waxy maize starch or HDP from waxy maize starch (degree of substitution 0.15, P content 0.004 %). Resting energy expenditure (REE) and blood concentrations of various biomarkers were measured at fasting and up to 180 min postprandially. Indirect calorimetry showed that the HDP meal caused higher REE and fat utilisation than the waxy maize starch meal. The HDP meal led to significantly lower postprandial glucose, insulin and GIP responses than the waxy maize starch meal. Both postprandial REE and fat utilisation were negatively correlated with the postprandial GIP response, but not with the glucose and insulin responses. In conclusion, dietary supplementation with HDP lowers postprandial GIP and increases postprandial REE and fat utilisation in healthy humans. An HDP-rich diet may therefore have beneficial implications in weight management [11399].

**Honey-sweetened beverage**

One study compared the effect of a honey-sweetened beverage with those of a commercial sports drink and a placebo on performance and inflammatory response to a 90-min soccer simulation. Ten experienced male soccer players randomly performed 3 trials (honey [H], sports drink [S], and placebo [P]), consuming the beverage before and during halftime for a total of 1.0 g/kg carbohydrate for H and S. Performance measures included 5 sets (T1-T5) of a high-intensity run and agility and ball-shooting tests followed by a final progressive shuttle-run test to exhaustion. Blood samples were drawn pretest, posttest (B2), and 1 hr posttest (B3) for markers of inflammation, oxygen radical absorbance capacity, and hormone response. T2-T5 were significantly slower than T1, and a decrease in progressive shuttle-run time was observed from baseline (-23 %) for all treatments. No significant effect of the interventions was observed for any performance measures. Plasma IL-1ra levels increased posttest for all treatments, but H was significantly less than S at posttest and P at B3. Other cytokines and ORAC increased at B2 with no difference by treatment. Acute ingestion of honey and a carbohydrate sports drink before and during a soccer-simulation test did not improve performance, although honey attenuated a rise in IL-1ra. Ingestion of carbohydrate and/or antioxidant-containing beverages at frequencies typical of a regulation match may not be beneficial for trained soccer players [09339].

**Low-carbohydrate diets and performance**

Athletes are continually searching for means to optimize their performance. Within the past 20 years, athletes and scientists have reported or observed that consuming a carbohydrate-restricted diet may improve performance. The original theories explaining the purported benefits centered on the fact that fat oxidation increases, thereby "sparing" muscle glycogen. More recent concepts that explain the plausibility of the ergogenicity of low-carbohydrate, or
high-fat, diets on exercise performance pertain to an effect similar to altitude training. We and others have observed that although fat oxidation may be increased, the ability to maintain high-intensity exercise (above the lactate threshold) seems to be compromised or at least indifferent when compared with consumption of more carbohydrate. That said, clinical studies clearly demonstrate that ad libitum low-carbohydrate diets elicit greater decreases in body weight and fat than energy-equivalent low-fat diets, especially over a short duration. Thus, although low-carbohydrate and high-fat diets appear detrimental or indifferent relative to performance, they may be a faster means to achieve a more competitive body composition [07299].

It has been speculated that dietary carbohydrate restriction is solely responsibly for mobilization of endogenous lipid stores, elevation of plasma free fatty acid (FFA) concentration, and an associated reduction in insulin sensitivity seen in starvation and low-carbohydrate diets. In 6 healthy men, dietary carbohydrate was eliminated but gluconeogenic substrate supply was maintained by 3 days of very low-carbohydrate/high-protein (HPLC) diet. Results were compared with 3-day starvation and 3-day mixed-carbohydrate diet. Intramyocellular lipid (IMCL) concentration was measured by \(^1\text{H}\) magnetic resonance spectroscopy, and insulin sensitivity was determined by intravenous glucose tolerance test. Fasting plasma glucose was significantly reduced, whereas IMCL to water ratio and fasting were significantly elevated after starvation but were unchanged after HPLC. Minimal model insulin sensitivity was significantly reduced after starvation. Plasma glucose, plasma FFAs, IMCLs, and insulin sensitivity are maintained when an HPLC diet is consumed, despite other forms of carbohydrate deprivation producing marked changes in these measures. It was conclude that dietary carbohydrate restriction does not cause circulating FFA to become elevated. However, it remains possible that circulating carbohydrate status has an important influence on plasma FFA and therefore insulin sensitivity in healthy people [10233].

Grain-based
To illustrate the effects of low-carbohydrate (LC) and grain-based (GB) diets on body composition, biomarkers, athletic training, and performance in an elite triathlete athlete followed 2 dietary interventions for 14 days while maintaining a prescheduled training program. Pre- and postintervention measurements for each diet included plasma and serum samples, resting energy expenditure, body composition, and a performance bike ride. Compared with the GB diet, the LC diet elicited more disruptions to training and unfavorable subjective experiences. Total cholesterol, HDL cholesterol, LDL cholesterol, ratings of perceived exertion, and heart rate were elevated on the LC diet. Blood insulin, resting lactate, postexercise lactate, and C-reactive protein were lowest on the LC diet. It was concluded that the LC diet resulted in both favorable and unfavorable outcomes. The primary observation was a disruption to scheduled training on the LC diet. Researchers should consider how the potential mediating effect of disruptions to training could influence pretest-posttest designs [07300].

Low energy
Energy availability is the amount of dietary energy remaining after exercise training for all other metabolic processes. Excessively low energy availability impairs reproductive and skeletal health, although genetics and age may alter an individual's initial conditions and sensitivity when low energy availability is imposed. Many marathon runners and other endurance athletes reduce energy availability either intentionally to modify body size and composition for improving performance; compulsively in a psychopathological pattern of disordered eating; or inadvertently because there is no strong biological drive to match energy intake to activity-induced energy expenditure. Inadvertent low energy availability is more extreme when consuming a low fat, high carbohydrate diet. Low energy availability, reproductive disorders, low bone mineral density and stress fractures are more common in
female than male athletes. Functional menstrual disorders caused by low energy availability should be diagnosed by excluding diseases that also disrupt menstrual cycles. To determine energy availability (in units of kilocalories or kilojoules per kilogram of fat-free mass), athletes can record their diets and use diet analysis software to calculate energy intake, measure energy expenditure during exercise using a heart monitor and measure fat-free mass using a bioelectrical impedance body composition scale. All are commercially available at consumer prices [07301].

**High and low glycaemic index recovery diets**

Intramyocellular lipid (IMCL) and plasma NEFA are important skeletal muscle fuel sources. By raising blood insulin concentrations, carbohydrate ingestion inhibits lipolysis and reduces circulating NEFA. We hypothesised that differences in the postprandial glycaemic and insulin response to carbohydrates (i.e. glycaemic index; GI) could alter NEFA availability and IMCL use during subsequent exercise. Endurance-trained individuals (n 7) cycled for 90 min at 70 % VO2peak and then consumed either high GI (HGI) or low GI (LGI) meals over the following 12 h. The following day after an overnight fast, the 90 min cycle was repeated. IMCL content of the vastus lateralis was quantified using magnetic resonance spectroscopy before and after exercise. Blood samples were collected at 15 min intervals throughout exercise and analysed for NEFA, glycerol, glucose, insulin, and lactate. Substrate oxidation was calculated from expired air samples. The 90 min cycle resulted in >2-fold greater reduction in IMCL in the HGI trial (3.5 (sem 1.0) mm/kg wet weight) than the LGI trial (1.6 mm/kg wet weight). During exercise, NEFA availability was reduced in the HGI trial compared to the LGI trial (area under curve 2.36 mEq/l per h vs 3.14 mEq/l per h, respectively). No other differences were significant. The findings suggest that HGI carbohydrates reduce NEFA availability during exercise and increase reliance on IMCL as a substrate source during moderate intensity exercise [07302].

**Carbohydrate loading effect of menstrual-cycle phase**

One study compared 3 d of carbohydrate loading (CHOL; 8.4 g carbohydrate per kg and day) in female eumenorrheic athletes with 3 d of an isoenergetic normal diet (NORM; 5.2 g carbohydrate per kg and day) and examined the effect of menstrual-cycle phase on performance, muscle-glycogen concentration [glyc], and substrate utilization. Nine moderately trained eumenorrheic women cycled in an intermittent protocol varying in intensity from 45 to 75 percent VO2max for 75 min, followed by a 16-km time trial at the midfollicular (MF) and midluteal (ML) phases of the menstrual cycle on NORM and CHOL. Time-trial performance was not affected by diet or menstrual-cycle phase. Resting [glyc] was lowest in the MF phase after NORM compared with the MF phase after CHOL and the ML phase after CHOL and NORM. No effect of phase on substrate utilization during exercise was observed. These data support previous observations of greater resting [glyc] in the ML than the MF phase of the menstrual cycle and suggest that lower glycogen storage in the MF phase can be overcome by carbohydrate loading [07303].

**Effects of carbohydrate beverage ingestion on the salivary IgA**

The purpose of one study was to establish if provision of carbohydrates altered the mucosal immune and salivary cortisol responses to intermittent exercise in the heat. In a double-blind design, 10 males undertook soccer-specific intermittent exercise on a motorized treadmill on 2 occasions, each over 90 min and separated by 1 week. During CHO and placebo trials, subjects were given either a carbohydrate solution (3 ml/kg body weight) or placebo drink, 5 min before the commencement of exercise, at 15, 30 min, at half time, 60 and 75 min into exercise. Salivary flow rate increased throughout the placebo trial and decreased throughout
the CHO treatment; the difference between conditions neared statistical significance. Neither s-IgA concentration nor s-IgA to osmolality ratio was affected by 2 conditions or differed at any time-point post-exercise. The s-IgA secretion rate increased, s-IgA to protein ratio decreased post-exercise and salivary cortisol decreased 24 h post-exercise compared to pre-exercise. Carbohydrate supplementation whilst exercising in the heat, does not influence rating of perceived exertion, thermal sensation, salivary flow rate, s-IgA concentration, s-IgA secretion rate, s-IgA to osmolality ratio or s-IgA to protein ratio and salivary cortisol but heart rate was increased [11385].

**Carbohydrate in young adolescents**

One study examined the effect of preexercise carbohydrate (CHO) feeding on performance on a Wingate anaerobic test (WAnT) in 11 boys (10 ± 1 years old). Four WAnTs with 2 min recovery were performed 30 min after consuming a CHO (1 g CHO/kg) or placebo drink. Peak power (PP) and mean power (MP) were similar between trials. The study found that the ingestion of a CHO solution before exercise did not influence power output during repeated performances of the WAnT [07304].

**Glucose polymers**

It was previously reported no difference in the oxidation rate of a high molecular weight glucose polymer (GP) versus maltodextrin (8 kDa) during exercise; however, the ingestion rate (1.8 g/min) was above the glucose absorption-oxidation maxima (about 1.0 g/min), possibly masking either faster gastric emptying of the GP and delivery to the circulation observed at rest or physical properties of the GP that might slow intestinal absorption. Therefore, we asked whether GP oxidation could be differentially affected when ingested at a lower rate (0.8 g/min). Eight cyclists performed three 150-min rides at 50 percent peak power while ingesting solutions containing 8 percent GP (500-750 kDa, 21 mosm/kg), 8 percent glucose (469 mosm/kg), or water. The exogenous carbohydrate oxidation rate was determined using stable isotope methodology and indirect calorimetry. Glucose and GP were oxidized on average at 0.54 g/min and 0.41 g/min, respectively, which equated to a moderate (effect size) reduction of 24 percent with GP. The endogenous carbohydrate oxidation rate with glucose (1.04 g/min) was not clearly different from GP (15 %) and total carbohydrate oxidation rate was not affected. Plasma glucose concentration was 8 percent lower and nausea 0.4 units higher with GP versus glucose. To conclude, the oxidation rate of GP when ingested below the glucose absorption-oxidation maxima is slower than glucose. Further work could determine the physical properties of the carbohydrate and (or) physiological mechanism determining this response. Meanwhile, utility of the glucose polymer over glucose or maltodextrin in energy beverages appears limited [11255].

**Carbohydrate effect on oxidative changes**

Carbohydrate administration during exercise diminishes stress hormone release, but the relationship of these hormones with oxidative stress has not been examined. Fifteen subjects functioned as their own controls and ingested carbohydrate (6 %) or placebo in a randomized design while cycling for 2.5-h approximately 75 % VO2peak). Blood and skeletal muscle samples were collected 30 min pre-exercise, immediately post-exercise, and 12-h post-exercise and analyzed for F (2)-isoprostanes, ferric reducing ability of plasma, glucose, insulin, cortisol, epinephrine, and muscle glycogen, respectively. Statistical design was a 2 (treatment) x 3 (time) repeated measures analysis of variance. Glucose, insulin, and ferric reducing ability of plasma were significantly higher and F (2)-isoprostanes, cortisol, and epinephrine significantly lower in carbohydrate versus placebo. The decrease in muscle
glycogen was not different. During cycling exercise, oxidative stress appears to be heavily influenced by carbohydrate ingestion and increased stress hormones [07294].

Increased lactate (decreased lipolysis) due to carbohydrates

The present study evaluated the acute effects of carbohydrate supplementation on heart rate (HR), rate of perceived exertion (RPE), metabolic and hormonal responses during and after sessions of high-intensity intermittent running exercise. Fifteen endurance runners (26 years) performed two sessions of intermittent exercise under carbohydrate (CHO) and placebo (PLA) ingestion. The sessions consisted of 12 x 800 m separated by intervals of 1 min 30 s at a mean velocity corresponding to the previously performed 3-km time trial. Both the CHO and PLA sessions were concluded within approximately 28 min. Blood glucose was significantly elevated in both sessions and mean blood lactate was significantly higher in the CHO than in the PLA condition. The metabolic stress induced by the exercise model used was confirmed by the elevated HR (approximately 182 bpm) and RPE (approximately 18 on the 15-point Borg scale) for both conditions. No significant differences in plasma insulin, cortisol or free fatty acids were observed during exercise between the two trials. During the recovery period, free fatty acid and insulin concentrations were significantly lower in the CHO trial. Supplementation with CHO resulted in higher lactate associated with lipolytic suppression, but did not attenuate the cortisol, RPE or HR responses [07295].

Lactate

For many years, the production and metabolism of lactate have been scrutinized by exercise physiologists. In the early days of study, lactate was considered to be a waste product formed when glucose-6-phosphate was catabolized by glycolysis under hypoxic conditions. Lactate production was also thought to release hydrogen ions, which promoted skeletal muscle fatigue by contributing to acidosis. Others suggested that high levels of lactate in the muscle cell could promote reductions in work capacity. Viewpoints on lactate started to change in the 1980s, when researchers began providing evidence that it was not a harmful waste product but an energy intermediate that could be further metabolized to provide substrate for the tricarboxylic acid cycle or gluconeogenesis. In either case, lactate could enhance energy substrate status during exercise by sparing muscle glycogen or by fortifying blood glucose stores. Close examination of glycolysis reveals that complete metabolism of glucose to lactate results in no net release of protons and, thus, does not contribute to acidosis. In fact, during the production of lactate from pyruvate, protons are consumed and acidosis is inhibited. Furthermore, lactate oxidation and lactate consumption via gluconeogenesis consume hydrogen ions and are alkalinizing processes. Lactate’s role as an energy substrate and mediator of pH balance has sparked interest into the metabolic and exercise performance effects of orally consumed lactate. Evidence has revealed no effects of lactate consumption on time to exhaustion during low- to moderate-intensity exercise, suggesting that it is ineffective as an energy supplement. Lactate ingestion has been shown to increase blood pH and bicarbonate levels and increase time to exhaustion in short, high-intensity work bouts. Future work should focus on determining optimal doses of lactate, temporal relationships between doses and exercise, and the efficacy of lactate as an ergogenic in different types of high-intensity exercise [12513].

Oral lactate as an energy substrate

Investigations have shown that orally consumed lactate is oxidized readily by working muscle and can maintain blood glucose levels during extended exercise bouts. Two studies have investigated the effects of lactate, consumed alone or with carbohydrate, on time to exhaustion during low- to moderate-intensity exercise and neither demonstrated ergogenic
The initial investigations of lactate consumption as an energy supplement evolved from previous works that studied the metabolic fates of intravenously infused lactate in exercising subjects. These studies of lactate metabolism demonstrated that substantial amounts of infused lactate were oxidized in exercising skeletal muscle and led some investigators to ponder the effects of oral lactate consumption on endurance exercise performance. Early attempts of lactate ingestion were unsuccessful because oral consumption of large boluses was found to result in extreme gastric distress. As lactate is not well tolerated by the gastrointestinal track it can be consumed only in relatively dilute solutions. Thus, the total caloric load that can be provided by oral lactate consumption is limited and relatively low. However, it was eventually identified tolerable oral doses of lactate and compared the effects of consuming beverages containing 7 percent lactate, 7 percent maltodextrin, or aspartame on various metabolic responses during 3 h of cycle ergometry at 50 percent of VO$_{2\text{max}}$. The beverages were provided in 250-mL doses 5 min before and every 20 min during exercise. Treatments were applied in a randomized, double-blind, crossover design. In each of the three treatments, blood glucose remained at basal levels through 100 min of exercise. During the final 60 min of exercise, blood glucose dropped significantly from preexercise levels, when subjects consumed the placebo, but was maintained at basal levels in the lactate and maltodextrin trials. No difference in blood glucose was observed between the lactate and maltodextrin trials. Blood pH and bicarbonate increased significantly over basal levels during the final 60 min of the lactate trial, while no differences in these variables were observed in the placebo and maltodextrin trials. While these results demonstrated positive metabolic responses to lactate ingestion, the investigation lacked key evidence to support the use of oral lactate consumption as an ergogenic aid. If acidosis is a major contributing factor to fatigue in their protocol, the consumption of lactate could provide an ergogenic effect by acting as a buffering agent [12392].

**Oral lactate as a buffering agent**

Numerous investigations have monitored the metabolic fates of infused or ingested lactate. During rest, approximately half of exogenous lactate has is oxidized while the remainder is used as substrate for numerous metabolic processes, most notably gluconeogenesis. Disposal of lactate by oxidation or gluconeogenesis consumes protons and thus has the potential to affect blood bicarbonate and pH levels [12392].

**Sucrose**

Sucrose is a disaccharide (two linked sugar molecules), which is broken down during digestion by the enzyme sucrase located in the intestinal microvilli, into the monosaccharides glucose and fructose; it has a glycaemic index of 64. Sucrose is odourless and has good palatability, it is not as sweet as fructose but is sweeter than glucose, and in solution it is colourless and very stable with a long shelf-life. Sucrose (table sugar) normally comes from sugar beet and cane, but is also found naturally in all fruits and vegetables, and even most herbs and spices. As a sweetener it has acquired a bad reputation for being associated with obesity, insulin resistance and dental caries. However, when consumed in moderation as a sport supplement it can have a positive effect on peak performance. There is much evidence for the use of carbohydrate ingestion before, during and after prolonged strenuous exercise to increase fuel availability for muscle and central nervous system. Carbohydrates, both sugars and starches, with moderate-to-high glycaemic index, such as glucose (high) and sucrose (moderate), are absorbed rapidly, providing a readily available source of carbohydrate and so are viewed as effective sources, particularly for intake during exercise. The two monosaccharide components of sucrose are absorbed by differing mechanisms: glucose is absorbed by a sodium co-transporter SGLUT-1 and rapidly enters the blood stream, while fructose is absorbed by glucose transporter (GLUT5) and has to be converted
to glucose by the liver before it is available for muscle metabolism. Therefore, the bioavailability of sucrose as an energy source is greater than by ingesting fructose or glucose alone. Because lower concentrations are required, there is less risk of gastro-intestinal slowing and stomach upset. While sucrose ingestion at rest can lead to rebound hypoglycaemia from a surge in insulin release, evidence suggests that serum insulin levels actually fall within minutes of commencing exercise and remain low for the duration of activity. Hypoglycaemia during prolonged exercise is more likely to occur as a consequence of glycogen depletion and is a cause of fatigue which can be prevented by sucrose ingestion before and during exercise. In summary, at a time when community health issues raise some concern about our daily consumption of sucrose, there may be value in, or at least room for, its inclusion in sports products targeting the provision of carbohydrate fuel during exercise [12382].

The consumption of carbohydrate before, during and after exercise is a central feature of the athlete's diet, particularly those competing in endurance sports. Sucrose is a carbohydrate present within the diets of athletes. Whether sucrose, by virtue of its component monosaccharide's glucose and fructose, exerts a meaningful advantage for athletes over other carbohydrate types or blends is unclear. This narrative reviews the literature on the influence of sucrose, relative to other carbohydrate types, on exercise performance or the metabolic factors that may underpin exercise performance. Inference from the research to date suggests that sucrose appears to be as effective as other highly metabolizable carbohydrates (e.g., glucose, glucose polymers) in providing an exogenous fuel source during endurance exercise, stimulating the synthesis of liver and muscle glycogen during exercise recovery and improving endurance exercise performance. Nonetheless, gaps exist in our understanding of the metabolic and performance consequences of sucrose ingestion before, during and after exercise relative to other carbohydrate types or blends, particularly when more aggressive carbohydrate intake strategies are adopted. While further research is recommended and discussed in this review, based on the currently available scientific literature it would seem that sucrose should continue to be regarded as one of a variety of options available to help athletes achieve their specific carbohydrate intake goals [13622].

**Ribose**

The amount of adenosine triphosphate (ATP) stored in the muscle available for immediate use is limited, and once used, must be resynthesized in the muscle. Ribose, a naturally occurring pentose sugar, helps resynthesize ATP for use in muscles. There have been claims that ribose supplements increase ATP levels and improve performance. Other studies have provided mixed results on the effectiveness of ribose as an ergogenic aid at high doses. None of these studies have compared the impact of the recommended dose of ribose on athletes and nonathletes under exercise conditions that are most conducive for effectiveness. The purpose of one study was to evaluate the effectiveness of ribose as an ergogenic aid at the dose recommended for supplements currently on the market during an exercise trial to maximize its efficacy. Male subjects (n=11) performed 2 trials 1 week apart. Each trial consisted of three 30-second Wingate tests with a 2-minute recovery between each test. Trials were counterbalanced, with 1 trial being performed with 625 mg of ribose and the other with a placebo. Peak power, mean power, and percent decrease in power were recorded during each Wingate test. Repeated-measures analysis of variance found no significant differences between ribose and placebo. These results suggest that ribose had no effect on performance when taken orally, at the dose suggested by the distributor [06255].

One article reviewed the current literature regarding the use of ribose as an ergogenic aid. Ribose manufacturers claim that it provides ergogenic benefit, but this has not been
substantiated through scientific investigations. Data have shown promise that ribose supplementation leads to enhanced restoration of ATP levels following exercise, but this has seldom translated into increased athletic performance. However, as with many ergogenic aids, additional research is needed to clarify its value as a supplement [07306].

ATP is the only directly usable source of energy for muscle contraction. At high-exercise intensities, the rate of ATP utilisation can exceed that of its regeneration from other energy sources and ATP levels in the muscle cells decrease. Most degraded ATP remains within the muscle as inosine monophosphate (IMP) which, after exercise, is used for resynthesis of ATP. However, a fraction of the IMP is degraded and lost from the muscle as purines. If intense exercise sessions are repeated, there may be an accumulated loss of ATP through the release of purines, whereby ATP stores in the muscle cells are reduced. The ATP lost from muscle may be replenished by de novo synthesis and D-ribose availability may be limiting for ATP de novo synthesis. Experimental studies have shown that ribose supplementation does not lower the loss in nucleotides with repeated intense exercise bouts but allows for a faster recovery of ATP levels within 72 h after termination of exercise training, most likely due to an improved rate of de novo ATP synthesis. However, despite a higher level of ATP after ribose supplementation, the study showed no effect on intense intermittent exercise performance. Accordingly, other studies examining the effect of ribose on performance during intense intermittent exercise and rowing8 have not been able to demonstrate improved performance in humans. Ribose dosages up to about 40 g/day, at times divided in 2–3 supplementations per day have been examined. There are no known adverse effects of ribose but reports on long-time use are lacking. It is concluded that, although ribose supplementation appears to improve the rate of de novo synthesis of ATP in muscle, existing scientific evidence do not support the use of ribose as an ergogenic aid in young, healthy individuals [12459].

**Galactose**

One study tested the hypothesis that supplementation with galactose before and during endurance exercise would spare carbohydrate (CHO), optimize fat utilization and improve performance compared with a typical sports drink formulation. Nine well-trained cyclists undertook three trials, each consisting of 120 min at 65 percent of VO$_{2\text{max}}$ followed immediately by a set work, self-paced time trial (TT). Three treatments, allocated as a randomized balanced design, consisted of the following: 8 percent (w/w) solution of galactose (Gal); 8 percent solution of 50 percent galactose/50 percent glucose (Gluc/Gal); and 8 percent solution of 80 percent glucose/20 percent fructose (Gluc/Fru). These were consumed as 0.67 g CHO per kg body wt 45-min pre-exercise; 1.0 g CHO per kg body wt per h for the first 120 min of exercise; 0.33 g CHO per kg body wt during the TT. Blood samples were collected before and during exercise; respiratory gas samples were collected only during fixed workload exercise. Mean TT power output was significantly less in Gal compared with Gluc/Gal (P=0.030). Blood glucose and insulin concentrations were lower, and free fatty acids higher in Gal compared with Gluc/Gal and Gluc/Fru. Respiratory exchange ratio was not significantly different between trials. It was concluded that ingestion of an 8 percent galactose-only solution (12.5 ml per kg body wt per h) is detrimental to endurance performance compared with equivalent volumes of iso-osmotic solutions containing 50 percent galactose/50 percent glucose or 80 percent glucose/20 percent fructose. This may reflect the inability of the liver to convert galactose into glucose at a rate required to support strenuous exercise intensity [07307].

A study was performed to determine beneficial effects of short-term galactose (GAL) supplementation over a 50:50 glucose-maltodextrin (GLUC) equivalent on self-paced
endurance cycling performance. On 2 separate occasions, subjects performed a 100-km self-paced time trial (randomized and balanced order). This was interspersed with four 1-km and four 4-km maximal efforts reflecting the physical requirements of racing. Before each trial 38 ± 3 g of GAL or GLUC was ingested in a 6 percent concentrate fluid form 1 hr preexercise and then during exercise at a rate of 37 ± 3 g/hr. Performance variables were recorded for all 1- and 4-km efforts, all interspersed intervals, and the total 100-km distance. Noninvasive indicators of work intensity (heart rate, HR, and rating of perceived exertion) were also recorded. Times taken to complete the 100-km performance trial were 8,298 ± 502 and 8,509 ± 578 s, with mean power outputs of 271 ± 37 and 256 ± 45 W, for GAL and GLUC, respectively. Mean HR did not differ. A main effect of carbohydrate (CHO) type on time to complete 4-km efforts occurred, with no CHO Type×Effort Order interaction observed. No main effect of CHO type or interaction of CHO Type×Sequential Order occurred for 1-km efforts. It was concluded that a 6 percent GAL drink does not enhance performance time during a self-paced cycling performance trial in highly trained endurance cyclists compared with a formula typically used by endurance athletes but may improve the ability to produce intermediate self-paced efforts [12460].

**Galactose and fructose**

Both liver and muscle glycogen stores play a fundamental role in exercise and fatigue, but the effect of different CHO sources on liver glycogen synthesis in humans is unclear. The aim was to compare the effect of maltodextrin (MD) drinks containing galactose, fructose, or glucose on postexercise liver glycogen synthesis. In a double-blind, triple crossover, randomized clinical trial, 10 well-trained male cyclists performed three experimental exercise sessions separated by at least 1 wk. After performing a standard exercise protocol to exhaustion, subjects ingested one of three 15 percent CHO solutions, namely, FRU (MD + fructose, 2:1), GAL (MD + galactose, 2:1), or GLU (MD + glucose, 2:1), each providing 69 g CHO per hour during 6.5 h of recovery. Liver glycogen changes were followed using $^{13}$C magnetic resonance spectroscopy. Liver glycogen concentration increased at faster rates with FRU and with GAL than with GLU. Liver volumes increased with FRU (9 % ± 2 %) and with GAL (10 % ± 2 %) but not with GLU (2 % ± 1%). Net glycogen synthesis appeared linear and was faster with FRU and with GAL than with GLU. When ingested at a rate designed to saturate intestinal CHO transport systems, MD drinks with added fructose or galactose were twice as effective as MD + glucose in restoring liver glycogen during short-term postexercise recovery [11587].

**Multiple transportable carbohydrates**

Since the 1980s, it has been known that carbohydrate intake during exercise can improve exercise performance lasting 2 h or longer. Soon after this discovery, it was established that not all carbohydrates are equal and carbohydrates ingested during exercise may be utilised at different rates. Until a landmark publication in 2004, it was believed that carbohydrate ingested during exercise could only be oxidised at a rate not higher than 1 g/min (60 g/h) independent of the type of carbohydrate. This is reflected in the guidelines published by the American College of Sports Medicine, which recommend that athletes should take between 30 and 60 g of carbohydrates during endurance exercise (>1 h) or 0.7 g/kg/h. Exogenous carbohydrate oxidation appears limited by the intestinal absorption of carbohydrates. It is believed that glucose uses a sodium dependent transporter (SGLT1) for absorption, which becomes saturated at a carbohydrate intake around 60 g/h (or 1 g/min). When glucose was ingested at this rate and another carbohydrate (fructose) that uses a different transporter was ingested simultaneously, oxidation rates well above 1 g/min (1.26 g/min) were observed. At present only two different intestinal carbohydrate transporters have been identified (SGLT1...
for glucose or glucose polymers and galactose, and glucose transporter type 5 for fructose). Interestingly, sucrose (a disaccharide of glucose and fructose), which is said to have its own disaccharide transporter, appears to behave more like glucose than glucose:fructose. Studies suggest that exogenous carbohydrate oxidation from sucrose is similar to glucose and does not reach the high oxidation rates observed with glucose and fructose (or other MTC). With the knowledge that a single carbohydrate could only be oxidised at a rate of 1 g/min, a series of studies was initiated in an attempt to find the combination that would result in the highest oxidation rates. In these studies, the rate of carbohydrate ingestion as well as the combinations of carbohydrates varied. All studies confirmed that MTC resulted in higher (up to 75 %) oxidation rates than carbohydrates that use the SGLT1 transporter only. Combinations of maltodextrin and fructose, and glucose and fructose or glucose, plus sucrose and fructose, seemed to produce the most favourable effects. From a practical perspective, it is important to note that such high oxidation rates can not only be achieved with carbohydrate ingested in a beverage but also as a gel or a low fat, low protein, low fibre energy bar. Strong evidence is now emerging of a dose–response relationship between carbohydrate intake and endurance performance. Studies have demonstrated that MTC can result in improved performance over and above the performance enhancing effect of a carbohydrate drink with one single carbohydrate. It has also been demonstrated that MTC may have advantages in fluid delivery and studies suggest less gastrointestinal discomfort. Recently published recommendations take these findings into account, acknowledging that there may be different carbohydrate needs for different durations of exercise as well as for different levels of athletes. MTC can be recommended at all durations but are most effective when the exercise is 2.5 h or longer. In those conditions, carbohydrate intakes of up to 90 g/h are recommended and these would only be oxidised to any significant degree if they are MTC in which glucose makes up no more than about 60 g [11523].

**Carbohydrates in tennis**

Carbohydrate supplementation in prolonged aerobic exercise has been shown to be effective in improving performance and deferring fatigue. However, there is confounding evidence with regard to carbohydrate supplementation and tennis performance, which may be due to the limited number of studies on this topic. One evidence based review, using database searches of Medline and SPORTDiscus, summarises the limited relevant literature to determine if carbohydrate supplementation benefits tennis performance, and, if so, the appropriate amounts and timing. Although more research is required, it appears that it may be beneficial in tennis sessions lasting more than 90 minutes [06257].

**Energy beverages/Sport drinks**

Energy drinks have become more and more popular since the late nineties. The manufactures claim that these drinks improve physical endurance, reaction speed and concentration. The main ingredients of energy drinks are caffeine, sugar, taurine and glucuronolactone. According to the manufacturers, the stimulating effects of these drinks are due to interaction between the various ingredients. To investigate whether energy drinks do indeed improve cognitive performance and to find out which ingredients are responsible for this effect and other benefits it was made a search of the literature for the period from 1997 to 2006 on the basis of Medline, by using the search term "energy drink or energy drinks" and restricting the search to "humans". results Not only did focused and sustained attention improve significantly but so did reaction speed in all sorts of reaction-time tasks. Memory improved too, but not to the same degree. The findings suggest that most of the effects of energy drinks on cognitive performance are related mainly to the presence of caffeine
The author examined gendered links among sport-related identity, endorsement of conventional masculine norms, risk taking, and energy-drink consumption by surveying 795 undergraduate students enrolled in introductory-level courses at a public university. Of participants, 39 percent consumed an energy drink in the past month, with more frequent use by men (2.5 d/month) than by women (1.2 d/month). Strength of jock identity was positively associated with frequency of energy-drink consumption; this relationship was mediated by both masculine norms and risk-taking behavior. The author concluded that sport-related identity, masculinity, and risk taking are components of the emerging portrait of a toxic jock identity, which may signal an elevated risk for health-compromising behaviors. College undergraduates’ frequent consumption of Red Bull and comparable energy drinks should be recognized as a potential predictor of toxic jock identity [08392].

Numerous studies have shown that ingesting carbohydrate in the form of a drink can improve exercise performance by maintaining blood glucose levels and sparing endogenous glycogen stores. The effectiveness of carbohydrate gels or jellybeans in improving endurance performance has not been examined. On 4 separate days and 1-2 hr after a standardized meal, 16 male and female athletes cycled at 75 percent VO$_{2\text{peak}}$ for 80 min followed by a 10-km time trial. Participants consumed isocaloric (0.6 g of carbohydrate per kg per hour) amounts of randomly assigned sports beans, sports drink, gel, or water only, before, during, and after exercise. Blood glucose concentrations were similar at rest between treatments and decreased significantly during exercise with the water trial only. Blood glucose concentrations for all carbohydrate supplements were significantly higher than water during the 80-min exercise bout and during the time trial. There were no significant differences in blood glucose between carbohydrate treatments. The 10-km time trials using all 3 carbohydrate treatments were significantly faster for sports beans, for sports drink, and for gel than water. All carbohydrate-supplement types were equally effective in maintaining blood glucose levels during exercise and improving exercise performance compared with water only [08393].

To investigate the frequency of energy-drink consumption and associated factors in a group of college students a cross-sectional study was conducted and included 439 students pursuing a career in medicine, sports, and arts. Only fourth-year students were approached. Data were collected using a self-administered standard questionnaire. In bivariate analyses, frequency of energy-drink consumption was higher in students of arts and sports and in those who did not have breakfast on a regular basis, ever smoked cigarettes, drank alcoholic beverages, and regularly engaged in sports compared with their counterparts. Many students who had “ever” tried an energy drink did so the first time because they wondered about its taste. Of regular users of energy drinks, reasons for using such drinks varied across the three selected groups of students and included obtaining getting energy, staying awake, boosting performance while doing sports, or mixing with alcoholic beverages. About 40 percent of all current users of energy drinks reported that they mixed those with alcoholic beverages. In multivariate analyses, statistically significant predictors of energy-drink consumption were faculty type, presence of any health insurance, use of alcoholic beverages, and monthly income, controlling for gender. Most students could not correctly define the ingredients of energy drinks or their potential hazardous health effects, and they could not distinguish energy and sports drinks when they were requested to select them from a list of commercial names of various drinks. It was concluded that consumption of energy drinks, despite the variation in the reason for choosing such drinks, is quite common in college students [10229].

Energy drinks are frequently marketed to individuals interested in athletics and an active
lifestyle. From 2001 to 2008, estimates of energy drink use in adolescent to middle-aged populations ranged from 24 to 56 percent. Most energy drinks feature caffeine and a combination of other components, including taurine, sucrose, guarana, ginseng, niacin, pyridoxine, and cyanocobalamin. This article examines the evidence for 2 commonly purported uses of energy drinks: athletic performance enhancement and weight loss. Observed ergogenic benefits of energy drinks are likely attributable to caffeine and glucose content. There is conflicting evidence regarding the impact of energy drinks on weight loss, although some data suggest that combining energy drink use with exercise may enhance body fat reduction. As with any pharmacologically active substance, energy drinks are associated with adverse effects. Combining energy drinks with alcohol exacerbates safety concerns and is an increasingly common practice contributing to toxic jock identity among college-aged male athletes. Practitioners should monitor identified populations likely to consume these loosely regulated beverages [10230].

The purpose of one study was to compare the effects of a carbohydrate-electrolyte plus caffeine, carnitine, taurine, and B vitamins solution (CE+) and a carbohydrate-electrolyte-only solution (CE) versus a placebo solution (PLA) on cycling performance and maximal voluntary contraction (MVC). In a randomized, double-blind, crossover, repeated-measures design, 14 male cyclists cycled for 120 min submaximally and then completed a 15-min performance trial (PT). Participants ingested CE+, CE, or PLA before (6 ml/kg) and every 15 min during exercise (3 ml/kg). MVC was measured as a single-leg isometric extension (70 degree knee flexion) before (pre) and after (post) exercise. Rating of perceived exertion (RPE) was measured throughout. Total work accumulated during PT was significantly greater in CE+ than PLA but not in CE versus PLA. MVC declined significantly from pre to post in PLA and CE but not in CE+. At Minutes 60, 90, 105, and 120 RPE was lower in CE+ than in PLA. CE+ resulted in greater total work than PLA. CE+, but not PLA or CE, attenuated pre-to-post MVC declines. Performance increases during CE+ may have been influenced by lower RPE and greater preservation of leg strength during exercise in part as a result of the hypothesized effects of CE+ on the central nervous system and skeletal muscle [10231].

Pre-exercise sports drinks (PRX) are commonly used as ergogenic aids in athletic competitions requiring aerobic power. However, in most cases, claims regarding their effectiveness have not been substantiated. In addition, the ingredients in PRX products must be deemed acceptable by the athletic governing bodies that regulate their use in training and competition. The purpose of one study was to examine the effects of a modified PRX formulation (known as EM.PACT) from earlier investigations on factors related to maximal aerobic performance during a graded exercise test. The modification consisted of removing creatine to meet the compliance standards set forth by various athletic organizations that regulate the use of nutritional supplements. Twenty-nine male and female college students varying in levels of aerobic fitness participated in a randomized crossover administration of PRX (containing 14 g/serving of fructose, medium-chain triglycerides, and amino acids mixed with 8 oz. of water) and placebo (PL) 30 minutes prior to performing a treadmill test with approximately one week separation between the trials. \( \text{VO}_{2\text{max}} \), maximal heart rate (HR), time to exhaustion (Time), and percentage estimated non-protein fat substrate utilization (FA) during two a priori submaximal stages of a graded exercise testing were evaluated. The \( \text{VO}_{2\text{max}} \) mean value of the PRX trial was significantly greater than the PL trial. The mean value for Time was also observed to be significantly greater for the PRX trial compared to PL. Additionally, percentage of FA during submaximal stages of the exercise test was significantly greater for PRX trial in comparison to PL. It was concluded that the modified PRX formulation utilized in this investigation supports the findings of the previous investigation and its efficacy for enhancing indices of aerobic performance during graded exercise testing [10232].
Exercise is making a resurgence in many countries, given its benefits for fitness as well as prevention of obesity. This trend has spawned many supplements that purport to aid performance, muscle growth, and recovery. Initially, sports drinks were developed to provide electrolyte and carbohydrate replacement. Subsequently, energy beverages (EBs) containing stimulants and additives have appeared in most gyms and grocery stores and are being used increasingly by “weekend warriors” and those seeking an edge in an endurance event. Long-term exposure to the various components of EBs may result in significant alterations in the cardiovascular system, and the safety of EBs has not been fully established. For one review, it was searched the MEDLINE and EMBASE databases from 1976 through May 2010, using the following keywords: energy beverage, energy drink, power drink, exercise, caffeine, red bull, bitter orange, glucose, ginseng, guarana, and taurine. Evidence regarding the effects of EBs is summarized, and practical recommendations are made to help in answering the patient who asks, “Is it safe for me to drink an energy beverage when I exercise?”

It has been reported that the frequency of cola intake (COLA) is positively associated with serum triglycerides and negatively associated with high-density-lipoprotein (HDL) cholesterol, both components of the metabolic syndrome (MetS). The question now is whether noncola soft drink intake (NCOLA) is associated with MetS. Among the 18770 participants in the Oslo Health Study, 5373 men and 6181 women had data on COLA and NCOLA and risk factors for MetS (except fasting glucose). Main MetS requirements are central obesity and 2 of the following: increased triglycerides, low HDL cholesterol, increased systolic or diastolic blood pressure, and elevated fasting blood glucose. The MetSRisk index was calculated to estimate many MetS components. Using regression analyses, the association between COLA (NCOLA) and MetS (MetSRisk) was studied. In young (aged 30 years), middle-aged (aged 40 and 45 years), and senior (aged 59 and 60 years) men and women, there was, in general, a positive correlation between COLA and MetSRisk, and between COLA and single MetS risk factors, except HDL cholesterol, which was negatively correlated. A less consistent picture was found for NCOLA. By regression analyses, after adjustment for sex, age, time since last meal, and use of sugar-sweetened soft drinks, a positive association between COLA (NCOLA) and MetSRisk (MetS) was still found. However, when also controlling for cheese, fatty fish, coffee, alcohol, smoking, physical activity, education, and birthplace, only the association with COLA remained significant, irrespective of the presence or absence of sugar. In conclusion, the self-reported intake frequency of soft drinks can be positively associated with MetS.

To review the effects, adverse consequences, and extent of energy drink consumption among children, adolescents, and young adults it was searched PubMed and Google using “energy drink,” “sports drink,” “guarana,” “caffeine,” “taurine,” “ADHD,” “diabetes,” “children,” “adolescents,” “insulin,” “eating disorders,” and “poison control center” to identify articles related to energy drinks. According to self-report surveys, energy drinks are consumed by 30 to 50 percent of adolescents and young adults. Frequently containing high and unregulated amounts of caffeine, these drinks have been reported in association with serious adverse effects, especially in children, adolescents, and young adults with seizures, diabetes, cardiac abnormalities, or mood and behavioral disorders or those who take certain medications. Of the 5448 US caffeine overdoses reported in 2007, 46 percent occurred in those younger than 19 years. Several countries and states have debated or restricted energy drink sales and advertising. It was concluded that energy drinks have no therapeutic benefit, and many ingredients are understudied and not regulated. The known and unknown pharmacology of agents included in such drinks, combined with reports of toxicity, raises concern for potentially serious adverse effects in association with energy drink use. In the short-term, pediatricians need to be aware of the possible effects of energy drinks in vulnerable populations and screen for consumption to educate families. Long-term research should aim to understand the effects in at-risk populations. Toxicity surveillance should be improved, and
regulations of energy drink sales and consumption should be based on appropriate research [11259].

The aim of one study was to examine sensory perceptions towards different formulations of sports drinks when consumed before, at various points during, and following exercise. Following familiarization 14 recreational runners underwent four trials in a single blind counterbalanced design. Each trial utilised one of four different solutions: 7.5 percent carbohydrate, 421 mg/L electrolyte (HiC-HiE); 7.5 percent carbohydrate, 140 mg/L electrolyte (HiC-LoE); 1.3 percent carbohydrate, 421 mg/L electrolyte (LoC-HiE) and water. Subjects were provided with 50 mL samples to ingest and then rate (using a 100 mm line scale) the intensity of sweetness, saltiness, thirst-quenching ability and overall liking before (-30 min), during (0, 30 and 60 min) and following (90 and 120 min) treadmill running exercise. Ratings of sweetness for all energy-containing drinks were significantly higher during exercise relative to pre- and post-exercise conditions (P<0.05); ratings also increased with duration of exercise. Sweetness ratings for LoC-HiE increased during exercise but remained the same for other beverages. Ratings of saltiness decreased for all energy-containing drinks during exercise relative to pre-exercise; ratings decreased with duration of exercise in these drinks. Ratings of thirst-quenching ability and overall liking increased with duration of exercise with all beverages. Significant changes in sensory perception occur when consuming sports drinks during exercise relative to non-exercise conditions. Temporal changes also occur during exercise itself which leads to enhanced liking of all beverages [11260].

Sports and energy drinks are being marketed to children and adolescents for a wide variety of inappropriate uses. Sports drinks and energy drinks are significantly different products, and the terms should not be used interchangeably. The primary objectives of this clinical report are to define the ingredients of sports and energy drinks, categorize the similarities and differences between the products, and discuss misuses and abuses. Secondary objectives are to encourage screening during annual physical examinations for sports and energy drink use, to understand the reasons why youth consumption is widespread, and to improve education aimed at decreasing or eliminating the inappropriate use of these beverages by children and adolescents. Rigorous review and analysis of the literature reveal that caffeine and other stimulant substances contained in energy drinks have no place in the diet of children and adolescents. Furthermore, frequent or excessive intake of caloric sports drinks can substantially increase the risk for overweight or obesity in children and adolescents. Discussion regarding the appropriate use of sports drinks in the youth athlete who participates regularly in endurance or high-intensity sports and vigorous physical activity was beyond the scope of this report [11261].

One study explored relationships regarding perceived stress, energy drink consumption, and academic performance among college students. Participants included 136 undergraduates attending a large southern plains university. Participants completed surveys including items from the Perceived Stress Scale and items to describe energy drink consumption, academic performance, and demographics. Positive correlations existed between participants' perceived stress and energy drink consumption. Participants' energy drink consumption and academic performance were negatively correlated. Freshmen and sophomores consumed a lower number of energy drinks yesterday than juniors. Males reported higher means than females for selected energy drink consumption items. Statistically significant interactions existed between gender and year in school for selected energy drink consumption items. Results confirm gender differences in energy drink consumption and illuminate a need for education regarding use of energy drinks in response to perceived stress [11262].

Energy drinks are attractive and readily available in every grocery store and gas station. While most youth verbalize an understanding that too much caffeine is bad for one's health,
at an age of multiple demands, an over-the-counter offer of increased energy and alertness is hard to ignore. What makes energy drinks different from regular coffee? Although the heavily caffeinated drinks promise increased energy and stamina and are loaded with healthy natural ingredients, excessive consumption is of concern on many levels. One article discussed some of the effects of excessive caffeine, as well as risks associated with energy drinks mixed with alcohol [11524].

The aim of one study was to investigate the effects of a naturally composed sports drink containing proteins and carbohydrates used during recovery in competitive badminton players. The hypothesis was that the use of a recovery drink would lead to positive subjective effects, enhanced physical performance and less signs of overtraining. During an indoor season 18 badminton players were instructed to drink at least 250 mL of a given sports drink immediately after each training or playing session. The study design was prospective double blind crossover with one active drink and one placebo. The active drink was based on natural products containing whey and orange juice, and the placebo was made of diluted apple juice. Evaluation of effects was done with laboratory tests, self-registered values and field tests. The players perceived statistically significant short-term subjective positive effects after using the active drink, compared with after using placebo. The blood hemoglobin concentration was also higher after the period with active drink. There were no other differences concerning other laboratory tests (leg strength, endurance, body fat percent, lean arm and leg masses), self-registered values (body weight, pulse, training amount and intensity) or field tests (speed, explosive effort, grip strength, endurance and POMS) between the periods with the different sports drinks. It was concluded that supplementation with a sports drink during recovery showed a significant short-term subjective positive effect compared with placebo. However, no effects were seen on physical performance or signs of overtraining [06258].

The term "energy drink" designates "any product in the form of a drink or concentrated liquid, which claims to contain a mixture of ingredients having the property to raise the level of energy and vivacity". The main brands, Red Bull, Dark Dog, Rockstar, Burn, and Monster, are present in food stores, sports venues, and bars among other soft drinks and fruit juices. Their introduction into the French market raised many reluctances, because of the presence of taurine, caffeine and glucuronolactone. These components present in high concentrations, could be responsible for adverse effects on health. The association of energy drinks and spirits is widely found among adolescents and adults who justify drinking these mixed drinks by their desire to drink more alcohol while delaying drunkenness. Given the importance of the number of incidents reported among the energy drinks consumers, it seemed appropriate to make a synthesis of available data and to establish causal links between the use of these products and the development of health complications. For a literature review, it was selected scientific articles both in English and French published between 2001 and 2011 by consulting the databases Medline, Embase, PsycINFO and Google Scholar. The words used alone or in combination are "energy drinks", "caffeine", "taurine", "toxicity", "dependence". An occasional to a moderate consumption of these drinks seems to present little risk for healthy adults. However, excessive consumption associated with the use of alcohol or drugs in amounts that far exceed the manufacturers recommended amount, could be responsible for negative consequences on health, particularly among subjects with cardiovascular disease [12398].

Sports drinks are increasingly regarded as an essential adjunct for anyone doing exercise, but the evidence for this view is lacking. It was investigated the links between the sports drinks industry and academia that have helped market the science of hydration: prehydrate; drink ahead of thirst; train your gut to tolerate more fluid; your brain doesn’t know you’re thirsty – the public and athletes alike are bombarded with messages about what they should
drink, and when, during exercise. But these drinking dogmas are relatively new. In the 1970s, marathon runners were discouraged from drinking fluids for fear that it would slow them down. At the first New York marathon in 1970, there was little discussion about the role of hydration – it was thought to have little scientific value. An investigation by the BMJ has found that companies have sponsored scientists, who have gone on to develop a whole area of science dedicated to hydration. These same scientists advise influential sports medicine organisations, which have developed guidelines that have filtered down to everyday health advice. These guidelines have influenced the European Food Safety Authority, the EU agency that provides independent advice on the evidence underpinning health claims relating to food and drink. And they have spread fear about the dangers of dehydration. Much of the focus on hydration can be traced back to the boom in road running, which began with the New York marathon. Manufacturers of sports shoes and the drink and nutritional supplement industries spotted a growing market. One drink in particular was quick to capitalise on the burgeoning market. Robert Cade, a renal physician from the University of Florida, had produced a sports drink in the 1960s that contained water, sodium, sugar, and monopotassium phosphate with a dash of lemon flavouring. Gatorade – named after the American Football team, the Gators, that it was developed to help – could prevent and cure dehydration, heat stroke, and muscle cramps, and improve performance, it was claimed. The first experimental batch of the sports drink cost GBP 28 to produce but has spawned an industry with sales of around GBP 260m a year in the UK alone – and consumption is increasing steadily. In the US the market is even bigger. In 2009, forecasters, Mintel, valued it at USD 1.6bn, and the market is projected to reach USD 2bn by 2016. PepsiCo bought Gatorade in 2001 and both Coca-Cola and GlaxoSmithKline (GSK) have their own sports drinks – Powerade and Lucozade respectively. The companies are a partner and service provider, respectively, to the London 2012 Olympics. The key behind the meteoric rise in consumption of sports drinks lies in the coupling of science with creative marketing. What started life as a mixture of simple kitchen food stuffs has become an “essential piece of sporting equipment.” The US National Athletic Trainers’ Association (NATA), a representative body of sports health professionals with over 35 000 members, works closely with Gatorade. The company has taken out advertisements in NATA’s newsheet that look like academic papers. These “research adverts” are just one example of how companies promote the idea that the benefits of their drinks are based on decades of thorough scientific research. Selling science. Indeed, just as drug companies have appointed key opinion leaders to influence doctors’ prescribing patterns, sports drink and supplement companies seek to work with gyms and instructors. Virgin Active has a partnership with Powerade, for example, and the GSK owned supplement brand, Maximuscle, has a partnership with LA Fitness. Perhaps one of Gatorade Sports Science Institute’s greatest successes was to undermine the idea that the body has a perfectly good homeostatic mechanism for detecting and responding to dehydration – thirst. “The human thirst mechanism is an inaccurate short-term indicator of fluid needs … Unfortunately, there is no clear physiological signal that dehydration is occurring.” The science of dehydration has led to another widely held belief that is not based on robust evidence – that the colour of urine is a good guide to hydration levels. Like athletes, British soldiers are told to check their urine. The Ministry of Defence signed a GBP 1.5m three year deal with GSK in 2005 to supply soldiers with Lucozade. The Mayo Clinic’s online guidance to patients also suggests urine is a good guide of hydration. “Unfortunately, thirst isn’t always a reliable gauge of the body’s need for water, especially in children and older adults. A better indicator is the color of your urine: Clear or light-colored urine means you’re well hydrated, whereas a dark yellow or amber color usually signals dehydration,” it says. However, a review of the evidence Oxford University’s Centre of Evidence Based Medicine linked to this investigation has assessed the predictive value of urine colour as a diagnostic test. “There is a lack evidence for the widely recommended practice of assessing hydration status by looking at the colour of urine,” it suggests. “The limited evidence shows that only first morning urine colour can be reliably used to assess dehydration and
rehydration," it adds. Studies suggest that thirst is a more reliable trigger. A meta-analysis of data from cyclists in time trials concluded that relying on thirst to gauge the need for fluid replacement was the best strategy. In 1993, a group of experts led by Ron Maughan, professor of sport and exercise nutrition at Loughborough University and a member of GSSI’s sports medicine review board since 1990, produced a consensus statement at a meeting funded by Isostar, a sports drink then owned by drug company, Novartis. “There is a need to make athletes more aware of the dangers of dehydration and of the importance of adequate fluid intake. Water is not the best fluid for rehydration, either during or after exercise,” they wrote in an article published in the British Journal of Sports Medicine. In America, the sports drinks industry also made a push into the area of clinical science. In 1992, the American College of Sports Medicine – the "premier organization in sports medicine and exercise science" with over 45 000 members – accepted a USD 250,000 donation from Gatorade. Four years later, in 1996, the American College of Sports Medicine produced guidelines that adopted a “zero percent dehydration” doctrine, advising athletes to “drink as much as tolerable.” Half the guideline’s authors either worked with the US military – the world’s biggest customer of Gatorade – or had a financial relationship with the Gatorade institute. The 1996 guidance stood until 2007, when in updated guidance the college acknowledged that people should drink according to the dictates of thirst. However, it still promoted the idea that people should lose no more than 2 percent of body weight during exercise, and this remains the position in the published literature. As sports drinks rise in popularity among children, there is concern their consumption is contributing to obesity levels. A 500 mL bottle of Powerade Ion4 contains 19.6 g of sugar, and the same sized bottles of Lucozade Sport and Gatorade Perform contain 17.5 g (3 g carbohydrate) and 30 g respectively (a teaspoon of sugar weighs about 4 g) [12399].

Energy drinks (EDs) contain caffeine and are a new, popular category of beverage. It has been suggested that EDs enhance physical and cognitive performance; however, it is unclear whether the claimed benefits are attributable to components other than caffeine. A typical 235 mL ED provides between 40 and 250 mg of caffeine, equating to doses that improve cognitive and, at the higher levels, physical performance. EDs often contain taurine, guaranã, ginseng, glucuronolactone, B-vitamins, and other compounds. A literature search using PubMed, Psych Info, and Google Scholar identified 32 articles that examined the effects of ED ingredients alone and/or in combination with caffeine on physical or cognitive performance. A systematic evaluation of the evidence-based findings in these articles was then conducted. With the exception of some weak evidence for glucose and guaranã extract, there is an overwhelming lack of evidence to substantiate claims that components of EDs, other than caffeine, contribute to the enhancement of physical or cognitive performance. Additional well-designed, randomized, placebo-controlled studies replicated across laboratories are needed in order to assess claims made for these products [12400].

Energy drink usage is common and contains caffeine or other stimulants. It was evaluated demographics, prevalence, reasons and adverse effects with consuming energy beverages. Cross-sectional study of a convenience sample of patients recruited from two US Emergency Departments from January to December 2009. 1298 subjects participated of which 53 percent were male. Ethnicity: Caucasian 48 percent, African American 17 percent, Hispanic 18 percent, and other 17 percent. Age ranges: 18-29 years (38 %), 30-54 years (50 %) and greater than 55 years (12 %). Reasons for use: 57 percent to "increase energy", 10 percent for studying/work projects, 2 percent while prolonged driving, improve sports performance 2 percent, with ethanol 6 percent, and "other" reasons 22 percent. Adverse reactions reported by 34 percent (429) patients. 280 report feeling "shaky/jittery", insomnia 136, palpitations 150, gastrointestinal upset 82, headache 68, chest pain 39, and seizures in 6. Eighty-five patients reported co-ingestion with illicit "stimulants" including cocaine and methamphetamine. It was identified one-third of patients reported at least one adverse effect.
Whilst most were not severe, a small number were serious e.g. seizures. In addition, some report purposely ingesting with illicit drugs [12401].

Consumption of energy drinks by both recreational and competitive athletes has increased dramatically in recent years. The primary ingredients in many energy drinks include caffeine (CAF) in various forms, as well as taurine. The purpose of this randomized, double-blind, crossover study was to examine the effect of sugar-free (SF) Red Bull (RB) containing CAF and taurine to a CAF only drink and a SF, CAF-free placebo (PL) on one repetition maximum (1RM) bench press (BP) and the volume load (VL; repetitions x kg at 70 % 1RM) during one BP set to failure in experienced weight lifters. Seventeen college-age men randomly received: (A) 500 ml of SF-RB containing CAF (160 mg) and taurine (2000 mg); (B) 500 ml of a SF drink containing CAF only (160 mg); or (C) a SF, CAF-free 500 ml PL drink 60 min prior to testing on three separate occasions. Following a standard warm-up, the 1RM was determined for each subject and, after 5 min rest, they completed repetitions to failure at 70% of their 1RM to assess VL. The results indicated that neither SF-RB nor the CAF drink had any effect on 1RM BP or VL compared to PL. Although the CAF content in the energy drinks used in the present study was low (2.0 mg/kg), the finding of no effect of the CAF containing energy drinks for 1RM BP are in agreement with previous studies using intakes up to 6.0 mg/kg. These findings suggest that SF-RB has no effect on upper body 1RM strength or VL in resistance trained men [12402].

The descriptive analytical study was conducted at a University Medical School from 2011 and 2012. A semi-structured questionnaire was filled by students who were asked about their socio-demographic status and their energy drink consumption. The mean age of the 390 students in the study was 21 years (range:16-27). Of them, 204 (52 %) were females. Overall 52 (13 %) were smoking regularly at least one cigarette per day; 122 (31 %) were consuming alcohol; 127 (33 %) had consumed energy drinks at least once and 73 (19 %) more than once. In terms of perception, 110 (28 %) students said energy drinks were similar to sports drinks, while only 121 (41 %) named the brands correctly; 96 (25 %) students did not answer this particular question. Although consumption of energy drinks was common among medical students, the knowledge of ingredients and knowledge of health risks of energy drinks among them was unsatisfactory [13632].

According to published research, energy drinks (ED) are the most popular dietary supplement besides multivitamins in the American adolescent and young adult population. ED are also reported to be the most popular supplement among British athletes. More recently, energy shots (ES) have also been purported to possess ergogenic value on mental focus and/or performance. It is important to make a distinction between ED, ES, and sports drinks. Sports drinks are a unique category within the beverage industry and are marketed to consumers with the primary function of promoting hydration, replacing electrolytes and sustaining endurance performance capacity. They typically provide a small amount of carbohydrate (e.g. 6-8 grams/100 mL) and electrolytes (sodium, potassium, calcium, magnesium). ED, on the other hand, typically contain higher amounts of carbohydrate along with nutrients purported to improve perceptions of attention and/or mental alertness. Low calorie ED are also marketed to increase mental alertness, energy metabolism, and performance. Energy shots are typically 2-4 oz. servings of concentrated fluid containing various purported ergogens. Since ED and ES contain carbohydrate, caffeine, and/or nutrients that may affect mental focus and concentration, they have the potential to affect exercise capacity and perceptions of energy and/or fatigue. One analysis represents a systematic review of the literature on the effects of “energy drinks” on exercise and cognitive performance as well as primary ingredients contained in popular energy drinks. A comprehensive literature search was performed by searching the Medline database of the US National Library of Medicine of the National Institutes of Health. The search strategy
involved entering "energy drinks" and commercial names of energy drinks and/or caffeinated beverages as well as a search of primary nutrients contained in popular energy drinks (e.g., caffeine, carbohydrate, taurine, glucuronolactone, Guarana, Yerba Mate, etc.). It is important to note, from a United States regulatory perspective, several of these ED are marketed as dietary supplements and not beverages, and the label on the product will indicate which category of Food and Drug Administration (FDA) authority the product falls under. Each category has its own set of governing laws and regulations. For example, depending on the category, the labels will include Supplement Facts (dietary supplements) or Nutrition Facts (beverages). Thus, the International Society of Sports Nutrition (ISSN) bases the following position stand on a critical analysis of the literature on the safety and efficacy of the use of energy drinks (ED) or energy shots (ES). The ISSN has concluded the following [13633]:

- Although ED and ES contain a number of nutrients that are purported to affect mental and/or physical performance, the primary ergogenic nutrients in most ED and ES appear to be carbohydrate and/or caffeine
- The ergogenic value of caffeine on mental and physical performance has been well-established but the potential additive benefits of other nutrients contained in ED and ES remains to be determined
- Consuming ED 10-60 minutes before exercise can improve mental focus, alertness, anaerobic performance, and/or endurance performance
- Many ED and ES contain numerous ingredients; these products in particular merit further study to demonstrate their safety and potential effects on physical and mental performance
- There is some limited evidence that consumption of low-calorie ED during training and/or weight loss trials may provide ergogenic benefit and/or promote a small amount of additional fat loss. However, ingestion of higher calorie ED may promote weight gain if the energy intake from consumption of ED is not carefully considered as part of the total daily energy intake
- Athletes should consider the impact of ingesting high glycemic load carbohydrates on metabolic health, blood glucose and insulin levels, as well as the effects of caffeine and other stimulants on motor skill performance
- Children and adolescents should only consider use of ED or ES with parental approval after consideration of the amount of carbohydrate, caffeine, and other nutrients contained in the ED or ES and a thorough understanding of the potential side effects
- Indiscriminant use of ED or ES, especially if more than one serving per day is consumed, may lead to adverse events and harmful side effects
- Diabetics and individuals with pre-existing cardiovascular, metabolic, hepatorenal, and neurologic disease who are taking medications that may be affected by high glycemic load foods, caffeine, and/or other stimulants should avoid use of ED and/or ES unless approved by their physician

The ingestion of nutrients prior to, during, and/or following exercise can affect exercise performance and/or training adaptations. ED typically contain water, carbohydrates (e.g. glucose, maltodextrin), vitamins, minerals, and "proprietary blends" of various nutrients purported to increase energy, alertness, metabolism, and/or performance (e.g., caffeine, taurine, amino acids, glucuronolactone, Guarana, Ginkgo biloba, Carnitine, Panax ginseng, Green Tea, Yerba Mate, etc.). Therefore, ingestion of ED or ES prior to, during, and/or following exercise could have some ergogenic value [13633].

The purpose of one study was to investigate the influence of an energy drink on cycling performance and immune-related variables. Eleven trained male cyclists consumed 500 mL of 1) energy drink (2.0g taurine, 1.2 g glucuronolactone, 160 mg caffeine, 56 g carbohydrate
(CHO), and B vitamins), 2) cola matched for caffeine and CHO (CC), or 3) flavored placebo (PL- sparkling water and flavoring) 50 min prior to racing in a randomized, cross-over design. Performance was measured as time to complete (TTC) a 25-mile simulated road race. Blood was collected at baseline (BASE), 30 minutes after drink consumption (PODR), during exercise at miles 5 (M5), 15 (M15), and immediately (POEX) and 30 min post-exercise (30 minPO). TTC was not different among trials. CC and ED elicited a mile hypoglycemia during cycling. POEX IL-6 was greatest after ED while CC IL-6 was greater than PL. Cycling increased leukocyte number in all conditions with energy drink leukocyte number greater than that of PL at M15. The drink induced an earlier recruitment of monocytes to the blood stream than CC. Mean fat oxidation was greater in PL compared to CC, but did not differ between energy drink and PL. Lactate was higher in energy drinkers compared to CC and PL at M5 and M15, but there was no significant influence of either energy drinkers or CC on performance. CHO and caffeine consumption prior to endurance cycling significantly increased the IL-6 release and leukocytosis, and the additional ingredients in energy drinkers appear to have further augmented these responses [13634].

Science on “sports drinks”

Exogenous carbohydrate oxidation was assessed in 6 male Category 1 and 2 cyclists who consumed CytoMax (C) or a leading sports drink (G) before and during continuous exercise (CE). C contained lactate-polymer, fructose, glucose and glucose polymer, while G contained fructose and glucose. Peak power output and VO\textsubscript{2} on a cycle ergometer were 408 ± 13 W and 67 mL O\textsubscript{2} per kg and minute. Subjects performed 3 bouts of CE with C, and 2 with G at 62 percent VO\textsubscript{2peak} for 90 min, followed by high intensity (HI) exercise (86 % VO\textsubscript{2peak}) to volitional fatigue. Subjects consumed 250 ml fluid immediately before (-2 min) and every 15 min of cycling. Drinks at -2 and 45 min contained 100 mg of [U-\textsuperscript{13}C]-lactate, -glucose or -fructose. Blood, pulmonary gas samples and \textsuperscript{13}CO\textsubscript{2} excretion were taken prior to fluid ingestion and at 5, 10, 15, 30, 45, 60, 75, and 90 min of CE, at the end of HI, and 15 min of recovery. HI after CE was 25 percent longer with C than G. \textsuperscript{13}CO\textsubscript{2} from the -2 min lactate tracer was significantly elevated above rest at 5 min of exercise, and peaked at 15 min. \textsuperscript{13}CO\textsubscript{2} from the -2 min glucose tracer peaked at 45 min for C and G. \textsuperscript{13}CO\textsubscript{2} increased rapidly from the 45 min lactate dose, and by 60 min of exercise was 33 percent greater than glucose in C or G, and 36 percent greater than fructose in G. \textsuperscript{13}CO\textsubscript{2} production following tracer fructose ingestion was greater than glucose in the first 45 minutes in C and G. Cumulative recoveries of tracer during exercise were: 92 percent for lactate in C and 25 percent for glucose in C or G. Recoveries for fructose in C and G were 75 percent and 26 percent respectively. Lactate was used more rapidly and to a greater extent than fructose or glucose. CytoMax significantly enhanced HI. In summary, this is the first report of greater fractional oxidation of lactate in comparison to other carbohydrate energy substrates during exercise. As well, the presence of fructose as an ingredient in an energy-electrolyte hydration beverage provides and advantage over glucose alone in terms of providing energy to an exercising athlete. By providing PolyLactate™ as well as fructose, glucose and glucose polymers as CHO-energy forms, CytoMax possesses clear advantages in terms of providing rapid and sustained energy allowing superior sprint finishing performance in comparison to a popular HFCS-based sports drink. It was concluded that intense endurance exercise promotes dehydration and depletion of blood glucose, muscle and liver glycogen, and electrolytes. Endurance athletes must satisfy the needs for fluids, energy, and electrolytes for optimal performance. Fluid-energy-electrolyte replacement beverages (i.e. sports drinks) improve endurance because they satisfy these needs, particularly in hot and humid environments [07308].

Substrates
Traditional sports drinks supply energy in the form of sugars (glucose, fructose, sucrose) and 1721
glucose polymers. Carbohydrate is the main energy source for prolonged physical activity, and of the dietary energy substrates, carbohydrates are most readily digested and absorbed. The drinks also contain electrolytes to replace those lost in sweat. Electrolytes also stimulate thirst, promote solute absorption in the gastrointestinal (GI) tract, and buffer endogenous acids. A sports drink containing a 6 percent (w/v) glucose solution is efficacious for promoting GI emptying and exercise performance. Consumption of 1 liter per hour in 250 ml aliquots delivers 1 g/min of glucose, which enhances fuel availability and provides other benefits. More, investigators have experimented with combinations of hexoses (e.g. 2 glucose/fructose) to raise drink solute content above 6 percent by taking advantage of specific intestinal transporters that promote solute absorption. The same investigators have used isotopically labeled solutes to track the oxidation of specific energy substrates in sports drinks. The results support the concept of increasing energy delivery by expanding the metabolite delivery profile of the beverages. Lactate is a dynamic substrate with great potential as an energy source in sports drinks. To date, however, the efficacy of adding lactate to these drinks has been sparsely assessed. Lactate was once considered a metabolic waste but is now recognized as an important energy substrate in the body. Lactate is the main product of carbohydrate metabolism and can be used as a fuel in working muscle cells shuttled to other tissues such as the heart where lactate is fuel, or to the liver were lactate serves as a gluconeogenic precursor. Lactate is transported across cell plasma and mitochondrial membranes by a family of proton-lactate anion-coupled symporter proteins, of which MCT1 is the predominant isof orm in muscle. Related, but from a different gene family is the sodium-coupled intestinal lactate transporter, sMCT, also known as the slc5a8. The presence of monocarboxylate (i.e. lactate) transport proteins in the GI tract, erythrocytes, myocytes, cardiocytes, hepatocytes, astrocytes and neurons provide a metabolic rationale for including lactate-containing food additives in sports drinks. PolyLactate™ in CytoMax™ (C) might hasten the delivery of substrate during prolonged intense exercise, which may improve sprint performance and delay prevent fatigue after prolonged, hard exercise. In one study it was evaluated rapidity and extent of use of substrates present in C and a popular brand (G). The main finding was that lactate was oxidized faster and to a greater extent than fructose or glucose, which are the principle nutrients contained in most sports drinks. Including lactate as a component of sports drinks is logical based on the research showing its role in carbohydrate utilization during exercise (i.e. the lactate shuttle) and the present results show that exogenous lactate is a readily available substrate in that it is rapidly transported and oxidized. On the bases of fractional oxidation rate and cumulative recovery of tracer in expired CO2 collected over more than 90 min of continuous followed by high intensity exercise, results showed that lactate was used as a fuel much faster and more completely than glucose, particularly in the drink formulations tested which are typical of sports drink platforms that are commonly used. Given the plethora of scientific evidence regarding endogenous lactate oxidation in humans in vivo, the results showing rapid and extensive oxidation of orally supplied lactate are not surprising. The rapid rate of lactate assimilation, distribution and oxidation could be predicted given its central role in linking glycolytic and oxidative metabolism, as well as the wide-spread expression of lactate-hydrogen ion symporters (referred as monocarboxylate transporters, or MCTs) in muscle and other tissues, and the presence of a sodium ion-mediated intestinal MCT. The initial oxidation rate of lactate was clearly superior to glucose and fructose at the outset of exercise, but equally or more impressive was the rapid assimilation and use of lactate during exercise. The oxidation rate of fructose was intermediate between lactate and glucose, actually tracking the pattern of lactate-derived tracer excretion in breath at the beginning of exercise. The results may indicate muscle fructose uptake and oxidation. However, a more likely route of entry for fructose is that it may undergo glycolysis in the intestinal mucosa or elsewhere in the splanchnic bed, causing a rapid entry of lactate into the systemic circulation [07308]
**Lactate oxidation**

Results obtained in one investigation indicate that various fuel energy substrates in sports drinks use different transport systems to gain access to the systemic circulation and cellular pathways of oxidative metabolism at different rates. Results can be interpreted to mean that the ideal sports drink should contain several different energy substrates because the absorption of any single substrate is limited by competition for its unique transporter sites. Including several substrates in a sports drink might accelerate the rate of energy absorption due to the utilization of various independent transport systems, and therefore the best way to provide energy substrate during prolonged exercise. In terms of the fractional oxidation rates of exogenously supplied fuel energy substrates, the results obtained in the present investigation are likely also attributable to the presence of muscle cell (sarcolemmal) transport systems as well as the intracellular pathways of energy substrate utilization. The preference of working muscle for lactate over glucose has been established in combined lactate clamp and tracer studies. The apparent preference for fructose over glucose is likely attributable to the conversion of fructose to lactate and subsequent use of lactate derived from exogenously supplied fructose. Lactate can be taken up by more than one type of tissue during exercise, so it is not known which tissue oxidized lactate in each study. However because working muscle accounts for most of the whole-body pulmonary oxygen consumption during exercise, it can reasonably assumed that working muscle accounted for most of the observed \([U^{13}C]lactate oxidation. Although it is well established that lactate is also the major gluconeogenic precursor that is taken up by the liver and converted to glucose which can be released to maintain blood glucose, if ingested lactate first went to the liver and was converted to glucose, an entirely different kinetic response from that observed would be expected. The appearance of \(^{13}CO_2\) from lactate in the breath would have been far slower than the rapid lactate fractional oxidation rates observed in the present study if gluconeogenesis was a major route of disposal of exogenously supplied lactate. Therefore, it is appropriate to conclude that the rapid oxidation of lactate in comparison to glucose is consistent with results of vascular lactate tracer and clamp procedures showing preferential and direct oxidation of lactate over glucose, and not lactate carbon converted to glucose and then oxidized. The results of the present investigation are important from another aspect because they indicate that at the exercise power output studied, splanchnic blood flow is sufficient for assimilation of the exogenous carbohydrate supply [07308].

**Buffering capacity**

Besides the well-established role of lactate as an essential intermediate between glycolytic and oxidative metabolism, the increased performance occurred could be partially explained by the enhanced buffering capacity of PolyLactate. It has been demonstrated that there is an increased bicarbonate and blood pH during exercise in subjects consuming a drink with PolyLactate as opposed to glucose polymer alone. The lactate in CytoMax is able to stoichiometrically scavenge protons because the lactate anion is the salt of the acid. By scavenging protons, the lactate in C acts to spare bicarbonate during periods of high proton efflux from skeletal muscle or intense exercise (which may explain why five of six subjects sprinted longer after prolonged, hard continuous exercise when consuming C than they did when consuming G). As well, the disposal of exogenous lactate as lactic acid via oxidation or gluconeogenesis results in stoichiometric removal of protons (H\(^+\)). That such a buffering, or other, effect of PolyLactate may have been in operation is suggested because the effect on sprint finishing performance can not be explained on the bases of respiratory gas exchange or blood metabolite levels [07308].

**With caffeine**

Caffeine is the most common ingredient utilized in energy drinks. Caffeine is extracted from the raw fruit of over sixty species of coffee plants (*coffea Arabica*), all part of the
methylxanthine family. Caffeine is also extracted from tea, kola nuts, and cocoa. After ingestion, caffeine is quickly absorbed and increases in plasma concentrations are generally observed between 30-60 minutes following ingestion. The difference in absorption time is dependent on the physicochemical formulation properties of the product dose. Caffeine is a strong cardiovascular stimulant that increases epinephrine output to a greater extent when ingested via its anhydrous formulation when compared to an equal amount of brewed or instant caffeinated coffee. In addition, caffeine’s half-life ranges from approximately 2 to 10 hours with 0.5-3.5 percent of its content excreted unchanged in urine and select amounts eliminated via perspiration. A recent position stand from the Journal of the International Society of Sports Nutrition summarized the effects of caffeine on exercise performance as follows [13633]:

- Caffeine is effective for enhancing sport performance in trained athletes when consumed in low-to-moderate dosages (3-6 mg/kg BM) and overall does not result in further enhancement in performance when consumed in higher dosages (≥ 9 mg/kg BM).
- Caffeine exerts a greater ergogenic effect when consumed in an anhydrous state as compared to coffee.
- It has been shown that caffeine can enhance vigilance during bouts of extended exhaustive exercise, as well as periods of sustained sleep deprivation.
- Caffeine is ergogenic for sustained maximal endurance exercise, and has been shown to be highly effective for time-trial performance.
- Caffeine supplementation is beneficial for high-intensity exercise, including team sports such as soccer and rugby, both of which are categorized by intermittent activity within a period of prolonged duration.
- The literature is equivocal when considering the effects of caffeine supplementation on strength-power performance, and additional research in this area is warranted.
- The scientific literature does not support caffeine-induced diuresis during exercise, or any harmful change in fluid balance that would negatively affect performance.

Several studies have reported significant improvements in both aerobic and resistance exercise with a relative dosage of approximately 2 mg/kg BM of caffeine. This is less than the amount recommended (3-6 mg/kg BM) to enhance performance, and may contribute to the hypothesis that the synergistic effects of the various ingredients contained in ED/ES are responsible for the reported improvements in exercise performance [13633].

With carbohydrate, protein and antioxidants

Another common ingredient in most ED is some type of carbohydrate source (e.g. glucose, sucrose, maltodextrin, etc.). Energy drinks also typically contain glucuronolactone, an ingredient which is involved in ascorbic acid synthesis and is metabolized into xylulose. Evidence from numerous studies indicates that carbohydrate feeding during exercise of about 45 minutes or longer can improve endurance capacity and performance. Mechanisms by which carbohydrate feeding prior to and during exercise improves endurance performance include maintaining blood glucose levels, maintaining high levels of carbohydrate oxidation, and the sparing of liver and possibly skeletal muscle glycogen. Peak rates of carbohydrate oxidation are commonly around 1 g of carbohydrate per minute or 60 g/h. Glucose, sucrose, maltodextrins and amyllopectin are oxidized at high rates, while fructose, galactose and amylose are oxidized at lower rates (approximately 25-50 % lower). Consequently, sports drinks typically contain a mixture of various types of carbohydrates designed to optimize exogenous carbohydrate oxidation. ED’s contain approximately 25-30 grams of carbohydrate per 240 mL (8 fluid ounces) serving. This amount nearly meets the lower value of 30 grams/hour recommended during endurance exercise, but falls short of the upper range of
60 g/hr. In order to meet this upper level of 60 grams of carbohydrate per hour during endurance exercise, approximately 530 mL (18 fluid ounces) of a typical ED per hour would need to be consumed. While the total carbohydrate content of typical ED is quite high, a shortcoming exists in regards to the concentration of commercially available energy drinks. The American College of Sports Medicine and the ISSN recommend ingesting carbohydrate in a 6-8 percent solution (6-8 grams per 100 ml of fluid) during endurance exercise. A typical ED provides carbohydrates at a greater concentration, typically around an 11-12 percent solution. Ingesting higher percentages (>10 %) of carbohydrate in fluids has been reported to delay gastric emptying and increase gastrointestinal distress. Consequently, athletes who want to use ED as sports drinks may need to dilute the beverage and/or alternate consumption of ED and water during exercise [13633].

Fourteen male cyclists were studied to compare the effect of carbohydrate-protein-antioxidant beverage (CHOPA) to an isocaloric carbohydrate-only (CHO) beverage on time to fatigue and muscle damage. Subjects performed two sets of rides to exhaustion on a cycle ergometer. In each set, the first ride was performed at 70 percent VO$_{2peak}$, and the second was performed 24 h later at 80 percent. CHO or CHOPA was consumed every 15 min during exercise and immediately afterward. Plasma CK and LDH and muscle soreness were measured pre- and postexercise. Time to fatigue was not different between CHO and CHOPA at 70 percent VO$_{2peak}$, 80 percent VO$_{2peak}$, or total performance time. Postexercise CK was increased from baseline in CHO but not with CHOPA. Similarly, LDH values increased over baseline in CHO, but not with CHOPA. Postexercise CPK and LDH were higher after the CHO trial than after the CHOPA trial. Median postexercise muscle soreness was higher in CHO than with CHOPA. Thus, no differences in time to fatigue were observed between the beverages, despite lower total carbohydrate content in the CHOPA beverage. The CHOPA beverage attenuated postexercise muscle damage, as evidenced by CK and LDH values, compared with an isocaloric CHO beverage [06259].

With other nutrients

Most ED and ES also contain a small amount of vitamins (e.g. thiamin, riboflavin, niacin, Vitamin B6, Vitamin B12, pantothenic acid, Vitamin C) and electrolytes (e.g. sodium, potassium, phosphorus, etc). While the addition of these nutrients may add to the nutrient density of these products, there is little evidence that ingestion of these vitamins and minerals in the amounts found in ED and ES would provide any ergogenic benefit during exercise performance in well-nourished individuals. Additionally, ED and ES typically contain nutrients purported to promote cognition and mental focus (e.g. Taurine, Ginkgo biloba, L-Tyrosine, Citocoline, 5-Hydroxy-L-Tryptophan [5-HTP], St. John’s Wort, etc), stimulants (e.g. caffeine, Guarana, Green Tea, Synephrine, Yerba mate, Yohimbine, Tyramine, Vinpocetine, etc.), and/or various purported ergogenic nutrients (e.g. panax ginseng, l-carnitine, d-ribose, beta-alanine, inositol, citrulline, quercetin, etc). While there are data to support the potential ergogenic value of some of these nutrients on cognitive function and/or exercise capacity; the amounts found in ED and ES are generally much lower than the typical concentrations associated with an ergogenic effect. Consequently, it is unclear whether adding these nutrients to ED and/or ES provides a synergistic or additive effect to the carbohydrate and caffeine found in these products. In addition, adding these nutrients to the caffeine found in ED and/or ES may change the adverse effect profile of these finished products, and warrant further study [13633].

Effect on exercise performance
Several studies have investigated the effects of ED consumption prior to exercise. The types of exercise that were evaluated include resistance exercise, anaerobic exercise, and aerobic/endurance exercise. Many of the studies investigating the effects of ED ingestion on anaerobic performance measures have been conducted within the past several years. ED (containing approximately 2 mg/kg BM caffeine) consumed 45 to 60 minutes prior to anaerobic/resistance exercise may improve upper- and lower- body total lifting volume, but has no effect on repeated high intensity sprint exercise, or on agility performance. ED containing approximately 2 mg/kg BM caffeine consumed 10 to 40 minutes prior to aerobic exercise improve cycling and running performance in both trained cyclists and recreationally active participants. In the one investigation in which no aerobic performance improvement was reported, the ED (containing 2 mg/kg BM caffeine) was ingested 60-minutes prior to the performance assessment. In light of the other findings, ingestion of the caffeine-containing ED 60-minutes prior to the exercise bout may be too long of a period to realize improvements in aerobic exercise performance. To date, most studies on ED have reported improvements in mood, reaction time, and/or markers of alertness, even though the relative importance of the various ingredients is not fully understood. The primary ergogenic value appears to be due to the caffeine and/or carbohydrate contained in these drinks. Individuals looking to enhance reaction time, mental alertness, and/or focus may benefit from consuming an ED prior to exercise [13633].

Energy drinks and their role in energy expenditure and weight loss

ED and some commercial beverages designed to increase metabolism typically contain a number of stimulants (e.g. caffeine, Guarana, Green Tea, synephrine, Yerba mate, Yohimbine, Tyramine, Vinocetine, etc). Several low-calorie ED and beverages have been marketed as “thermogenic blends” with a focus on increasing metabolism. Theoretically, ingestion of ED prior to exercise may increase energy expenditure which over time could help manage and/or promote weight loss. In support of this theory, studies have shown that ingestion of caffeine (e.g. 200-500 mg) can increase acute (1-24 hours) energy expenditure, chronic (28 days) energy expenditure, and elevate plasma free-fatty acid and glycerol levels. Collectively, these findings suggest that the stimulant properties of caffeine contained in ED can elevate an individual's metabolic rate as well as elevate the rate of lipolysis in the body. However, these studies used various types of caffeine/stimulant/vitamin-enriched coffee, a caffeine/stimulant blend supplement, and various calorie-free thermogenic ED. Consumption of low calorie ED and thermogenic beverages have been reported to increase resting energy expenditure and fat metabolism on an acute basis. Preliminary studies suggest that ingesting some types of ED and thermogenic beverages prior to exercise during training could promote positive adaptations in body composition. However, more research is needed to determine whether daily use of ED would affect long-term energy balance and body composition [13633].

Gastric emptying

The purpose of one study was to examine the gastric emptying and rehydration effects of hypotonic and hypertonic glucose-electrolyte drinks after exercise-induced dehydration. Eight healthy males lost about 1.8 percent body mass by intermittent cycling and rehydrated (150 % of body mass loss) with a hypotonic 2 percent (2 % trial) or a hypertonic 10 percent (10 % trial) glucose-electrolyte drink over 60 min. Blood and urine samples were taken at pre-exercise, post-exercise, and 60, 120, 180 and 240 min post-exercise. Gastric and test drink volume were determined 15, 30, 45, 60, 90 and 120 min post-exercise. At the end of the gastric sampling period 0.3 percent (2 % trial) and 42 percent (10 % trial) of the drinks remained in the stomach. Plasma volume was lower and serum osmolality was greater at 60
and 120 min during the 10 percent trial. At 240 min, 52 percent (2% trial) and 64 percent (10% trial), a statistically significant difference, of the drinks were retained. Net fluid balance was greater from 120 min during the 10 percent trial. When net fluid balance was corrected for the volume of fluid in the stomach, it was greater at 60 and 120 min during the 2 percent trial. These results suggest that the reduced urine output following ingestion of a hypertonic rehydration drink might be mediated by a slower rate of gastric emptying, but the slow gastric emptying of such solutions makes rehydration efficiency difficult to determine in the hours immediately after drinking, compromising the calculation of net fluid balance [13637].

Safety considerations

ED have had a negative connotation in the media and more recently medical community, mostly related to potential concerns about excessive caffeine intake and/or potential deleterious effects of mixing ED with alcohol. While safety concerns and use of alcohol go beyond the scope of this paper, the reader is referred to a recent viewpoint published in the Journal of the American Medical Association related to safety concerns of mixing ED with alcohol. In terms of use of ED in the traditional sense, most concerns have been based on case studies or adverse event reports that have serve only to document a potential association, but does not establish causality. In reality, there are currently only a few studies (acute or long term) that have investigated the side effects of ED. There appear to be two primary active nutrients in most ED and ES (i.e. carbohydrate and caffeine) that may possess safety concerns in some populations. Many ED contain 25-50 g of simple sugars, therefore, ingestion of ED prior to exercise are likely to rapidly increase insulin in order to maintain normal blood glucose levels. For this reason, diabetics and pre-diabetics should avoid high glycemic load ED or consider consuming low carbohydrate versions of ED. Very often, ED also contain various stimulants with the most common being caffeine. Some concern has been raised about excessive caffeine intake that could be obtained from consuming too many ED and/or from a lack of knowledge that that some ingredients contained in ED may contain caffeine. However, if caffeine is added to a food, it must then be listed; therefore many individuals may consume more caffeine than they realize. In Canada, caffeine levels are limited to 180 mg per drink. The caffeine content of common ED and ES has been reported to range from about 100 to 286 mg. As a comparison, the average cup of coffee or contains between 40 and 150 mg caffeine, while a 20 oz. cup of Starbucks regular drip coffee has been found to contain as much as 480 mg of caffeine. The potential side effects of caffeine include: insomnia, nervousness, restlessness, gastric irritation, nausea, vomiting, tachycardia, tremors, and anxiety; which have been reported at doses as low as 250 to 300 mg. Caffeine availability is ubiquitous and it is one of the most extensively studied substances in the food supply with a long history as generally regarded as safe when consumed in moderation. However, all substances may be toxic under the right conditions, with toxicity being a function of the interaction of many physiologic variables that include the following: acute and chronic dosing, route of administration, genetics, age, sex, environment, and intrinsic health of the individual being exposed. Young adults have been found to have subclinical coronary atherosclerosis. In addition, post-mortem assessment of sudden cardiac death in young persons (<35 years) reveals a variety of anatomic abnormalities of the coronary arteries, myocardium, valves and the conduction system. A lethal dose of caffeine has been typically in excess of 5 g, which equates to about 42 cups of coffee at 120 mg of caffeine per cup. Individuals with certain medical conditions (e.g. metabolic syndrome or diabetes mellitus) should avoid consumption of high glycemic drinks and/or foods and therefore should not consume the high caloric versions of ED. It would be prudent for individuals with known cardiovascular disease to avoid altogether their use of ED and/or ES, or other products with known cardio-stimulant effects. While ED containing caffeine and other stimulants may have negative effects upon health and cardiac parameters in individuals with such pre-existing health conditions, the current evidence (although small) suggests that
consumption of ED and ES are safe in healthy populations and similar to ingesting other foods and beverages containing caffeine [13633].

**Consumption pattern**

Sales of sports and energy drinks have increased dramatically, but there is limited information on regular consumers of sports and energy drinks. Characteristics associated with sports and energy drink intake were examined among a sample representing the civilian noninstitutionalized US adult population. The 2010 National Health Interview Survey data for 25,492 adults (18 years of age or older; 48 % males) were used. Nationwide, 31 percent of adults were sports and energy drink consumers during the past 7 days, with 22 percent consuming sports and energy drinks one or more times per week and 11.5% consuming sports and energy drinks three or more times per week. Based on multivariable logistic regression, younger adults, males, non-Hispanic blacks and Hispanics, not-married individuals, adults with higher family income, those who lived in the South or West, adults who engaged in leisure-time physical activity, current smokers, and individuals whose satisfaction with their social activities/relationships was excellent had significantly higher odds for drinking sports and energy drinks one or more times per week. In this model, the factor most strongly associated with weekly sports and energy drink consumption was age. Lower odds for consuming sports and energy drinks one or more times per week were associated with other/multiracial (OR=0.80 vs non-Hispanic white) and obesity (OR=0.87 vs underweight/normal weight). Separate modeling of the association between other beverage intake and sports and energy drink intake showed that higher intake of regular soda, sweetened coffee/tea drinks, fruit drinks, milk, 100 percent fruit juice, and alcohol were significantly associated with greater odds for drinking sports and energy drinks one or more times per week. These findings can help medical care providers and public health officials identify adults most in need of encouragement to reduce sports and energy drink intake and increase healthier beverage intake [13635].

**Beverage after aerobic activity**

Recovery beverages are commonly used by endurance and team-sport athletes during the time between exercise sessions. Practical recommendations on the optimal nutrient composition of these drinks and timing of their consumption are therefore needed. One article summarized research to date on the use of recovery beverages after aerobic activities and provides the following recommendations for practitioners on the optimal formula and timing of use for endurance and team-sport athletes. Current evidence suggests that, to maximize glycogen resynthesis, athletes should consume about 1.2 g carbohydrate per kilogram body weight as glucose and sucrose immediately after exercise and each hour thereafter for 4-6 hours postexercise. Alternatively, they may consume 0.8 g/kg/h in combination with 0.4 g/kg/h amino acids or protein. Liquids provide valuable fluids for rehydration, and an ideal recovery beverage should not only contain carbohydrate and protein but also contain electrolytes, including about 0.3-0.7 g sodium-per liter fluid to help restore sodium lost through sweat. Commercial beverages with this type of nutrient composition are effective, and recent work indicates that chocolate milk may be as effective as or superior to these in promoting recovery. Research regarding the effects of specific types of amino acids and antioxidants on recovery is mixed; thus, further investigation is needed before specific recommendations about these nutrients can be made. Future studies that include women and athletes representing a variety of sports, ages, and training levels and that use consistent methodology will lead to a better understanding of the effects of postexercise intake on recovery [11525].
In school children

High intake of sugar-sweetened beverages in childhood is linked to increased risk of obesity and type II diabetes later in life. Using three nationally representative surveys of dietary intake, we investigated beverage patterns and trends among US school-aged children from 1989/91 to 2007/08. 3, 583 participants ages 6-11 years old were included. It was reported per capita trends in beverage consumption, percent consuming, and amount per consumer for the following categories of beverages: sugar-sweetened beverages (SSB), caloric nutritional beverages (CNB) and low calorie beverages (LCB). While per capita kcal contribution from total beverages remained constant over the study period, per capita consumption of SSBs increased and CNBs decreased in similar magnitude. The substantial increase in consumption of certain SSBs, such as fruit drinks and soda, high fat high sugar milk, and sports drinks, coupled with the decrease in consumption of high fat low sugar milk was responsible for this shift. The percent consuming SSBs as well as the amount per consumer increased significantly over time. Per capita intake of total milk declined, but the caloric contribution from high fat high sugar milk increased substantially. Among ethnicities, important differences in consumption trends of certain SSBs and 100 percent juice indicate the complexity in determining strategies for children's beverage calorie reduction. It was concluded that as upward trends of SSB consumption parallel increases in childhood obesity, educational and policy interventions should be considered [11526].

Organized sport provides one option for children to be physically active. However, there is a paucity of information about the relationship between children's participation in organized sport and their diet, and specifically their sports drink consumption. Therefore, the relationship between sports participation in children and the consumption of sports drinks, sugar-sweetened beverages (SSBs) and other components of diet was examined. A cross-sectional descriptive study was conducted using baseline data from the Action Schools! BC Dissemination study cohort (n=1421; 10 years; 736 girls, 685 boys). The differences between the dietary behaviours of children participating in organized sport (sport) versus those that did not participate (non-sport) was examined. A modified Physical Activity Questionnaire for Older Children (PAQ-C) was used to measure physical activity levels and participation in organized sport. A Food Frequency Questionnaire (FFQ) and 24-hour dietary recall were used to assess eating behaviour and macronutrient intake (including protein, fat, and carbohydrate as well as sugar, fibre and total calories). Fruit, vegetable and beverage quantities were hand-tallied from the dietary recall. Fruit, vegetable and beverage frequency was assessed using the FFQ. Analysis of covariance (ANCOVA) was used to analyse differences between groups and a chi-square test of association was use to determine if participation in sport was significantly associated with the proportion of children consuming sports drinks and SSBs, and with gender. Children involved in sport had a lower body mass index (BMI) and were more physically active than children in the non-sport group. Only a small number (n = 20/1421) of children consumed sports drinks and no difference in consumption of sports drink between sport and non-sport participants was observed. However, children involved in organized sport consumed more total calories, fat, fibre, fruit, vegetables and non-flavoured milk than non-sport children. It was concluded that children involved in organized sport were more physically active, consumed a healthier diet than non-participants and on average had lower BMI's despite consuming more calories. As consumption of sports drinks among this age group was low, this may be an ideal time to begin educating children and their parents about the appropriate consumption of sports drinks and the perils of consuming too many SSBs, specifically [13636].

Adverse effects
It was reported a case of a 17-year-old boy who suffered acute renal failure after consuming 3 L of energy drink in combination with 1 L of vodka amounting to 4600 mg of taurine and 780 mg of caffeine mixed with 380 g of alcohol. The consumption of this mixture is extremely popular in adolescents, because the joint effects of caffeine and taurine reduce the effect of alcohol. Although there have been case reports of deaths linked to the consumption of energy drinks with and without alcohol, awareness of the possible dangers is still low. The fact that athletes and major sports events are sponsored by energy drink manufacturers imply that they may even be healthy and performance-enhancing [11395].

**Sweet, non-alcoholic beverages**

One study aimed to analyse the contribution of Australian print news coverage to the public profile of sweet, non-alcoholic beverages. News media portrayal of health contributes to individuals' decision-making. The focus on sugar-sweetened beverages reflects their contribution to excessive energy intake. One year's coverage of sweet, non-alcoholic beverages by major Australian newspapers was analysed using content and frame analysis. Research questions addressed which sweet drinks are most prominently covered, what makes sweet drinks newsworthy and how are the health aspects of sweet drinks framed? Fruit juice was the most widely covered sweet drink, closely followed by carbonated, sugar-sweetened soft drinks. Overall coverage was positively oriented towards sweet drinks, with fruit juice primarily portrayed as having health benefits. Some coverage mentioned risks of sweet drinks, such as obesity, tooth decay, metabolic syndrome and heart attack. Sweet drinks often enjoy positive coverage, with their health benefits and harms central to their ability to attract journalists' attention. However, the mix of coverage may be contributing to consumer confusion about whether it is safe and/or healthy to consume sweet non-alcoholic drinks. Framing of sweet drinks as healthy may undermine efforts to encourage individuals to avoid excess consumption of energy-dense drinks which offer few or minimal health benefits [11396].

**Red bull**

Energy drinks are frequently consumed by athletes prior to competition to improve performance. This study examined the effect of Red Bull® on repeated sprint performance in women athletes. Fifteen collegiate soccer players participated. After performing a familiarization trial, subjects performed three sets of eight bouts of the modified t test after ingestion of 255 mL of placebo or Red Bull 1 h pre-exercise in a randomized, placebo-controlled crossover design. Throughout testing, sprint time, heart rate (HR), and rating of perceived exertion (RPE) were continuously obtained. Repeated measures analysis of variance was used to examine differences in variables between drink conditions. Across athletes, t test time ranged from 10.4 to 12.7 s. Mean sprint time was similar between Red Bull and placebo. HR and RPE increased during the bouts, but there was no effect of Red Bull on either variable versus placebo. Findings indicate that 255 mL of Red Bull containing 1.3 mg/kg of caffeine and 1 g of taurine does not alter repeated sprint performance, RPE, or HR in women athletes versus placebo. One serving of this energy drink provides no ergogenic benefit for women athletes engaging in sprint-based exercise [11263].

The purpose of one study was to determine the effects of Red Bull energy drink on Wingate cycle performance and muscle endurance. Healthy young adults (n=15, 11 men, 4 women, 21 years old) participated in a crossover study in which they were randomized to supplement with Red Bull (2 mg/kg body mass of caffeine) or isoenergetic, isovolumetric, noncaffeinated placebo, separated by 7 days. Muscle endurance (bench press) was assessed by the maximum number of repetitions over 3 sets (separated by 1-min rest intervals) at an intensity
corresponding to 70 percent of baseline 1-repetition maximum. Three 30-s Wingate cycling tests (load = 0.075 kp/kg body mass), with 2 min recovery between tests, were used to assess peak and average power output. Red Bull energy drink significantly increased total bench-press repetitions over 3 sets, but had no effect on Wingate peak or average power. It was concluded that Red Bull energy drink significantly increased upper body muscle endurance but had no effect on anaerobic peak or average power during repeated Wingate cycling tests in young healthy adults [07311].

Energy drinks (ED) such as Red Bull (RB) are marketed to enhance metabolism. Secondary ingredients of EDs (e.g., taurine) have been purported to improve time-trial performance; however, little research exists on how such secondary ingredients affect aerobic metabolism during heavy exercise. The purpose of one study was to investigate the effect of the secondary ingredients of RB on aerobic metabolism during and subsequent to heavy exercise. In double-blind, counterbalanced, and crossover fashion, 8 recreationally trained individuals completed a graded exercise test to determine the gas exchange threshold (GET). Subjects returned on two separate occasions and ingested either an 245 ml serving of RB or a control (CTRL) drink with the equivalent caffeine prior to engaging in two, 10-min constant-load cycling bouts, at an intensity equivalent to GET, with 3 min of rest between bouts. Accumulated liters of O\textsubscript{2} (10 min) was higher for the first (17.1 ± 3.5 L) versus the second (16.7 ± 3.5 L) bout but did not differ between drinks. Similarly, excess post-exercise oxygen consumption was higher following the initial versus second bout but did not differ between drinks. No differences occurred between drinks for measures of heart rate or rating of perceived exertion. These results indicate that the secondary ingredients contained in a single serving of RB do not augment aerobic metabolism during or subsequent to heavy exercise [12403].

Energy drinks are frequently consumed by athletes prior to competition to improve performance. One study examined the effect of Red Bull™ on repeated sprint performance in women athletes. Fifteen collegiate soccer players participated, with mean age, height, and body mass equal to 20 year, 168 cm, and 63 kg, respectively. After performing a familiarization trial, subjects performed three sets of eight bouts of the modified t test after ingestion of 255 mL of placebo or Red Bull 1 h pre-exercise in a randomized, placebo-controlled crossover design. Throughout testing, sprint time, heart rate (HR), and rating of perceived exertion (RPE) were continuously obtained. Repeated measures analysis of variance was used to examine differences in variables between drink conditions. Across athletes, t test time ranged from 10.4 to 12.7 s. Mean sprint time was similar between Red Bull and placebo. HR and RPE increased during the bouts, but there was no effect of Red Bull on either variable versus placebo. Findings indicate that 255 mL of Red Bull containing 1.3 mg/kg of caffeine and 1 g of taurine does not alter repeated sprint performance, RPE, or HR in women athletes versus placebo. One serving of this energy drink provides no ergogenic benefit for women athletes engaging in sprint-based exercise [12404].

To investigate the effects of a caffeine-containing energy drink on soccer performance during a simulated game 19 semiprofessional soccer players ingested 630 ± 52 mL of a commercially available energy drink (sugar-free Red Bull®) to provide 3 mg of caffeine per kg of body mass, or a decaffeinated control drink (0 mg/kg). After sixty minutes they performed a 15-s maximal jump test, a repeated sprint test (7 × 30 m; 30 s of active recovery) and played a simulated soccer game. Individual running distance and speed during the game were measured using global positioning satellite devices. In comparison to the control drink, the ingestion of the energy drink increased mean jump height in the jump test, mean running speed during the sprint test and total distance covered at a speed higher than 13 km/h during the game. In addition, the energy drink increased the number of sprints during the whole game. Post-exercise urine caffeine concentration was higher after the
energy drink than after the control drink. A caffeine-containing energy drink in a dose equivalent to 3 mg/kg increased the ability to repeatedly sprint and the distance covered at high intensity during a simulated soccer game. In addition, the caffeinated energy drink increased jump height which may represent a meaningful improvement for headers or when players are competing for a ball [12274].

Preworkout products - unregulated dietary supplements - typically contain "proprietary blends" of multiple ingredients, including caffeine, dimethylamylamine, creatine, arginine, beta-alanine, taurine, and phosphates. While some dietary supplement labels instruct consumers to seek the advice of a health care professional before using the products, the labels usually do not disclose all ingredients or their precise amounts, and evidence to support the purported performance-enhancing benefits is generally lacking. There is limited evidence to support the use of some preworkout supplement ingredients. For example, in one small placebo-controlled study (n=12), the use of the energy drink Red Bull (containing caffeine and taurine) 40 minutes before a simulated cycling time trial appeared to provide a meaningful ergogenic benefit; in another small study (n=12), the use of a similar caffeine-containing product (Redline) by strength-trained athletes was found to improve reaction time, energy, and mental focus relative to placebo use. However, published evidence on the use of the other ingredients listed above is scant, inconclusive, or conflicting. Adverse effects reported in association with preworkout supplements include gastrointestinal symptoms, cardiac arrhythmia, blood pressure increases, and potential effects on lipids and blood glucose. Thus, although evidence exists to support the performance-enhancement efficacy of some preworkout ingredients as standalone agents, published data on combination products are scant, inconclusive, or conflicting. The safety of these products may be compromised if users consume larger-than-recommended amounts or use more than one product [13623].

Consumption of energy drinks by both recreational and competitive athletes has increased dramatically in recent years. The primary ingredients in many energy drinks include caffeine (CAF) in various forms and taurine. The purpose of this randomized, double-blind, crossover study was to examine the effect of sugar-free (SF) Red Bull (RB) containing CAF and taurine to a CAF only drink and a SF CAF-free placebo (PL) on 1 repetition maximum (1RM) bench press (BP) and the volume load (VL; repetitions x kg at 70 % 1RM) during one BP set to failure in experienced lifters. Seventeen college-age men randomly received the following: (A) 500 mL of SF-RB containing CAF (160 mg) and taurine (2000 mg); (B) 500 mL of a SF drink containing CAF only (160 mg); or (C) a SF CAF-free 500 mL PL drink 60 minutes before testing on 3 separate occasions. After a standard warm-up, the 1RM was determined for each subject and, after 5 minutes rest, they completed repetitions to failure at 70 percent of their 1RM to assess VL. Differences between trials for 1RM BP and the VL were identified using repeated measures analysis of variance. The results indicated that neither SF-RB nor the CAF drink had any effect on 1RM BP or VL compared with PL. Although the CAF content in the energy drinks used in the present study was low (2.0 mg/kg), the finding of no effect of the CAF containing energy drinks for 1RM BP are in agreement with previous studies using intakes up to 6.0 mg/kg. These findings suggest that SF-RB has no effect on upper body 1RM strength or VL in resistance trained men [13624].

Energy drinks (EDs) such as Red Bull (RB) are marketed to enhance metabolism. Secondary ingredients of EDs (e.g. taurine) have been purported to improve time trial performance; however, little research exists on how such secondary ingredients affect aerobic metabolism during heavy exercise. The purpose of one study was to investigate the effect of the secondary ingredients of RB on aerobic metabolism during and subsequent to heavy exercise. In double-blind, counterbalanced, and crossover fashion, 8 recreationally trained individuals completed a graded exercise test to determine the gas exchange threshold (GET). Subjects returned on 2 separate occasions and ingested either a 245 ml serving of
RB or a control (CTRL) drink with the equivalent caffeine before engaging in two 10-minute constant-load cycling bouts, at an intensity equivalent to GET, with 3 minutes of rest between bouts. Accumulated liters of O₂ (10 minutes) were higher for the first bout (17.1 ± 3.5 L) versus the second bout (16.7 ± 3.5 L) but did not differ between drinks. Similarly, excess postexercise oxygen consumption was higher after the initial bout (RB mean, 2.6 ± 0.85 L; CTRL mean, 2.9 ± 0.90 L) versus the second bout (RB mean, 1.5 ± 0.85 L; CTRL mean, 1.9 ± 0.87 L) but did not differ between drinks. No differences occurred between drinks for measures of heart rate or rating of perceived exertion. These results indicate that the secondary ingredients contained in a single serving of RB do not augment aerobic metabolism during or subsequent to heavy exercise [13625].

Canada

Although many of us regularly enjoy tea, coffee and other caffeinated beverages, most of us have enough common sense not to willingly allow children to consume 10 cans of cola in one sitting — the amount of caffeine in 500 mL of some energy drinks. Owing to inadequate labelling requirements, a lack of awareness of caffeine’s harmful effects and marketing campaigns that appeal to children and youth, this is precisely what we are unwittingly allowing in Canada and elsewhere. Energy drinks are very effective high-concentration caffeine delivery systems. These sugar-loaded syrups typically contain 80 to 140 mg of caffeine per 250 mL — the equivalent caffeine in one cup of coffee or two cans of cola. However, beverage companies are offering formulations with caffeine concentrations as high as 500 mg per can in US products such as Wired X505™ and Fixx™. Caffeine can also be purchased in 100- and 200-mg tablets in Canada and the United States. However, even tablets with two and one-half to five times less caffeine have mandatory health warnings guarding against use in children and cautions to limit use because too much caffeine may cause nervousness, irritability, sleeplessness and, occasionally, rapid heart rate. Caffeine-loaded energy drinks have now crossed the line from beverages to drugs delivered as tasty syrups. Health Canada has taken steps to regulate many energy drinks as natural health products with warnings on labels. However, many energy drinks are still considered foods that only list ingredients. Neither natural health products nor foods list total caffeine content or easily understood equivalents in terms of cups of coffee because caffeine-containing herbal extracts are listed separately. People who are inclined to downplay such concerns might argue that caffeine has been safely consumed in foods for centuries. Consumers of more traditional caffeinated beverages indicate that acute intoxication lasts only a few hours, with seemingly few serious short- or long-term health consequences. Moreover, popular coffee retailers sell products with large amounts of caffeine. For instance, a 16-oz “grande” coffee at Starbucks contains 330 mg of caffeine. However, marketing of energy drinks is distinctly different from that of other highly caffeinated beverages. Energy drinks are often targeted toward children and youth through carefully designed advertising campaigns as well as sponsorship of events such as snowboarding and skateboarding competitions. Children and youth are notorious for making poor health choices. They can hardly be expected to make appropriate decisions about consuming energy drinks when information on caffeine concentration and appropriate safe amounts is not visible on these products. In children, effects of high concentrations of caffeine should concern us. A study of 100 US adolescents aged 12 to 18 found that 73 percent consumed 100 mg or more of caffeine per day, with most consumption in the evening, the time of day most likely to negatively affect sleep. Poor sleep quality and quantity in adolescents has been associated with mood disorders, exacerbation of asthma, obesity, lower sense of well-being and poor school performance. Given the potential for harm, regulatory authorities such as Health Canada should step in. Regulations could include government-mandated restrictions on labelling, sales and marketing, or self-imposed industry-wide standards with clear labelling accompanied by public education. Many countries have either imposed or tried to impose strict regulations
because of potential health risks of caffeine. Until 2008, France did not even allow the sale of Red Bull™, and in Denmark, sale was prohibited as of 2009 [10523].

In Canada, energy drinks are formulated, labelled and marketed in accordance with Health Canada’s Natural Health Product Regulation and policies. They are not regulated or labelled as foods. Energy drinks must be marketed in compliance with the Consumer Advertising Guidelines for Marketed Health Products. Energy drinks are intended for adults; the labels clearly indicate that this category of beverage is not recommended for children and people who are sensitive to caffeine, and they include warnings not to mix the beverage with alcohol. Unlike coffee and iced coffee beverages, which have no warnings or quantitative labelling, all energy drinks declare levels of caffeine from all sources (natural and synthetic).

In Canada, mainstream energy drinks contain less caffeine by volume than a cup of filter drip coffee (80–140 mg per energy drink v. 179 mg per 237 mL cup of coffee). Labels on energy drinks advise consumers to have only one drink per day. Nowhere in this country are there energy drinks with caffeine levels approaching those suggested in the editorial. Energy drinks have been the subject of extensive review and analysis by regulatory authorities worldwide. Without exception, these reviews have confirmed the safety of these products in markets around the world [10524].

USA

To examine the dietary and activity correlates of sugar-sweetened beverage consumption by children in middle and high school data were obtained from a cross-sectional survey of 15,283 children in middle and high schools in Texas. Consumption of sodas and noncarbonated flavored and sports beverages (FSBs) were examined separately for their associations with the level of (1) unhealthy food (fried meats, French fries, desserts) consumption, (2) healthy food (vegetables, fruit, and milk) consumption, (3) physical activity including usual vigorous physical activity and participation in organized physical activity, and (4) sedentary activity, including hours spent watching television, using the computer, and playing video games. For both genders, consumption of soda and FSBs was systematically associated with a number of unhealthy dietary practices and with sedentary behaviors. However, consumption of FSBs showed significant positive graded associations with several healthy dietary practices and level of physical activity, whereas soda consumption showed no such associations with healthy behaviors. It was concluded that consumption of FSBs coexists with healthy dietary and physical activity behaviors, which suggests popular misperception of these beverages as being consistent with a healthy lifestyle. Assessment and obesity-prevention efforts that target sugar-sweetened beverages need to distinguish between FSBs and sodas [10525].

Effects of cold drinks

The aims of one study were to determine the effect of cold (4 °C) and thermoneutral (37 °C) beverages on thermoregulation and performance in the heat and to explore sensory factors associated with ingesting a cold stimulus. Seven males completed cold, thermoneutral, and thermoneutral + ice trials in randomized order. Participants cycled for 90 min at 65 percent of $\text{VO}_{2\text{peak}}$, followed by a 15-min performance test at 28 °C and 70 percent relative humidity. They ingested 2.3 ml per kg of a 7.4 percent carbohydrate-electrolyte solution every 10 min during the 90-min steady-state exercise including 30 ml ice puree every 5 min in the ice trial. Absolute changes in skin temperature, mean body temperature, and heat storage were lower across the 90-min exercise bout for the cold compared with the thermoneutral trial. Significant improvements in performance were observed with cold but no significant differences were detected with ice. Consumption of cold beverages during prolonged exercise in the heat improves body temperature measures and performance. Consumption of
ice did not reveal a sensory response, but requires further study. Beverages consumed by athletes exercising in the heat should perhaps be cold for performance and safety reasons [10404].

Proteins

Skeletal muscle protein turnover rates are 1-2 percent/d and exist in dynamic, usually balanced, equilibrium between muscle protein breakdown (MPB) and muscle protein synthesis (MPS). For example, in the fasted state, MPB>MPS, whereas in response to ingestion of protein-containing meals, MPS>MPB. Thus, in healthy adults, muscle mass remains relatively stable due to “fed-gain” being balanced by fasted-loss, so daily protein flux, while it may be 3-4 times greater than net intake and loss, is in tight balance. Fasted-state protein losses are typically about 40-60 g/d for a sedentary person weighing 70-90 kg and it is debatable what the losses would be in athletes, be they aerobically or resistance trained. Dietary protein for athletic populations can serve as signal and substrate for MPS, resulting in protein accretion for hypertrophy, repair of damaged proteins or assisting the maintenance of lean mass. There are important messages for athletes, who differ from sedentary individuals, in terms of quantity, timing and quality of protein intake in relation to an athlete's training stimulus. The molecular changes underpinning these adaptations are gene transcription and mRNA translational signalling and are highlighted in a review. The general consensus is that adults need no more than 0.8-0.9 g/kg/d of protein to meet their needs. However, the notion of consumption of “extra” protein above these levels to cover the needs of increased physical activity is not considered. Dietary guidelines for athletes typically recommend protein intakes of 1.2–1.7 g/kg/d, based on maintaining nitrogen (i.e. protein) balance. By all accounts, nitrogen balance is a flawed method, measuring the minimum amount of protein required to balance losses. Given the functional demands of training and performance, an optimal protein intake for athletes might exist beyond merely satisfying a minimal requirement and thus being in nitrogen balance. Indeed, protein intakes of 0.86 g/kg/d have been shown to reduce whole-body protein synthesis rates in strength-trained athletes, suggesting that current recommendations for athletes may be insufficient if synthetic rates of proteins are adversely affected. Recently, it was demonstrated a protein dose response following resistance exercise. Specifically, resistance exercise-induced MPS increased in a curvilinear fashion with ingestion of graded amounts of isolated egg protein, reaching a plateau at 20 g, with no further increase at 40 g of protein. The amino acids supplied beyond 20 g of postexercise protein were not assimilated into new muscle protein but instead were directed toward oxidation. Interestingly, 10 g of essential amino acids (EAA), equivalent to 25 g of most high-quality intact protein, has been shown to maximally stimulate MPS at rest also [12405].

There is no clear consensus as to whether protein ingestion before, during or after exercise promotes the greatest adaptive response. With respect to pre-exercise feeding, acute and long-term studies, comparing pre- and post-training protein feeding have yielded equivocal results. Consumption of protein during exercise may serve to provide amino acids required to improve protein balance during and after exercise. However, in these studies, carbohydrate and amino acids were provided: given the profound impact of insulin for the suppression of MPB, it may be that the greater net balance is simply an artefact of energy intake suppressing MPB and not a protein-mediated rise in MPS. In addition to adaptation, ingestion of additional protein during endurance exercise does not improve performance, reduce proxy markers of muscle damage or hasten the recovery of muscle function. The potency of postexercise protein ingestion for potentiating MPS is unequivocal. After exercise, the energy status of the cell is returning to resting levels, signalling that pathways are still active, the muscle is prone to greater rates of MPS, and all of these effects are enhanced.
with feeding. Resistance exercise specifically targets a synthetic response of myofibrillar proteins, it is therefore not surprising that protein ingestion augments this response. Interestingly, protein ingestion also potentiates the acute muscle protein synthetic response to endurance exercise. Surprisingly, despite acute increases in mitochondrial protein synthesis with endurance exercise, protein ingestion following a prolonged cycle did not potentiate this response, but instead increased the synthesis of myofibrillar proteins. Thus, protein ingestion may assist in maintaining muscle structural integrity and power-generating capacity, rather than influencing muscle aerobic capacity [12405].

High volume resistance exercise appears to sensitise the muscle to amino acid provision beyond the so called ‘window of opportunity’; a period thought to induce the greatest muscle anabolic effect. Thus, while there is some debate about the critical nature of the timing of postexercise protein consumption, it was recommend that the sooner athletes consume protein after exercise the better. In addition, relatively frequent protein ingestion (i.e. every 3-4 h) over 24 h after exercise to sustain the elevation in MPS is also recommended. A protein digestibility corrected amino acid score close to 1.0 is defined as “high quality”. This includes animal protein sources such as milk (composed of whey and casein protein), eggs, isolated soya protein and most meats. Habitual consumption of high-quality protein sources has a pronounced effect on muscle recovery and adaptation. For example, milk proteins result in a pronounced increase in MPS after resistance exercise, compared with equivalent amounts of isolated soya protein which, over time, promotes greater hypertrophy and is likely to be due to the whey protein constituent in milk. Whey proteins stimulate greater rates of MPS over isonitrogenous amounts of casein and soy protein at rest and after exercise. The mechanisms underpinning the anabolic advantage of whey protein are not entirely clear, but maybe due to the relative amount of the branched-chain amino acids, in particular, leucine. Leucine occupies a position of prominence in that it alone can act as a stimulatory signal for MPS. Milk proteins and whey, in particular, are highly enriched with leucine. More importantly perhaps, the rapid absorption kinetics of whey proteins (or hydrolysed slow digested proteins) induces a greater rate of leucine appearance in the circulation than soy and casein proteins and may be important for stimulating MPS. Thus, although rapid leucinemia may be important in activating MPS, provision of other EAAs may be required to sustain the anabolic response [12405].

Based on the current evidence, the following strategies are proposed which should be very effective at allowing repair, remodelling and adaptation, and gains in lean mass in athletes [12405]:

- daily intakes higher than the RDA (1.2–1.6 g/kg/d)
- emphasise dairy source proteins enriched in leucine
- consume protein in doses of 20-25 g/serving to maximise adaptive responses
- equally spaced protein meals throughout the day
- consumption of protein immediately after exercise.

Carbohydrate ingestion after prolonged strenuous exercise enhances recovery, but protein might also be important. In a crossover with 2-week washout, 10 cyclists completed 2.5 h of intervals followed by 4-h recovery feeding, provided 218 g protein, 435 g carbohydrate, and 79 g fat (protein enriched) or 34 g protein, 640 g carbohydrate, and 79 g fat (isocaloric control). The next morning, cyclists performed 10 maximal constant-work sprints on a Velotron cycle ergometer, each lasting approximately 2.5 min, at approximately 5-min intervals. Test validity was established and test reliability and the individual response to the protein-enriched condition estimated by 6 cyclists' repeating the intervals, recovery feeding, and performance test 2 week later in the protein-enriched condition. During the 4-h recovery, the protein-enriched feeding had unclear effects on mean concentrations of plasma insulin,
cortisol, and growth hormone, but testosterone was 25 percent higher (90 % confidence limits ± 14 %). Protein enrichment also reduced plasma creatine kinase by 33 percent (90 % confidence limits ± 38 %) the next morning and reduced tiredness and leg-soreness sensations during the sprints, but effects on mean sprint power were unclear. To conclude, protein-enriched recovery feeding had no clear effect on next-day performance [08385].

Muscle protein turnover following resistance exercise and amino acid availability are relatively well described. By contrast, the beneficial effects of different sources of intact proteins in relation to exercise need further investigation. The objective of one report was to compare muscle anabolic responses to a single bolus intake of whey or casein after performance of heavy resistance exercise. Young male individuals were randomly assigned to participate in two protein trials (n = 9) or one control trial (n = 8). Infusion of L-[1-13C]leucine was carried out, and either whey, casein (0.3 g/kg lean body mass), or a noncaloric control drink was ingested immediately after exercise. L-[1-13C]leucine-labeled whey and casein were used while muscle protein synthesis (MPS) was assessed. Blood and muscle tissue samples were collected to measure systemic hormone and amino acid concentrations, tracer enrichments, and myofibrillar protein synthesis. Western blots were used to investigate the Akt signaling pathway. Plasma insulin and branched-chain amino acid concentrations increased to a greater extent after ingestion of whey compared with casein. Myofibrillar protein synthesis was equally significantly increased 1-6 h postexercise after whey and casein intake, both of which were higher compared with control. Phosphorylation of Akt and p70(S6K) was increased after exercise and protein intake, but no differences were observed between the types of protein except for total 4E-BP1, which was higher after whey intake than after casein intake. In conclusion, whey and casein intake immediately after resistance exercise results in an overall equal MPS response despite temporal differences in insulin and amino acid concentrations and 4E-BP1 [10515].

Amino acids are commonly described as the building blocks of protein. In chemistry, an amino acid is a molecule containing both amine and carboxyl functional groups. The majority of amino acids ingested by humans are in a combined form as dietary proteins from both animal and vegetable sources. Not all proteins in the diet have the same nutritional value, because they contain different proportions of essential amino acids. Essential and non-essential amino acids refers to whether or not the amino acid in question can be synthesised by the body at a rate sufficient to meet normal requirements for protein synthesis. On this basis, sufficient essential amino acids are contained in “first-class proteins” or “complete proteins”, for example dairy products, eggs, fish and meat. However, proteins from plant sources, known as “second-class proteins” can be combined with each other to make complete proteins. For example, grains plus legumes, grains plus nuts or seeds, and legumes plus nuts or seeds, with legumes including both pulses (peas and beans) and peanuts, can be consumed during the day to ensure adequate amounts of essential amino acids are obtained. Strict vegetarian athletes and, in particular, vegans, need to plan their diet to ensure that their daily combination of plant foods provides them with all the essential amino acids. During intensive training they may need to consider supplementing the diet with essential amino acids. Recent research suggests that the timing of the intake of protein related to exercise may be more important than the total amount of protein consumed in a day. In the case of resistance training, an intake of approximately 20-25 g of a high quality protein source in the hour after exercise appears to produce the maximum rate of protein synthesis. More work is needed to examine the case for other types of exercise. However, it is only the essential amino acids that are needed to achieve this effect, so athletes who need to be energy conscious may opt to consume simply 6-8 g of essential amino acids rather than a whole protein source. Several exercise studies have looked at changes in total plasma amino acids; this seems unhelpful, because some do not change, some go up and some go down. Rather than simply considering amino acids as a precursor for protein
synthesis, it is likely to be of more benefit to look at individual amino acids and specific roles they may play in metabolism and cell signalling pathways. Those that have so far been researched either individually or in combination within supplements will each be the focus of a short, separate section in this review series. Concerns arise over the consumption of single amino acids, due to the impact it may have on an imbalance of other amino acids. In general, therefore, it is better to opt for an even mix of all or just essential amino acids. Nevertheless, there may sometimes be a sound biochemical argument in favour of the repletion of specific nutrients, certainly during long-term depletion, and sometimes during transient depletion, in a variety of situations.

Ingesting protein (PRO) with CHO during prolonged exercise is purported to improve performance compared with CHO alone by altering the regulation of skeletal muscle energy provision. However, no study has directly investigated this issue. It was therefore tested the hypothesis that compared with CHO alone, coingestion of PRO would alter markers of metabolic control, including the magnitude of glycogen use and the net expansion of the tricarboxylic acid cycle intermediate pool, which has been linked to the capacity for oxidative energy delivery. Eight trained men cycled at 69 % VO2peak for 90 min on two occasions, and biopsy samples (vastus lateralis) were obtained before and after exercise. In a randomized, double-blind manner, subjects ingested one of two drinks during exercise that contained either 6 percent CHO or 6 percent CHO + 2 percent PRO (CHO + PRO) at a rate of 1 L per hour. CHO + PRO ingestion significantly increased the plasma concentration of branched chain and essential amino acids after exercise versus CHO. However, net muscle glycogen use and tricarboxylic acid cycle intermediate expansion were similar between trials. Blood creatine kinase activity and 20-km time trial performance measured approximately 24 h after the first exercise bout were not different between treatments. It was concluded that coingestion of protein does not alter specific markers proposed to reflect an enhanced capacity for skeletal muscle energy delivery.

One study was designed to compare the acute response of mixed muscle protein synthesis (MPS) to rapidly (i.e. whey hydrolysate and soy) and slowly (i.e. micellar casein) digested proteins both at rest and after resistance exercise. Three groups of healthy young men (n=6 per group) performed a bout of unilateral leg resistance exercise followed by the consumption of a drink containing an equivalent content of essential amino acids (10 g) as either whey hydrolysate, micellar casein, or soy protein isolate. Mixed MPS was determined by a primed constant infusion of l-[ring-13C(6)]phenylalanine. Ingestion of whey protein resulted in a significant larger increase in blood essential amino acid, branched-chain amino acid, and leucine concentrations than either casein or soy. Mixed MPS at rest (determined in the nonexercised leg) was higher with ingestion of faster proteins. MPS after consumption of whey was approximately 93 percent greater than casein and approximately 18 percent greater than soy, which was a significant difference. A similar result was observed after exercise (whey > soy > casein); MPS following whey consumption was approximately 122 percent greater than casein and 31 percent greater than soy, which also was significant differences. MPS was also greater with soy consumption at rest (64 %) and following resistance exercise (69 %) compared with casein. It was conclude that the feeding-induced simulation of muscle protein synthesis in young men is greater after whey hydrolysate or soy protein consumption than casein both at rest and after resistance exercise; moreover, despite both being fast proteins, whey hydrolysate stimulated muscle protein synthesis to a greater degree than soy after resistance exercise. These differences may be related to how quickly the proteins are digested (i.e. fast vs slow) or possibly to small differences in leucine content of each protein.

Protein supplements commonly are ingested by athletes to improve strength, agility, and speed. While athletes require a higher amount of protein (g/kg body weight) than nonathletes
do to support protein synthesis, they do not necessarily need to consume protein from supplemental sources. To date, no studies have shown an advantage of ingesting protein supplements over natural, protein-containing foods; therefore, dietary sources of protein may be just as effective as protein supplemental sources in the regulation of muscle protein synthesis. Misconceptions regarding protein supplement effectiveness may originate from athletes' nutrition information sources. A survey questionnaire queried high school football players about sources of information and measured protein supplement misconceptions by using scores on a Protein Supplement Misconceptions Index. Sixty-one high school football players participated in the study; 39 were protein supplementers, and 22 were non-protein supplementers. There was a significant difference between index scores of protein supplementers and non-protein supplementers, indicating that protein supplementers had a greater level of misconceptions than non-protein supplementers did. Bonferroni post hoc procedures used with individual index items revealed that protein supplementers were significantly more likely than non-protein supplementers to agree that "athletes should take protein supplements" and needed them "to gain as much muscle as possible". Greater misconceptions for protein supplementers may have resulted from the sources chosen for information and advice. Since coaches, parents, and friends were the primary sources of advice about protein supplements for protein supplementers, it would be valuable to provide nutrition education to these groups concurrently with educating young athletes to dispel ongoing misconceptions regarding the need for and effectiveness of protein supplements [08386].

The purpose of one study was to examine the impact of increased protein intake on short-term decrements in endurance performance during a block of high intensity training. Trained male cyclists completed two, three-week trials, both divided equally into normal (NOR), intensified (INT) and recovery (REC) training. In a counter-balanced, crossover experimental design, cyclists received either a high protein (PRO) (3 g protein per kg body mass (BM) per day) or a normal diet (CON) (1.5 g protein per kg BM per day) during INT and REC. Dietary carbohydrate content remained constant at 6 g per kg BM per day. Energy balance was maintained during each training week. Endurance performance was assessed with a VO$_{2\text{max}}$ test and a pre-loaded time trial. Alterations in blood metabolite responses to exercise were measured at rest, during and following exercise. Cyclists completed the Daily Analysis of Life Demands for Athletes (DALDA) questionnaire each day. Increased dietary protein intake led to a possible attenuation (4.3) in the decrement in time trial performance following a block of high-intensity training compared with normal diet. Restoration of endurance performance during recovery training possibly benefited (2.0 %) from additional protein intake. Frequency of symptoms of stress described as "worse than normal" reported following a block of high-intensity training was very likely (97 %) attenuated by increasing the protein content of the diet. No discernable changes in blood metabolite concentrations were observed in PRO. It was concluded that additional protein intake reduced symptoms of psychological stress, and may result in a worthwhile amelioration of the performance decline experienced during a block of high-intensity training [10405].

Fashion for a healthy lifestyle, muscular and athletic silhouette change our approach to diet. Sports sculpture highlighting the body forces the consumption of larger quantities of proteins than the commonly recommended optimum protein, which is 1 g/kg b.w./24 hours. Raises the question whether or not damages the kidneys? A protein-rich diet has the same haemodynamic effects on the kidneys as well as starvation. In diabetic nephropathy consumed a moderate reduction of protein slows the progression of renal failure, but such actions are not significant restrictions protein. It seems that the protein intake has adverse effects contained in them, salt (up to 3 % by weight). Persons undergoing dialysis should consume at least 1.5 g protein/kg b.w./24 hours. Even more is recommended for sport's people. Both resistance exercise and aerobic are necessary to maintain proper physical
fitnness and muscle mass, which provides better and longer survival. Everything is banned in competitive sports is recommended in dialysis: EPO, anabolic steroids, growth hormone [10219].

One study investigated effects of a high protein (PROT) versus a high carbohydrate (CHO) diet on performance and physiological responses during an ultraendurance climbing race at moderate altitude. On two different periods, in a randomised crossover design, ten climbers (30 years of age) participated in the race (duration 29 h approximately, energy expenditure 44 MJ/day) and were fed either with the PROT (30 % protein content) or the CHO diet (68 % carbohydrate) each providing 17 MJ. Mental performance was assessed by the Stroop test and we estimated maximal voluntary strength of quadriceps muscle. It was quantified metabolic and hormonal circulating concentrations. Mental performance was unaffected after the two races, while muscular performance and body weight were decreased with no diet effects. Significant decreases were measured for IGF-I concentration and its binding protein IGFBP-3, and increases for cortisol and norepinephrine with no diet effects. Glucose concentration decreased without diet effects, while amino acids (leucine, isoleucine, valine, and tyrosine) decreased in CHO group. Leptin concentration decreased without diet effects, whereas total ghrelin increased in CHO group. The results showed that a high PROT or high CHO intake during physical exertion at moderate altitude maintained mental performance, but did not limit muscle force reduction and body weight loss. There was decreased glucose availability, and hormonal responses indicated both catabolism and extreme energy deficiency induced by exercise with opposite responses of ghrelin and leptin. The ghrelin response was additionally indicative of macronutrient intake during the race [10220].

The effect of 42 g of protein ingested pre- and post-exercise on recovery from an acute resistance exercise session was examined in 15 male strength/power athletes who were randomly divided into a supplement (SUP) or placebo (PL) group. Subjects reported to the Human Performance Laboratory (HPL) on four separate occasions (T1-T4). Maximal strength (one repetition-maximum, 1-RM) testing was performed during T1. During T2 subjects performed four sets of ten repetitions at 80 percent of their 1-RM in the squat, dead lift and barbell lunge exercises with 90 s of rest between each set. Blood draws occurred at baseline (BL), immediate and 15 min post-exercise to determine testosterone, cortisol and creatine kinase (CK) concentrations. Subjects reported back to the HPL 24 (T3) and 48 h (T4) post-exercise for a BL blood draw and to perform four sets of ten repetitions with 80 percent of 1-RM for the squat exercise only. No differences in the number of repetitions performed in the squat exercise were seen between the groups at T2. Relative to T2, PL performed significantly fewer repetitions than SUP at T3 and T4. No differences in hormonal measures were seen between the groups. CK concentrations were significantly elevated at T3 for both groups, but continued to elevate at T4 for PL only. No significant group differences were noted for CK at any time point. Results indicate that a proprietary protein supplement consumed before and after a resistance training session significantly contributes to improvements in exercise recovery 24 and 48 h post-exercise [10221].

The purpose of the one study was to examine the effect of increased protein intake on short-term decrements in endurance performance during a block of high-intensity training. Trained male cyclists completed two 3-wk trials both divided equally into normal (NOR), intensified (INT), and recovery (REC) training. In a counterbalanced crossover experimental design, cyclists received either a high-protein (PRO; 3 g protein/kg body mass (BM)/d) or a normal diet (CON; 1.5 g protein/kg BM/d) during INT and REC. Dietary carbohydrate content remained constant at 6 g/kg BM/d. Energy balance was maintained during each training week. Endurance performance was assessed with a VO2max test and a preloaded time trial. Alterations in blood metabolite responses to exercise were measured at rest, during, and after exercise. Cyclists completed the Daily Analysis of Life Demands for Athletes (DALDA)
questionnaire each day. Increased dietary protein intake led to a possible attenuation (4.3%) in the decrement in time trial performance after a block of high-intensity training compared with NOR. Restoration of endurance performance during recovery training possibly benefited from additional protein intake. Frequency of symptoms of stress described as "worse than normal" reported after a block of high-intensity training was very likely (97%) attenuated by increasing the protein content of the diet. No discernable changes in blood metabolite concentrations were observed in PRO. It was concluded that additional protein intake reduced symptoms of psychological stress and may result in a worthwhile amelioration of the performance decline experienced during a block of high-intensity training [11264].

The effect of dietary protein ingested after exercise on recovery in women athletes is unknown. Therefore, it was asked whether there is a meaningful difference between high- and low-protein recovery diets on the subsequent performance of well-trained female cyclists. In a crossover, 12 female cyclists completed three high-intensity rides composed of 2.5-h intervals on day 1, followed by repeat-sprint performance tests on days 2 and 4, interspersed with a rest day. During the 4-h recovery on days 1 and 2, cyclists ingested 1.4/0.7/0.26 or 2.1/0.1/0.26 g/kg/h of CHO/protein/fat in high-protein or isocaloric control conditions, respectively. At other times, cyclists ingested an isoenergetic high-CHO diet. No effect of protein dose on the mean power during repeat sprint was evident on day 2 or on day. Furthermore, fatigue effects (slope) were unclear. Perceptions of leg tiredness and soreness were increased, and leg strength was reduced in the high-protein condition relative to control. In the high-protein condition, plasma glucose concentrations were lower during recovery, and plasma lactate concentrations were lower during the sprints. Effects on circulating creatine kinase activity were trivial. Net nitrogen balance during the experiment was positive in the high-protein condition but negative in the control condition; the estimated protein requirement was 1.28 g/kg/d. In contrast with the previous findings in males, it was observed no clear influence of dietary protein quantity on the subsequent performance in females. The findings on nitrogen balance suggest that female cyclists training intensely have daily protein requirements approximately 1.6 times the recommended daily allowance but 0.65 times that of males [11265].

The purpose of one review was to determine whether past research provides conclusive evidence about the effects of type and timing of ingestion of specific sources of protein by those engaged in resistance weight training. Two essential, nutrition-related, tenets need to be followed by weightlifters to maximize muscle hypertrophy: the consumption of 1.2-2.0 g protein/kg of body weight, and ≥44-50 kcal/kg of body weight. Researchers have tested the effects of timing of protein supplement ingestion on various physical changes in weightlifters. In general, protein supplementation pre- and post-workout increases physical performance, training session recovery, lean body mass, muscle hypertrophy, and strength. Specific gains, differ however based on protein type and amounts. Studies on timing of consumption of milk have indicated that fat-free milk post-workout was effective in promoting increases in lean body mass, strength, muscle hypertrophy and decreases in body fat. The leucine content of a protein source has an impact on protein synthesis, and affects muscle hypertrophy. Consumption of 3-4 g of leucine is needed to promote maximum protein synthesis. An ideal supplement following resistance exercise should contain whey protein that provides at least 3 g of leucine per serving. A combination of a fast-acting carbohydrate source such as maltodextrin or glucose should be consumed with the protein source, as leucine cannot modulate protein synthesis as effectively without the presence of insulin. Such a supplement post-workout would be most effective in increasing muscle protein synthesis, resulting in greater muscle hypertrophy and strength. In contrast, the consumption of essential amino acids and dextrose appears to be most effective at evoking protein synthesis prior to rather than following resistance exercise. To further enhance muscle hypertrophy and strength, a
resistance weight-training program of at least 10-12 weeks with compound movements for both upper and lower body exercises should be followed [12407].

The pattern of protein intake following exercise may impact whole-body protein turnover and net protein retention. It was determined the effects of different protein feeding strategies on protein metabolism in resistance-trained young men. Participants were randomly assigned to ingest either 80 g of whey protein as 8x10 g every 1.5h (PULSE; n=8), 4x20 g every 3 h (intermediate, INT; n=7), or 2x40g every 6h (BOLUS; n=8) after an acute bout of bilateral knee extension exercise (4x10 repetitions at 80 % maximal strength). Whole-body protein turnover (Q), synthesis (S), breakdown (B), and net balance (NB) were measured throughout 12h of recovery by a bolus ingestion of [15N]glycine with urinary [15N]ammonia enrichment as the collected end-product. PULSE Q rates were greater than BOLUS (19 %) with a trend towards being greater than INT (9 %). Rates of S were 32 and 19 percent greater and rates of B were 51 percent and 57 percent greater for PULSE as compared to INT and BOLUS, respectively, with no difference between INT and BOLUS. There were no statistical differences in NB between groups; however, magnitude-based inferential statistics revealed likely small and moderate increases in NB for PULSE and INT compared to BOLUS and possible small increase for INT versus PULSE. It was concluded that the pattern of ingested protein, and not only the total daily amount, can impact whole-body protein metabolism. Individuals aiming to maximize NB would likely benefit from repeated ingestion of moderate amounts of protein (20g) at regular intervals (3h) throughout the day [12408].

To prospectively evaluate the effects of resistance training combined with increased energy intake or protein-supplementation on lean body-mass, resting metabolic-rate (RMR) and cardiovascular risk factors 24 healthy males (aged 19-32 years) performed resistance exercise for 12 weeks aiming for at least 1 hour training-sessions 3 times a week. The participants were randomized to consume extra protein (33 g whey protein/day) or a meal of fast-food/day (1350 kcal, 41 g protein). Body-composition was measured with Dual-Energy X-ray Absorptiometry (DEXA) and RMR by indirect calorimetry. Fasting blood samples were drawn before and after the 3-month training period and after 12 months. The body weight increased from 75.1 ± 6.9 kg to 78.7 ± 7.2 kg, without differences between the groups. RMR increased from 1787 ± 143 kcal/24 h to 1954 ± 187 kcal/24 h (n=24), which was more than expected from the increase in lean body-mass (increase from 59.7 ± 4.3 kg to 61.8 ± 4.1 kg). Fasting serum-insulin levels increased in the fast-food group compared with the extra-protein group. ApoB increased from 0.691 ± 0.14 g/L to 0.768 ± 0.17 g/L. in the fast-food group only. Long-term follow up after 12 months showed that RMR, body weight, total fat and lean body-masses did not differ from baseline (n=19). It was concluded that resistance training for 12 weeks increased RMR and lean body-mass similarly when based on either an increased energy-intake or protein supplement. However, the increase in RMR was higher than expected from the increase in lean body-mass. Thus resistance training could potentially decrease the risk of obesity by induction of increased RMR [12409].

One study investigated the impact of protein coingestion with carbohydrate on muscle protein synthesis during endurance type exercise. Twelve healthy male cyclists were studied during 2 h of fasted rest followed by 2 h of continuous cycling at 55 percent W_max. During exercise, subjects received either 1.0 g/kg/h carbohydrate (CHO) or 0.8 g/kg/h carbohydrate with 0.2 g/kg/h protein hydrolysate (CHO+PRO). Continuous intravenous infusions with l-(ring-13C3)phenylalanine and l-(ring-3H2)tyrosine were applied, and blood and muscle biopsies were collected to assess whole body protein turnover and muscle protein synthesis rates at rest and during exercise conditions. Protein coingestion significantly stimulated whole body protein synthesis and oxidation rates during exercise. Whole body protein breakdown rates did not differ between experiments. As a consequence, whole body net protein balance was slightly negative in CHO and positive in the CHO+PRO treatment, respectively. Mixed
muscle protein fractional synthetic rates were higher during exercise compared with resting conditions. Fractional synthetic rates during exercise did not differ between experiments. It was concluded that muscle protein synthesis is stimulated during continuous endurance type exercise activities when carbohydrate with or without protein is ingested. Protein coingestion does not further increase muscle protein synthesis rates during continuous endurance type exercise [11266].

Protein nutrition for athletes has long been a topic of interest. From the legendary Greek wrestler Milo – purported to eat copious amounts of beef during his five successive Olympic titles – to modern athletes consuming huge amounts of supplements, protein intake has been considered paramount. Recommendations for protein intake for athletes has not been without controversy, however. In general, scientific opinion on this controversy seems to divide itself into two camps—those who believe participation in exercise and sport increases the nutritional requirement for protein and those who believe protein requirements for athletes and exercising individuals are no different from the requirements for sedentary individuals. There seems to be evidence for both arguments. Although this issue may be scientifically relevant, from a practical perspective, the requirement for protein—as most often defined—may not be applicable to most athletes [07312].

The accretion of muscle protein as a result of resistance exercise occurs because of successive periods of positive muscle protein balance. Periods of positive protein balance are due to a synergistic interaction of an exercise and feeding-induced stimulation of muscle protein synthesis (MPS). Protein ingestion provides essential amino acids for protein synthesis, which also act, in the case of leucine, to stimulate the translational machinery. Protein ingestion also increases systemic insulin, which has a mild stimulatory effect on MPS. Rather than a dose-dependent stimulation, a minimal threshold of insulin is required to allow MPS to proceed unabated; however, further stimulation of MPS is not seen at higher doses [07309].

Consuming protein following exercise has been shown to stimulate protein synthesis acutely in skeletal muscle and has been recommended to prevent sarcopenia. It is not known, however, whether acute stimulation persists long term or includes muscle cell division. It was asked whether consuming protein following exercise during aerobic training increases long-term protein and DNA synthesis rates in skeletal muscle of adult humans. Sixteen previously untrained participants (50 ± 8 years) consumed either a carbohydrate or carbohydrate and protein drink following each session during 6 week of treadmill training. A younger untrained group provided a nonexercising comparison. Participants were administered heavy water (²H₂O; deuterium oxide) continuously for 6 weeks to isotopically label newly synthesized skeletal muscle proteins and DNA. Muscle biopsies were performed after 6 wk of training. Contrary to acute studies, consuming protein after exercise did not increase skeletal muscle protein synthesis rates. In contrast, muscle protein synthesis, DNA, and phospholipid synthesis were significantly higher in the older exercise groups than the younger sedentary group. The higher DNA replication rate could not be attributed to mitochondrial DNA and may be due to satellite cell activation. It was concluded that postexercise protein supplementation does not increase rates of mixed protein synthesis over 6 week and that aerobic exercise may stimulate long-term cell division (DNA synthesis) in skeletal muscle of humans. Measurements of long-term synthesis rates provide important insights into aging and exercise adaptations [11388].

Variability in protein consumption may influence muscle mass changes induced by resistance exercise training (RET). It was sought to administer a post-exercise protein supplement and determine if daily protein intake variability affected variability in muscle mass
gains. Men (n=22) and women (n=30) ranging in age from 60 to 69 y participated in a 12-week RET program. At each RET session, participants consumed a post-exercise drink (0.4 g/kg lean mass protein). RET resulted in significant increases in lean mass (1.1 ± 1.5 kg), similar between sexes. Variability in mean daily protein intake was not associated with change in lean mass. The group with the highest protein intake (1.35 g/kg/day, n=8) had similar changes in lean mass as the group with the lowest daily protein intake (0.72 g/kg/day, n=9). These data suggest that variability in total daily protein intake does not affect variability in lean mass gains with RET in the context of post-exercise protein supplementation [06260].

Protein supplement use is common among athletes, active adults, and military personnel. This review provides a summary of the evidence base that either supports or refutes the ergogenic effects associated with different mechanisms that have been proposed to support protein supplementation. It was clear that if carbohydrate delivery was optimal either during or after an acute bout of exercise that additional protein will not increase exercise capacity. Evidence was also weak to substantiate use of protein supplements to slow the increase in brain serotonin and onset of central fatigue. It was also evident that additional research is warranted to test whether the benefits of protein supplements for enhancing recovery of fluid balance after exercise will affect subsequent work in the heat. In contrast, with repeated exercise, use of protein supplementation was associated with reductions in muscle soreness and often a faster recovery of muscle function due to reductions in protein degradation. There was also good supportive evidence for long-term benefits of protein supplementation for gains in muscle mass and strength through accelerated rates of protein synthesis, as long as the training stimulus was of sufficient intensity, frequency, and duration. However, studies have not examined the impact of protein supplements under the combined stress of a military environment that includes repeated bouts of exercise with little opportunity for feeding and recovery, lack of sleep, and exposure to extreme environments. Both additional laboratory and field research is warranted to help provide evidence-based guidance for the choice of protein supplements to enhance soldier performance [13643].

**Peroxiredoxins (PRDXs)**

Peroxiredoxins (PRDXs) are multifunctional proteins that have recently received much attention. They are part of the endogenous antioxidative capacity and function as efficient scavengers, especially for hydrogen peroxides. Studies show that physical training can induce an upregulation of PRDX isoform contents in the long term. This might help counteract chronic diseases that are causally linked to a high amount of free radicals, e.g., diabetes mellitus. Furthermore, it has been demonstrated that PRDX can overoxidize under pathological conditions during acute exercise. Overoxidized PRDXs could be useful because they act as protective chaperones. Taken together, it can be speculated that physical activity has a positive effect on the PRDX system and thereby prevents cells from free radical-induced damage [13642].

**Protein turnover**

Skeletal muscle is the major deposit of protein molecules. As for any cell or tissue, total muscle protein reflects a dynamic turnover between net protein synthesis and degradation. Noninvasive and invasive techniques have been applied to determine amino acid catabolism and muscle protein building at rest, during exercise and during the recovery period after a single experiment or training sessions. Stable isotopic tracers (13C-lysine, 15N-glycine, ²H5-phenylalanine) and arteriovenous differences have been used in studies of skeletal muscle and collagen tissues under resting and exercise conditions. There are different fractional synthesis rates in skeletal muscle and tendon tissues, but there is no major difference between collagen and myofibrillar protein synthesis. Strenuous exercise provokes increased proteolysis and decreased protein synthesis, the opposite occurring during the recovery period. Individuals who exercise respond differently when resistance and endurance types of
contractions are compared. Endurance exercise induces a greater oxidative capacity (enzymes) compared to resistance exercise, which induces fiber hypertrophy (myofibrils). Nitrogen balance (difference between protein intake and protein degradation) for athletes is usually balanced when the intake of protein reaches 1.2 g/kg/day compared to 0.8 g/kg/day in resting individuals. Muscular activities promote a cascade of signals leading to the stimulation of eukaryotic initiation of myofibrillar protein synthesis. As suggested in several publications, a bolus of 15-20 g protein (from skimmed milk or whey proteins) and carbohydrate (± 30 g maltodextrine) drinks is needed immediately after stopping exercise to stimulate muscle protein and tendon collagen turnover within 1 h [12411].

**Recommended daily allowance in athletes**

There is likely no other dietary component that inspires as much debate, insofar as athletes are concerned, as protein. How much dietary protein is required, optimal, or excessive? Dietary guidelines from a variety of sources have settled on an adequate dietary protein intake for those over the age of 19 of about 0.8-0.9 g protein/kg body weight and day. According to US and Canadian dietary reference intakes, the recommended allowance for protein of 0.8 g protein/kg and day is "the average daily intake level that is sufficient to meet the nutrient requirement of nearly all [i.e. 98 %] . . . healthy individuals". The panel also stated, "in view of the lack of compelling evidence to the contrary, no additional dietary protein is suggested for healthy adults undertaking resistance or endurance exercise". Currently, no group or groups of scientists involved in establishing dietary guidelines see a need for any statement that athletes or people engaging in regular physical activity require more protein than their sedentary counterparts. Popular magazines, numerous Web sites, trainers, and many athletes decry protein intakes even close to those recommended. Even joint position stands from policy-setting groups state that "protein recommendations for endurance athletes are 1.2 to 1.4 g/kg body weight per day, whereas those for resistance and strength-trained athletes may be as high as 1.6 to 1.7 g/kg body weight per day". The divide between those setting dietary protein requirements and those who might be making practical recommendations for athletes appears substantial, but ultimately, most athletes indicate that they consume protein at levels beyond even the highest recommendations. Thus, one might conclude that any debate on protein "requirements" for athletes is inconsequential; however, a critical analysis of existing and new data reveals novel ideas and concepts that may represent some common ground between these apparently conflicted groups. The goal of one review was to provide a critical and thorough analysis of current data on protein requirements in an attempt to provide some guidance to athletes, trainers, coaches, and sport dietitians on athletes' protein intake. In addition, an effort was made to clearly distinguish between "required" dietary protein, "optimal" intakes, and intakes that are likely "excessive," perhaps not from the standpoint of health, but certainly from the standpoint of potentially compromised performance [07313].

**High protein feeding**

The effect of dietary protein ingested following exercise on recovery in woman athletes is unknown. Therefore, it was asked whether there is a meaningful difference between high-versus low-protein recovery diets on subsequent performance in well-trained female cyclists. In a cross-over, 12 female cyclists completed 3 high-intensity rides comprising 2.5 h of intervals on day 1, followed by repeat-sprint performance tests on days 2 and 4, interspersed with a rest day. During the 4-h recovery on days 1 and 2, cyclists ingested 1.4/0.7/0.26 or 2.1/0.1/0.26 g.kg.h carbohydrate/protein/fat in high-protein or isocaloric control conditions, respectively. At other times, cyclists ingested an isoenergetic high-carbohydrate diet. No effect of protein dose on repeat-sprint mean power was evident on day 2 or day 4;
furthermore, fatigue effects (slope) were unclear. Perception of leg tiredness and soreness were increased, and leg strength reduced in the high-protein condition relative to control. In the high-protein condition, plasma-glucose concentrations were lower during recovery, and plasma-lactate concentrations were lower during the sprints. Effects on circulating creatine-kinase activity were trivial. Net nitrogen balance over the experiment was positive in the high-protein condition but negative in control, estimated protein requirement was 1.28 ± 0.57 g/kg/d. It was concluded that in contrast with previous findings in males, it was observed no clear influence of dietary protein quantity on subsequent performance in females. The nitrogen balance findings suggest that female cyclists training intensely have daily protein requirements approximately 1.6 times the recommended daily allowance, but 0.65 times that of males [10222].

**Egg white protein**

The aim of one study was to evaluate the effects of egg white protein compared to carbohydrate intake prior to exercise on fat free mass (FFM), one repetition maximum (1RM) muscle strength and blood biochemistry in female athletes. Thirty healthy female collegiate athletes were recruited for this study and matched by sport type, body fat percentage and 1RM leg curl muscle strength. Participants were randomly divided into two groups: protein group (15.0 g egg white protein; 75 kcal) and carbohydrate group (17.5 g maltodextrin, 78 kcal). Supplements were administered daily at the same time in a double-blind manner prior to training during an 8-week period. Measurements were performed before and after the 8-week regimen. The mean dietary energy intake did not change throughout the study period. FFM and 1RM assessments (i.e. leg curl, leg extension, squat, and bench press) increased in both groups. Furthermore, serum urea and serum citrulline levels after the 8-week regimen increased significantly only in the protein group. Our findings indicated that compared to the carbohydrate supplement, the protein supplement was associated with some changes in protein metabolites but not with changes in body composition or muscle strength. Fifteen grams of egg white protein contain 1341 mg of leucine (Leu), 837 mg of isoleucine (Ile), and 1096 mg of valine (Val), and there is also an abundant source of branched amino acids (BCAA) and aromatic amino acids (AAA) [12410].

**Whey**

Previously, we have shown that consuming carbohydrate plus whey protein hydrolysates (WPHs) replenished muscle glycogen after exercise more effectively than consuming intact whey protein or branched-chain amino acids (BCAAs). The mechanism leading to superior glycogen replenishment after consuming WPH is unclear. In this 5 week intervention, ddY mice were fed experimental diets containing WPH, a mixture of whey amino acids (WAAs), or casein (control). After the intervention, gastrocnemius muscle glycogen levels were significantly higher in the WPH group (4.35 mg/g) than in the WAA (3.15 mg/g) or control (2.51 mg/g) groups. In addition, total glycogen synthase (GS) protein levels were significantly higher in the WPH group (153 %) than in the WAA (89 %) or control groups, and phosphorylated GS levels were significantly decreased in the WPH group (51 %). These results indicate that dietary WPH may increase the muscle glycogen content through increased GS activity [12413].

To determine the effects of whey protein before and during resistance exercise (RE) on body composition and strength in young adults participants were randomized to ingest whey protein (PRO; 0.3 g/kg protein; n=9, 25 years, 88 kg) or placebo (PLA; 0.2 g/kg cornstarch maltodextrin + 0.1 g/kg sucrose; n=8) during RE (3 sets of 6-10 repetitions for 9 whole-body exercises), which was performed 4 d/week for 8 weeks. PRO and PLA were mixed with
water (600 ml); 50 percent of the solution containing 0.15 g/kg of PRO or PLA was consumed immediately before the start of exercise, and about 2 percent of the remaining solution containing 0.006 g/kg of PRO or PLA was consumed immediately after each training set. Before and after the study, measures were taken for lean-tissue mass (dual-energy X-ray absorptiometry), muscle size of the elbow and knee flexors and extensors and ankle dorsiflexors and plantar flexors (ultrasound), and muscle strength (1-repetition-maximum chest press). There was a significant increase in muscle size of the knee extensors, knee flexors, and ankle plantar flexors and chest-press strength over time, with no differences between groups. It was concluded that the ingestion of whey protein immediately before the start of exercise and again after each training set has no effect on muscle mass and strength in untrained young adults [12412].

Different dietary proteins affect whole body protein anabolism and accretion and therefore, have the potential to influence results obtained from resistance training. This study examined the effects of supplementation with two proteins, hydrolyzed whey isolate and casein (C), on strength, body composition, and plasma glutamine levels during a 10 wk, supervised resistance training program. In a double-blind protocol, 13 male, recreational bodybuilders supplemented their normal diet with either whey or C (1.5 gm/kg body wt/d) for the duration of the program. Strength was assessed by 1-RM in three exercises (barbell bench press, squat, and cable pull-down). Body composition was assessed by dual energy X-ray absorptiometry. Plasma glutamine levels were determined by the enzymatic method with spectrophotometric detection. All assessments occurred in the week before and the week following 10 wk of training. Plasma glutamine levels did not change in either supplement group following the intervention. The whey group achieved a significantly greater gain in lean mass than the C group and a significant change in fat mass compared to the C group. The whey group also achieved significantly greater improvements in strength compared to the C group in each assessment of strength. When the strength changes were expressed relative to body weight, the whey group still achieved significantly greater improvements in strength compared to the C group [06263].

The purpose of one study was to examine the effects of whey protein supplementation on body composition, muscular strength, muscular endurance, and anaerobic capacity during 10 weeks of resistance training. Thirty-six resistance-trained males (31 years) followed a 4 days-per-week split body part resistance training program for 10 weeks. Three groups of supplements were randomly assigned, prior to the beginning of the exercise program, in a double-blind manner to all subjects: 48 g per day (g/day carbohydrate placebo (P), 40 g/day of whey protein + 8 g/day of casein (WC), or 40 g/day of whey protein + 3 g/day branched-chain amino acids + 5 g/day L-glutamine (WBG)). At 0, 5, and 10 weeks, subjects were tested for fasting blood samples, body mass, body composition using dual-energy x-ray absorptiometry (DEXA), 1 repetition maximum (1RM) bench and leg press, 80 percent 1RM maximal repetitions to fatigue for bench press and leg press, and 30-second Wingate anaerobic capacity tests. No changes were noted in all groups for energy intake, training volume, blood parameters, and anaerobic capacity. WC experienced the greatest increases in DEXA lean mass and DEXA fat-free mass. Significant increases in 1RM bench press and leg press were observed in all groups after 10 weeks. In the study, the combination of whey and casein protein promoted the greatest increases in fat-free mass after 10 weeks of heavy resistance training. Athletes, coaches, and nutritionists can use these findings to increase fat-free mass and to improve body composition during resistance training [06264].

Whey protein is a widely consumed dietary supplement purported to enhance weight loss or support gains in skeletal muscle mass after a programme of resistance exercise training. Whey is the liquid portion of coagulated milk and represents about 20 percent of the total protein content of milk. It is a high-quality protein source that contains all the building blocks...
(amino acids) for the synthesis of new skeletal muscle proteins. An innovative study, using intrinsically labelled protein sources, demonstrated that whey is rapidly digested and absorbed, and resulted in a greater postprandial muscle protein accretion as compared to other dietary protein sources (casein or hydrolysed casein). The greater anabolic potential of whey is probably attributable to its faster protein digestion and amino acid absorption rates and a higher leucine content; this ultimately results in a large and transient spike in plasma leucine concentrations. The latter appears to be fundamental in maximising the feeding-mediated stimulation of muscle protein synthesis rates during resistance exercise recovery. However, further work is required to establish firmly whether the leucine content of a meal is of equal value in the postexercise phase, as it appears to be in the postprandial phase in absence of exercise. Some evidence suggests that ingestion of whey promotes greater satiety when compared against casein. It was speculated that ingestion of whey protein prior to meals may be a helpful in weight management strategy. In recent work, however, demonstrated that whey protein supplementation during a 9-month exercise training programme does not further enhance strength or changes in body composition in the middle-aged overweight and obese adults. Overall, these data showed that increasing habitual physical activity is a simple and effective lifestyle strategy to promote healthy aging. Whey protein supplements (or proteins in food) appear to be safe to consume with no direct evidence to suggest that excessive protein intake, as commonly seen in athletes, is harmful to healthy kidneys. Moreover, whey protein ingestion appears to be a highly effective protein source for the stimulation of postprandial muscle protein synthesis rates [13694].

It is well known that ingestion of a protein source is effective in stimulating muscle protein synthesis after exercise. In addition, there are numerous reports on the impact of leucine and leucine-rich whey protein on muscle protein synthesis and mammalian target of rapamycin (mTOR) signalling. However, there is only limited information on the effects of whey protein hydrolysates (WPH) on muscle protein synthesis and mTOR signalling. The aim of one study was to compare the effects of WPH and amino acids on muscle protein synthesis and the initiation of translation in skeletal muscle during the post-exercise phase. Male Sprague-Dawley rats swam for 2 h to depress muscle protein synthesis. Immediately after exercise, the animals were administered either carbohydrate (CHO), CHO plus an amino acid mixture (AA) or CHO plus WPH. At 1 h after exercise, the supplements containing whey-based protein (AA and WPH) caused a significant increase in the fractional rate of protein synthesis (FSR) compared with CHO. WPH also caused a significant increase in FSR compared with AA. Post-exercise ingestion of WPH caused a significant increase in the phosphorylation of mTOR levels compared with AA or CHO. In addition, WPH caused greater phosphorylation of ribosomal protein S6 kinase and eukaryotic initiation factor 4E-binding protein 1 than AA and CHO. In contrast, there was no difference in plasma amino acid levels following supplementation with either AA or WPH. These results indicate that WPH may include active components that are superior to amino acids for stimulating muscle protein synthesis and initiating translation [13695].

The purpose of one study was to investigate the effects of a controlled typical one day diet supplemented with two different doses of whey protein isolate on blood amino acid profiles and hormonal concentrations following the final meal. Nine males (age: 30 ± 6 years) completed four conditions in random order: a control (C) condition of a typical mixed diet containing about 10 percent protein (0.8 g/kg), 65 percent carbohydrate and 25 percent fat; a placebo (P) condition calorically matched with carbohydrate to the whey protein conditions; a low dose condition of 0.8 grams of whey protein isolate per kilogram body mass per day (g/kg/d; W1) in addition to the typical mixed diet; or a high dose condition of 1.6 g/kg/d (W2) of supplemental whey protein in addition to the typical mixed diet. Following the final meal, significant increases in total amino acids, essential amino acids (EAA), branch-chained amino acids (BCAA), and leucine were observed in plasma with whey protein
supplementation while no changes were observed in the control and placebo conditions. There was no significant group difference for glucose, insulin, testosterone, cortisol, or growth hormone. In conclusion, supplementing a typical daily food intake consisting of 0.8 g of protein/kg/d with a whey protein isolate (an additional 0.8 or 1.6 g/kg/d) significantly elevated total amino acids, EAA, BCAA, and leucine but had no effect on glucose, insulin, testosterone, cortisol, or growth hormone following the final meal. Future acute and chronic supplementation research examining the physiological and health outcomes associated with elevated amino acid profiles is warranted [13696].

The purpose of this study was to examine the effects of 2 different types of protein supplementation on thigh muscle cross-sectional area (CSA), blood markers, muscular strength, endurance, and body composition after 8 weeks of low- or moderate-volume resistance training in healthy, recreationally trained, college-aged men. One hundred and six men were randomized into 5 groups: low-volume resistance training with bioenhanced whey protein (BWPLV; n=22), moderate-volume resistance training with BWP (BWPMV; n=20), moderate-volume resistance training with standard whey protein (SWPMV; n=22), moderate-volume resistance training with a placebo (PLA; n=21), or moderate-volume resistance training with no supplementation (CON; n=21). Except for CON, all groups consumed 1 shake before and after each exercise session and one each on the nontraining day. The BWPLV, BWPMV, and SWPMV groups received approximately 20 g of whey protein per shake, whereas the BWP groups received 5 g of additional polyethylene glycosylated (PEG) leucine. Resistance training sessions were performed 3 times per week for 8 weeks. There were no interactions for muscle strength and endurance variables, body composition, muscle CSA, and safety blood markers, but the main effects for training were observed. However, the Albumin:Globulin ratio for SWPMV was lower than BWPLV and BWPMV. Relative protein intake (PROREL) indicated a significant interaction with no differences across groups at pre; however, BWPLV, BWPMV, and SWPMV had a greater intake than did PLA or CON at post. This study indicated that 8 weeks of resistance training improved muscle performance and size similarly among groups regardless of supplementation [13697].

Wheat germ

The wheat germ (Triticum vulgare, Gramineae), a by-product of the flour-milling industry, represents about 2.5-3.8 percent of the total seed weight. Wheat germ and the oil extracted from wheat germ contain significant quantities of bioactive compounds and, in particular, are known to be the richest plant origin source of tocopherols (vitamin E), with the antioxidant activity of tocopherols being well-documented. Wheat germ contains mainly alpha-tocopherols and beta-tocopherols, alongside other bioactive compounds such as phytosterols, polycosanols (POC), carotenoids, particularly lutein and zeaxanthin, thiamine (vitamin B₁) and riboflavin (vitamin B₂). Wheat germ is also a source of α-linolenic-rich polyunsaturated fatty acids, and contains several minerals, principally potassium, magnesium, calcium, zinc and manganese. Wheat germ contains about 10-15 percent lipids (oil), with oil extraction being primarily achieved by mechanical pressing or solvent extraction, which retrieves about 50 percent or >90 percent of the total lipids, respectively. Crude wheat germ oil (WGO) is usually dark coloured and may have a strong odour and flavour depending on the oxidative conditions of the oil. To produce high-quality, stable oils, undesirable compounds must be eliminated while retaining as much of tocopherols and other key nutritional compounds as possible. WGO is widely utilised for vitamin production (e.g. alpha-tocopherol) in medication and in the cosmetic industry, as well as in food, animal feed and as a biological insect control agent. WGO has been purported to improve human physical endurance/fitness, an effect attributed to its high POC, specifically its high octacosanol content, alongside other marketed health benefits such as reducing plasma and liver
cholesterol levels and possibly helping to delay the effects of aging. Despite these claims, studies investigating WGO supplementation effects in humans, on exercise performance and health, are sparse. A study investigating the effects of phytosterols from wheat germ on cholesterol absorption in humans found that consuming a meal containing 80 g of original wheat germ containing 328 mg phytosterols resulted in less cholesterol absorption when compared to ingestion of phytosterol-free wheat germ. This suggests that phytosterols found in wheat germ may have an important role in cholesterol metabolism. Another study recruited 32 patients with hypercholesterolaemia to examine the effects of 2 months of daily WGO supplementation or a placebo (maize oil) on oxidative stress. Although no differences in serum lipid profile was observed in both the groups, oxidative stress and platelet CD40L, a protein with inflammatory and prothrombotic properties, were reduced. This suggests that WGO supplementation had positive anti-inflammatory effects in patients with hypercholesterolaemia. Despite the lack of supporting evidence regarding a role for WGO in sports performance, it does provide an excellent source of vitamin E, with the high fat content of the wheat germ enabling its transport. The recommended dietary intake for vitamin E of 15 mg/day could be reached through consumption of 100 g of wheat germ – that's a pretty large handful! A more practical alternative source would therefore be WGO, being more concentrated in vitamin E than raw wheat germ [13694].

Soy-protein

The aim of one study was to determine in what areas the therapeutic application of soy predominates in clinical trials and to assess the emerging fields of its use by means of an analysis of bibliographic resources. A search was performed in the MEDLINE database up to 31 december 2004, limited to the Title/Abstract field, and Clinical Trials as the type of publication. The abstracts from the publications selected (n=86) were reviewed and different variables were assessed. A total of 3280 subjects were included: 15 percent men and 59 percent women (71 % postmenopausal). The studies were performed basically in healthy individuals (71 %). Twenty five percent of the studies investigated plasma levels of different metabolites and 21% determined hormone or lipid profiles. After the year 2000 a new population focus was detected, with the publication of two studies in elite gymnasts and judoists, with positive results. The present observations indicate that soy supplementation in the competitive sports elite may be an emerging application [06262].

To determine the changes in endurance capacity as well as in metabolic, hormonal and inflammatory markers induced by endurance training combined with a soy-been protein based supplement a randomized controlled study consisting of moderate endurance training without (GO) or with (G1) a soy protein based supplement. Two groups of 15 subjects (10 males and 5 females in each group): healthy sports students aged 24 years. Body composition (body mass (BM), body density (BD) by air displacement) and physical fitness (determined by treadmill ergometry) were measured at baseline and after 6 weeks of the intervention; changes in circulating metabolic and hormonal parameters (glucose, lactate, urea, uric acid, ammonia, cortisol, insulin, IGF-1), and exercise-induced stress and inflammatory markers (CK, LDH, myoglobin, hs-CRP, IL-6, IL-10, blood cell counts) were determined after the intervention period in afield test (12 km running on hilly ground). Thirty participants completed the 6-week study; 28 students were able to perform the field test. No significant changes in BM and BD were noted after intervention with only slight increases in running performance and maximum aerobic capacity in the total group (2 %). Subjects in the G1 group showed significant improvements in running velocity and lower lactate values following the intervention (-12 %). In addition, the G1 group showed significantly lower differences in the exercise-induced increase of metabolic parameters (triglycerides, uric acid) and insulin in the post-exercise recovery period. The data suggest that moderate endurance
training in combination with a soy-based protein supplement improves aerobic energy supply and metabolic function in healthy sports students, even without changes in body composition and without changes in the exercise-induced stress and inflammatory reaction [12406].

**Combination of sago and soy-protein**

The purpose of one study was to investigate whether a combination of sago and soy protein ingested during moderate-intensity cycling exercise can improve subsequent high-intensity endurance capacity compared with a carbohydrate in the form of sago and with a placebo. The participants were 8 male recreational cyclists. The design of the study was a randomized, double-blind placebo-controlled crossover comprising 60 min of exercise on a cycle ergometer at 60 percent VO$_{2\text{max}}$ followed by a time-to-exhaustion ride at 90 percent VO$_{2\text{max}}$. The sago feeding provided 60 g of carbohydrate, and the sago-soy combination provided 53 g of carbohydrate and 15 g of protein, both at 20-min intervals during exercise. Times to exhaustion for the placebo, sago, and sago-soy supplementations were 4.1 ± 1.3, 5.5 ± 1.2, and 7.5 ± 2.0 min, respectively. Sago-soy supplementation increased endurance by 84 percent and by 37 percent relative to placebo and sago, respectively. The plasma insulin response was elevated above that with placebo during sago and sago-soy supplementations. The authors conclude that a combination of sago and soy protein can delay fatigue during high-intensity cycling [10249].

**Wheat gluten hydrolysate**

Wheat gluten hydrolysate (WGH) is reported to suppress the muscle injuries associated with exercise in long distance running and weight training. In one study, it was investigated the effects of WGH consumption on suppression of muscle injury after soccer training in a double-blind crossover study. Immediately after a mini soccer game, six soccer players consumed 18.0 g of WGH, and muscle injury was investigated using serum creatine kinase (CK) as an indicator. The results showed a significant increase in serum CK from immediately after exercise to 12 h after exercise stress in the placebo group, while serum CK decreased during this same time period in the WGH group, and the difference between the two groups was significant. This suggests that WGH consumption suppresses delayed-onset muscle injury after exercise in soccer [12433].

**Building muscles in fed and fasted state**

The purpose of one investigation was to assess mixed-muscle fractional synthesis rate (FSR) and the expression of genes involved in skeletal muscle remodeling after aerobic exercise in the fasted and fed states. Eight recreationally active males (25 ± 1 yr; VO$_{2\text{max}}$: 52 ± 2 ml/kg and min) performed 60-min of cycle ergometry at 72 ± 1 percent VO$_{2\text{max}}$ on two occasions in a counter-balanced design. Subjects ingested a noncaloric placebo (EX-FAST) or a beverage containing (per kg body wt): 5 kcal, 0.83 g carbohydrate, 0.37 g protein, and 0.03 g fat (EX-FED) immediately and 1 h after exercise. FSR was assessed at rest and following exercise with the use of a l-[ring (2)H(5)]-phenylalanine infusion combined with muscle biopsies at 2 and 6 h postexercise. mRNA expression was assessed at 2 and 6 h postexercise via real-time RT-PCR. FSR was significantly higher after exercise in both EX-FAST (0.112 ± 0.010 % per hour) and EX-FED (0.129 ± 0.014 % per hour) compared with rest (0.071 ± 0.005 % per hour). Feeding attenuated the mRNA expression of proteolytic factors MuRF-1 (6 h) and calpain-2 (2 and 6 h) postexercise but did not alter FOXO3A, calpain-1, caspase3, or myostatin mRNA expression compared with EX-FAST. Myogenic regulatory factor (MRF4) mRNA was also significantly attenuated at 2 and 6 h postexercise in EX-FED compared with EX-FAST. These data demonstrate that a nonexhaustive bout of
aerobic exercise stimulates skeletal muscle FSR in the fasted state and that feeding does not measurably enhance FSR between 2 and 6 h after aerobic exercise. Additionally, postexercise nutrient intake attenuates the expression of factors involved in the ubiquitin-proteosome and Ca^{2+}-dependent protein degradation pathways. These data provide insight into the role of feeding on muscle protein metabolism during recovery from aerobic exercise [10516].

Research measuring whole-body protein turnover (WBPT) after both exercise and nutrition has generally focused on resistance exercise; however, there is a paucity of data regarding the effect of postaerobic exercise nutrition, especially in older adults. It is not known if postexercise protein feeding has a beneficial effect on protein turnover after low- to moderate-intensity exercise. We investigated whether consuming protein plus carbohydrate (PRO) immediately after an acute bout of aerobic exercise has an additive effect over carbohydrate alone (CHO) on WBPT in older individuals. Twelve healthy older adults (age, 59 ± 4 years) were studied on 2 separate occasions after 1 h of exercise at approximately 50% of maximal rate of oxygen uptake, followed by 4 h of recovery. Immediately following exercise, subjects ingested a CHO (60 g) or an isocaloric PRO beverage (40 g carbohydrate, 20 g whey protein). Whole-body protein metabolism was determined using [1-^{13}C]leucine infusion (60 mg prime; 75 mg/h continuous), and sampling blood and expired breath. Rates of whole-body leucine appearance and oxidation, and nonoxidative leucine disposal during the third and fourth hours of postexercise recovery were higher in the PRO group, than in the CHO group. The results indicate that consumption of a PRO beverage after aerobic exercise increased WBPT to a greater extent than a CHO beverage [10517].

Protein drinks

Post-workout nutrient timing and macronutrient selection are essential for recovery, glycogen replenishment and muscle protein synthesis (MPS). Performance repeatability, particularly after strenuous activity, can be influenced by substrate availability, recovery markers and perceived rate of exertion. One study compared the differential effects of a complex protein ready-to-drink drink (VPX) and isocaloric carbohydrate drink (iCHO) on performance – agility T-test, push-up test, 40-yard sprint, and rate of perceived exertion (RPE), following high-intensity resistance training (HIRT). In a randomized, double blind two-arm crossover controlled trial, 15 subjects performed a 15-18 minute (2:1 work to rest) HIRT and then immediately drank one of the two treatments. After a 2-hour fast, subjects returned to execute the field tests and report RPE. The protocol was repeated one week later with the other treatment. There were no significant main effect differences in the agility T-test, push-up, sprint, average agility RPE, average push-up RPE or average sprint RPE between the two trials and the two treatments. The multivariate analysis yielded a cumulative significant interaction effect amongst the three performance variables after consuming VPX. These results suggest a complex protein drink is a better post-workout choice compared to an isocaloric carbohydrate drink for repeated performance for activities that require multiple energy demands and athletic skills; however, this outcome was not observed for each single performance event or RPE. Thus, when considering the collective physical effects of the agility T-test, push-up and sprint tests, a complex protein drink may provide a recovery advantage as it relates to repeated-bout performance compared to an iCHO-only drink. Additional research examining the chronic effects of post-exercise protein versus iCHO drinks on performance repeatability, particularly in special populations (e.g. tactical and elite athletes), is warranted to further develop these findings [13648].
Effect on endurance and muscles

Dietary CHO intake is fundamental to an athlete’s nutritional program to provide fuel to the working muscle and to facilitate muscle glycogen synthesis between exercise sessions. As a consequence of this, a wide range of CHO-based sports supplements are commercially available. During the last 20 years, evidence has been emerging that the addition of protein to CHO-based supplements may be beneficial; however, this remains a controversial topic. This challenging view will present the evidence that demonstrates the favorable metabolic consequences of consuming protein along with CHO. The majority of studies in this area have focused on CHO-protein intake either during exercise or in recovery from exercise, but of course, nutritional strategies during recovery also become pre-exercise nutritional strategies in many instances. Before any such strategy can be truly advocated, published evidence should first reveal consistently positive effects on practically meaningful outcomes (i.e. performance) across multiple independent laboratories, ideally also isolating plausible mechanisms of action. With due consideration of the precise context and nature of these effects, it is proposed that a “worthwhile” nutritional supplement must fulfill at least one of the following interrelated criteria:

- true “supplementation” of the diet (i.e. either compensating for deficiencies or meeting increased requirements)
- supplement formulation facilitates digestion, absorption, and/or metabolism to more effectively deliver active ingredients than possible via whole foods
- optimal timing for supplement ingestion renders it impractical to acquire active ingredients via whole foods as a meal (e.g. effects are either short-lived or situation dependent).

A relevant example to illustrate these contextual issues is CHO-based sports supplements per se (i.e. without added protein), the use of which is supported in a recent joint position statement both because of the increased CHO requirements of many athletes beyond the typical diet and the efficacy of specially formulated CHO supplements, which are easily incorporated into athletes' preexercise, midexercise, or postexercise regimen. In contrast, that same position statement attests that even the slightly elevated protein requirements of athletes are almost always met by diet alone and that “protein or amino acid supplementation has not been shown to positively impact athletic performance”. The perspective set out below will therefore defend the prevailing view that CHO without protein should be included in sports supplements; initially by presenting the balance of published data that have yet to reveal any ergogenic or mechanistic benefit of added protein or amino acids during exercise, before questioning whether the positive effects evident in recovery can legitimately be interpreted as supporting any practical value of these supplements when considered in a real-world context (e.g. in the fed state and/or when following existing guidelines for CHO intake) [11389].

One study examined 10 weeks of resistance training and the ingestion of supplemental protein and amino acids on muscle performance and markers of muscle anabolism. Nineteen untrained males were randomly assigned to supplement groups containing either 20 g protein (14 g whey and casein protein, 6 g free amino acids) or 20 g dextrose placebo ingested 1 h before and after exercise for a total of 40 g/d. Participants exercised 4 times/wk using 3 sets of 6-8 repetitions at 85-90 percent of the one repetition maximum. The protein supplement resulted in greater increases in total body mass, fat-free mass, thigh mass, muscle strength, serum IGF-1, IGF-1 mRNA, MHC I and IIa expression, and myofibrillar protein. Ten weeks of resistance training with 20 g protein and amino acids ingested 1 h before and after exercise is more effective than carbohydrate placebo in up-regulating markers of muscle protein synthesis and anabolism along with subsequent improvements in
muscle performance [07314].

One investigation examined the effect of variations in protein intake on whole-body protein turnover (WBPTO) after exercise in endurance-trained males. Five male runners (21 years) participated in a randomized, crossover-design diet intervention, where they consumed either a low- (0.8 g/kg; LP), moderate- (1.8 g/kg; MP), or high-protein (3.6 g/kg; HP) diet for 4 weeks. WBPTO (Ra, leucine rate of appearance; NOLD, nonoxidative leucine disposal; and Ox, leucine oxidation) were assessed after a 75-min run at 70 percent VO\textsubscript{2peak} after each diet-intervention period. Leucine Ra (indicator of protein breakdown) and leucine Ox were greater on the HP diet than on the LP diet. No differences were noted in NOLD (an indicator of protein synthesis) across diets. Plasma branched chain amino acids (BCAA) at rest were greater for MP and HP than for LP, and nonessential amino acids (NEA) were greater for LP than MP at rest and greater than MP and HP after exercise. Findings from this study show that variations in protein intake can alter plasma amino acid levels and modulate rates of WBPTO after exercise. Additionally, a lower protein intake was associated with decreased rates of WBPTO after exercise [07315].

One study compared a training diet recommended for endurance athletes (H-CHO) with an isoenergetic high protein (whey supplemented), moderate carbohydrate (H-Pro) diet on endurance cycling performance. Over two separate 7-d periods subjects (n=7) ingested either H-CHO (8 g/kg/day carbohydrate; 1.2 g/kg/day fat; 1.3 g/kg/day protein) or H-Pro (5 g/kg/day, 1.3 g/kg/day, or 3.3 g/kg/day, respectively) diet in a randomized, balanced order. On day 8 subjects cycled (self-paced) for a body weight dependent (60 kJ/bm) amount of work. No differences occurred between energy intake or fat intake during the two dietary conditions. Performance was significantly impaired following H-Pro compared with H-CHO. No differences between treatments were observed for physiological measures taken during the performance trials. These results indicate an ergolytic effect of a 7-d high protein diet on self-paced endurance cycling performance [06261].

The effects of protein intake timing in relation to strength training

Protein timing is a popular dietary strategy designed to optimize the adaptive response to exercise. The strategy involves consuming protein in and around a training session in an effort to facilitate muscular repair and remodeling, and thereby enhance post-exercise strength- and hypertrophy-related adaptations. It is generally accepted that protein should be consumed just before and/or immediately following a training session to take maximum advantage of a limited anabolic window. Proponents of the strategy claim that, when properly executed, precise intake of protein in the peri-workout period can augment increases in fat-free mass. Some researchers have even put forth the notion that the timing of food intake may have a greater positive effect on body composition than absolute daily nutrient consumption. A number of studies support the superiority of protein timing for stimulating increases in acute protein synthesis pursuant to resistance training when compared to placebo. Protein is deemed to be the critical nutrient required for optimizing post-exercise protein synthesis. The essential amino acids, in particular, are believed primarily responsible for enhancing this response, with little to no contribution seen from provision of non-essential amino acids. It was found that a 6 g dose of essential amino acids (EAAs) consumed immediately post-exercise produced an approximate twofold increase in net protein balance compared to a comparable dose containing an approximately equal mixture of essential and non-essential amino acids, indicating a dose–response relationship up to 6 g EAAs. However, increasing EAA intake beyond this amount has not been shown to significantly heighten post-exercise protein synthesis. There is limited evidence that carbohydrate has an additive effect on enhancing post-exercise muscle protein synthesis when combined with amino acid ingestion with a majority of studies failing to demonstrate any such benefit. The
The purpose of one paper therefore was to conduct a multi-level meta-regression of randomized controlled trials to determine whether protein timing is a viable strategy for enhancing post-exercise muscular adaptations. The strength analysis comprised 478 subjects and 96 ESs, nested within 41 treatment or control groups and 20 studies. The hypertrophy analysis comprised 525 subjects and 132 ESs, nested with 47 treatment or control groups and 23 studies. A simple pooled analysis of protein timing without controlling for covariates showed a small to moderate effect on muscle hypertrophy with no significant effect found on muscle strength. In the full meta-regression model controlling for all covariates, however, no significant differences were found between treatment and control for strength or hypertrophy. The reduced model was not significantly different from the full model for either strength or hypertrophy. With respect to hypertrophy, total protein intake was the strongest predictor of ES magnitude. These results refute the commonly held belief that the timing of protein intake in and around a training session is critical to muscular adaptations and indicate that consuming adequate protein in combination with resistance exercise is the key factor for maximizing muscle protein accretion. The Recommended Dietary Allowance (RDA) for protein is 0.8 g/kg/day. However, these values are based on the needs of sedentary individuals and are intended to represent a level of intake necessary to replace losses and hence avert deficiency; they do not reflect the requirements of hard training individuals seeking to increase lean mass. Studies do in fact show that those participating in intensive resistance training programs need significantly more protein to remain in a non-negative nitrogen balance. Position stands from multiple scientific bodies estimate these requirements to be approximately double that of the RDA. Higher levels of protein consumption appear to be particularly important during the early stages of intense resistance training. The average protein intake for controls in the unmatched studies was 1.33 g/kg/day while average intake for treatment was 1.66 g/kg/day. Since a preponderance of these studies involved untrained subjects, it seems probable that a majority of any gains in muscle mass would have been due to higher protein consumption by the treatment group. The findings also support previous recommendations that a protein consumption of at least 1.6 g/kg/day is necessary to maximize muscle protein accretion in individuals involved in resistance training programs. In conclusion, current evidence does not appear to support the claim that immediate (≤ 1 hour) consumption of protein pre- and/or post-workout significantly enhances strength- or hypertrophic-related adaptations to resistance exercise. The results of this meta-analysis indicate that if a peri-workout anabolic window of opportunity does in fact exist, the window for protein consumption would appear to be greater than one-hour before and after a resistance training session. Any positive effects noted in timing studies were found to be due to an increased protein intake rather than the temporal aspects of consumption, but a lack of matched studies makes it difficult to draw firm conclusions in this regard. The fact that protein consumption in non-supplemented subjects was below generally recommended intake for those involved in resistance training lends credence to this finding. Since causality cannot be directly drawn from our analysis, however, we must acknowledge the possibility that protein timing was in fact responsible for producing a positive effect and that the associated increase in protein intake is merely coincidental. Future research should seek to control for protein intake so that the true value regarding nutrient timing can be properly evaluated. Particular focus should be placed on carrying out these studies with well-trained subjects to better determine whether resistance training experience plays a role in the response [13644].

Plus maltodextrine
One study investigated the ingestion of maltodextrin, fructose, and protein on exogenous carbohydrate oxidation (CHOEXO) and exercise performance. Seven trained cyclists and (or) triathletes (maximal oxygen consumption, 59 ± 9 mL/kg/min) performed 3 exercise trials that consisted of 150 min of cycling at 50 percent maximal power output (160 ± 11 W), followed by a 60 km time trial. One of 3 beverages were randomly assigned during each trial and consumed at 15-min intervals: 0.84 g/min maltodextrin + 0.52 g/min fructose + 0.34
g/min protein (MD+F+P); 1.10 g/min maltodextrin + 0.60 g/min fructose (MD+F); or 1.70 g/min maltodextrin (MD). CHOEXO and fuel utilisation were assessed via measurement of expired air $^{13}$C content and indirect calorimetry, respectively. Mean total CHO oxidation (CHOTOT) rates were 2.35 ± 0.18, 2.76 ± 0.08, and 2.61 ± 0.17 g/min with MD, MD+F, and MD+F+P, respectively, although not significantly different. Peak CHOEXO rates with MD+F were significantly greater by 41 percent and 45 percent compared with MD+F+P and MD, respectively. Performance times were 2.2 percent and 5.0 percent faster with MD+F compared with MD+F+P and MD, respectively; however, they were not statistically significant. Ingestion of an MD-fructose-protein commercial sports beverage significantly reduced peak and mean CHOEXO rates compared with MD+F, but did not significantly influence CHOTOT. The addition of protein to an MD+F beverage did not enhance performance times [13645].

**Influence on muscle synthesis**

Hyperaminoacidemia stimulates myofibrillar fractional synthesis rate (myoFSR) transiently in resting skeletal muscle. It was investigated whether light-load resistance exercise can extent this responsiveness. Ten healthy males exercised one leg with a light-load resistance-like exercise at 16 percent of 1 repetition maximum and received oral protein boluses every hour for a 10-h period. Their myoFSR was determined by $[1^{-13}$C$]$-leucine incorporation. Muscle biopsies were obtained from the resting (REST) and exercised (EXC) muscles every 2.5-h in the protein-fed period. Protein feeding significantly elevated plasma leucine and essential amino acids by an average of 39 ± 9 percent (mean ± SEM) and 20 ± 4 percent, respectively, compared to the basal concentrations: 197 ± 12 micromol/L and 854 ± 35 micromol/L, respectively. The myoFSR was similar in EXC and REST muscles in the first 8 h. After 8 h the myoFSR dropped in the REST muscle to 0.041 ± 0.005 percent/h, which was 65 ± 5 percent of the rate in EXC leg at the same time point (0.062 ± 0.004 %/h) and 80 ± 14 percent of the level in REST leg from 0.5 to 8 h. Thus, compared to rest, light-load exercise prolonged the stimulatory effect of dietary protein on muscle biosynthesis providing perspectives for a muscle restorative effect in clinical settings where strenuous activity is intolerable [13646].

High-quality proteins such as soy, whey, and casein are all capable of promoting muscle protein synthesis postexercise by activating the mammalian target of rapamycin (mTORC1) signaling pathway. It was hypothesized that a protein blend of soy and dairy proteins would capitalize on the unique properties of each individual protein and allow for optimal delivery of amino acids to prolong the fractional synthetic rate (FSR) following resistance exercise (RE). In this double-blind, randomized, clinical trial, 19 young adults were studied before and after ingestion of about 19 g of protein blend (PB) or about 18 g whey protein (WP) consumed 1 h after high-intensity leg RE. It was examined mixed-muscle protein FSR by stable isotopic methods and mTORC1 signaling with western blotting. Muscle biopsies from the vastus lateralis were collected at rest (before RE) and at 3 postexercise time points during an early (0-2 h) and late (2-4 h) postingestion period. WP ingestion resulted in higher and earlier amplitude of blood branched-chain amino acid (BCAA) concentrations. PB ingestion created a lower initial rise in blood BCAA but sustained elevated levels of blood amino acids later into recovery. Postexercise FSR increased equivalently in both groups during the early period, however, FSR remained elevated only in the PB group during the late period. mTORC1 signaling similarly increased between groups, except for no increase in S6K1 phosphorylation in the WP group at 5 h postexercise. It was concluded that a soy-dairy PB ingested following exercise is capable of prolonging blood aminoacidemia, mTORC1 signaling, and protein synthesis in human skeletal muscle and is an effective postexercise nutritional supplement [13647].

**Proteins before exercise**
Adaptations to exercise training are determined by the response of metabolic and molecular mechanisms that determine changes in proteins. The type, intensity, and duration of exercise, as well as nutrition, determine these responses. The importance of protein, in the form of intact proteins, hydrolysates, or free amino acids, for exercise adaptations is widely recognized. Exercise along with protein intake results in accumulation of proteins that influence training adaptations. The total amount of protein necessary to optimize adaptations is less important than the type of protein, timing of protein intake, and the other nutrients ingested concurrently with the protein. Acute metabolic studies offer an important tool to study the responses of protein balance to various exercise and nutritional interventions. Recent studies suggest that ingestion of free amino acids plus carbohydrates before exercise results in a superior anabolic response to exercise than if ingested after exercise. However, the difference between pre- and postexercise ingestion of intact proteins is not apparent. Thus, the anabolic response to exercise plus protein ingestion seems to be determined by the interaction of timing of nutrient intake in relation to exercise and the nutrients ingested. More research is necessary to delineate the optimal combination of nutrients and timing for various types of training adaptations. Protein and amino acid intake have long been deemed important for athletes and exercising individuals. Olympic athletes, from the legendary Milo to many in the 1936 Berlin games, reportedly consumed large amounts of protein. Modern athletes may consume slightly less than these historical figures, yet protein is deemed extremely important by most. Protein is important as a source of amino acids for recovery from exercise and repair of damaged tissues, as well as for adaptations to exercise training, such as muscle hypertrophy and mitochondrial biogenesis [07316].

**Protein-rich intake for recovery after exercise**

Protein, protein hydrolysates, and amino acids have become popular ingredients in sports nutrition. The use of protein, protein hydrolysates, and amino acid mixtures has multiple applications when aiming to improve postexercise recovery. After exhaustive endurance-type exercise, muscle glycogen repletion is the most important factor determining the time needed to recover. Coingestion of relatively small amounts of protein and/or amino acids with carbohydrate can be used to augment postprandial insulin secretion and accelerate muscle glycogen synthesis rates. Furthermore, it has been well established that ingesting protein, protein hydrolysates, and amino acid can stimulate protein synthesis and inhibit protein breakdown and, as such, improve net muscle protein balance after resistance- or endurance-type exercise. The latter has been suggested to lead to a more effective adaptive response to each successive exercise bout. To augment net muscle protein accretion, athletes involved in resistance-type exercise generally ingest both protein and carbohydrate during postexercise recovery. However, carbohydrate ingestion after resistance-type exercise does not seem to be warranted to further stimulate muscle protein synthesis or improve whole-body protein balance when ample protein has already been ingested. Because resistance-type exercise is also associated with a substantial reduction in muscle glycogen content, it would be preferred to coingest some carbohydrate when aiming to accelerate glycogen repletion. More research is warranted to assess the impact of ingesting different proteins, protein hydrolysates, and/or amino acids on muscle protein accretion after exercise [07317].

The purpose of one study was to determine whether resistance exercise performance and postexercise muscle damage were altered when consuming a carbohydrate and protein beverage (CHO-PRO; 6.2 % and 1.5 % concentrations). Thirty-four male subjects (age: 22) completed 3 sets of 8 repetitions at their 8 repetition maximum to volitional fatigue. The exercise order consisted of the high pull, leg curl, standing overhead press, leg extension, lat pull-down, leg press, and bench press. In a double-blind, posttest-only control group design,
subjects consumed 355 ml of either CHO-PRO or placebo (electrolyte and artificial sweetener beverage) 30 minutes prior to exercise, 177 ml immediately prior to exercise, 177 ml halfway through the exercise bout, and 355 ml immediately following the exercise bout. There were no significant differences between groups relative to exercise performance. Cortisol was significantly elevated in the placebo group compared to the CHO-PRO group at 24 hours postexercise. Insulin was significantly elevated immediately pre-exercise, after the fourth lift, immediately postexercise, 1 hour, and 6 hours postexercise in CHO-PRO compared to the placebo group. Myoglobin levels in the placebo group approached significance halfway through the exercise bout and at 1 hour postexercise (p = 0.06 and 0.07, respectively) and were significantly elevated at 6 hours postexercise compared to the CHO-PRO group. Creatine kinase levels were significantly elevated in the placebo group compared to the CHO-PRO group. The CHO-PRO supplement did not improve performance during a resistance exercise bout, but appeared to reduce muscle damage, as evidenced by the responses of both myoglobin and creatine kinase. These results suggest the use of a CHO-PRO supplement during resistance training to reduce muscle damage and soreness [07318].

Carbohydrate ingestion after prolonged strenuous exercise enhances recovery, but protein might also be important. In a crossover with 2-week washout, 10 cyclists completed 2.5 h of intervals followed by 4-h recovery feeding, provided 218 g protein, 435 g carbohydrate, and 79 g fat (protein enriched) or 34 g protein, 640 g carbohydrate, and 79 g fat (isocaloric control). The next morning, cyclists performed 10 maximal constant-work sprints on a Velotron cycle ergometer, each lasting approximately 2.5 min, at approximately 5-min intervals. Test validity was established and test reliability and the individual response to the protein-enriched condition estimated by 6 cyclists repeating the intervals, recovery feeding, and performance test 2 wk later in the protein-enriched condition. During the 4-h recovery, the protein-enriched feeding had unclear effects on mean concentrations of plasma insulin, cortisol, and growth hormone, but testosterone was 25 percent higher (90 % confidence limit ± 14 %). Protein enrichment also reduced plasma creatine kinase by 33 percent (± 38 %) the next morning and reduced tiredness and leg-soreness sensations during the sprints, but effects on mean sprint power were unclear (-1.4 %). The between-subjects trial-to-trial coefficient of variation in overall mean sprint power was 3.1 percent, whereas the variation in the protein-enriched condition was 5.9 percent, suggesting that individual responses to the protein-enriched treatment contributed to the unclear performance outcome. To conclude, protein-enriched recovery feeding had no clear effect on next-day performance [07319].

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the unclear performance outcome. To conclude, protein-enriched recovery feeding had no clear effect on next-day performance [07320].

Ingestion of carbohydrate (CHO) and protein (PRO) following intense exercise has been reported to increase insulin levels, optimize glycogen resynthesis, enhance PRO synthesis, and lessen the immuno-suppressive effects of intense exercise. Since different forms of CHO have varying glycemic effects, the purpose of this study was to determine whether the type of CHO ingested with PRO following resistance-exercise affects blood glucose availability and insulin levels, markers of anabolism and catabolism, and/or general immune markers. Forty resistance-trained subjects performed a standardized resistance training workout and then ingested in a double blind and randomized manner 40 g of whey PRO with 120 g of sucrose (S), honey powder (H), or maltodextrin (M). A non-supplemented control group (C) was also evaluated. Blood samples were collected prior to and following exercise as well as 30, 60, 90, and 120 min after ingestion of the supplements. Glucose concentration 30 min following ingestion showed the H group to be greater than S; M and C groups. No significant differences were observed among groups in glucose area under the curve (AUC) values, although the H group showed a trend versus control. Insulin response for each treatment was significant by time, treatment and AUC. 30-min peak post-feeding insulin for S, H, and M were greater than C as was AUC with no significant differences observed among types of CHO. No significant group x time effects were observed among groups in testosterone, cortisol, the ratio of testosterone to cortisol, muscle and liver enzymes, or general markers of immunity. It was concluded that CHO and PRO ingestion following exercise significantly influences glucose and insulin concentrations. Although some trends were observed suggesting that H maintained blood glucose levels to a better degree, no significant differences were observed among types of CHO ingested on insulin levels. These findings suggest that each of these forms of CHO can serve as effective sources of CHO to ingest with PRO in and attempt to promote post-exercise anabolic responses [07321].

**Protein-carbohydrate-antioxidant**

The authors investigated the effects of postexercise carbohydrate-protein-antioxidant (CHO+P+A) ingestion on plasma creatine kinase (CK), muscle soreness, and subsequent cross-country race performance. Twenty-three runners consumed 10 mL/kg body weight of CHO or CHO+P+A beverage immediately after each training session for 6 d before a cross-country race. After a 21-d washout period, subjects repeated the protocol with the alternate beverage. Postintervention CK and soreness were significantly lower after CHO+P+A intervention than after CHO, despite no differences in baseline measures. There were no overall differences in running performance after CHO and CHO+P+A interventions. There were, however, significant correlations between treatment differences and running mileage, with higher mileage runners having trends toward improved attenuations in CK and race performance after CHO+P+A intervention than lower mileage runners. We conclude that muscle damage incurred during training was attenuated with postexercise CHO+P+A ingestion, which could lead to performance improvements in high-mileage runners [07322].

**Combined CHO-protein ingestion during exercise**

Although some studies have shown ergogenic benefits when combined CHO–protein supplements are ingested during exercise, one of these actually reflects an alternative but ultimately no more effective choice of pacing strategy, and the majority of evidence on this topic conforms to the prevailing view that there is no benefit. Could it then be argued that positive findings emerging from even only a minority of studies nonetheless demonstrate that added protein can enhance performance, albeit if that effect may be specific to the experimental setup in certain laboratories? On the contrary, even those researchers observing performance benefits have also reported negative outcomes when conducting similar studies. In fact, the only discernible methodological factor to explain why certain
studies have observed a benefit of added protein is that all these studies have compared CHO–protein supplements relative to control supplements providing CHO alone at a lower energy content and/or below the recommended intake for the exercise tests used. Whereas these findings are undoubtedly of mechanistic interest, the only logical interpretation from a practical viewpoint is that protein is an unnecessary addition to an otherwise adequate CHO supplementation regimen. In summary, the case against including protein in CHO supplements to be ingested during exercise is fundamentally grounded in the numerical balance of just four published studies demonstrating any benefit of added protein opposed to nine showing no effects on physical performance with the few extant reports of benefit explained by the insufficient quantities of CHO ingested in these studies. Lastly, another major problem facing the challenging view is the absence of any empirically supported mechanism to explain why ingesting protein during exercise would be expected to impart performance gains, with the most plausible proposed mechanisms (e.g. altered skeletal muscle metabolism) having recently been comprehensively examined and largely discounted. Several studies have investigated the effect of consuming protein with CHO compared with CHO only during prolonged exercise. One reported a 36 percent improvement in time to fatigue after 3 h of variable intensity cycling when cyclists ingested a mixture of CHO and protein compared with a matched quantity of CHO. Subsequently, several others have reported improvements in time to fatigue when including protein in a CHO supplement during exercise. The studies that have shown no performance improvement after a mixture of CHO and protein over CHO only are those that have provided sufficient CHO to satisfy oxidative requirements during exercise or when the addition of protein does not increase the energy content of the supplement. Although the evidence for an improvement in performance from adding protein to a CHO supplement during exercise seems to be equivocal, it is unlikely that consuming protein with CHO during prolonged endurance exercise will have a detrimental effect on performance. Indeed, there are other physiological effects that may be of benefit to the exercising individual. Coingestion of protein with CHO during prolonged endurance exercise has been shown to improve protein balance by increasing synthesis and decreasing breakdown, resulting in a positive net protein balance during exercise and during recovery. The inclusion of protein in a CHO supplement consumed during exercise has also been shown to reduce indices of postexercise muscle disruption compared with CHO supplements matched for CHO content or total calories. It is therefore clear that, although the benefits of consuming a CHO-protein mixture during exercise on immediate performance are still uncertain, this nutritional strategy can have a positive effect on the recovery process and therefore may improve exercise performance on subsequent days [11389].

In the fasted state, resistance exercise does not result in net protein accretion because muscle protein breakdown exceeds muscle protein synthesis; however, consumption of protein and/or essential amino acid provision shifts net protein balance to positive and promotes muscle protein accretion during the acute recovery period after resistance exercise. Recent reports suggest that hyperinsulinemia stimulated by the co-ingestion of CHO with protein does not seem to provide an even greater stimulus for muscle protein accretion. However, hyperinsulinemia is reported to stimulate rates of muscle protein breakdown and/or inhibit leg protein breakdown, both of which could augment protein accretion. These effects are equivocal, however, in terms of their beneficence for postexercise protein accretion; in particular, they are not seen when protein or amino acid consumption is sufficient to maximally stimulate rates of muscle protein synthesis. To date, no workers have examined rates of MPS or MPB after the consumption of a bolus of protein with and/or without CHO. Studies have examined the effects of a mixed amino acid infusion and repeated feedings of small doses of protein on MPS, neither of which would yield practical information for athletes or those concerned with gaining muscle mass. In previous works, the methods used resulted in a sustained hyperaminoacidemia and hyperinsulinemia,
which is not typical of what happens when food and/or drinks are consumed in a normal feeding pattern. Thus, the primary purpose of the present study was to use direct measures of muscle protein turnover and examine whether a bolus dose of high-quality whey protein, which contains sufficient essential amino acids to maximally stimulate rates of MPS, coingested with CHO (50 g) would augment rates of MPS and/or inhibit rates of MPB compared with protein alone. Further, it was examined intramuscular signaling events shown to be involved in the regulation of mRNA translation and elongation. This approach would provide practical insight on the relevance of CHO to a single-dose protein beverage on postexercise muscle protein turnover and intramuscular signaling events. It was hypothesized that insulin, if it were to be stimulatory for rates of MPS, might stimulate limb blood flow and enhance amino acid delivery to the limb at rest; however, insulin would not further stimulate limb blood flow or rates of MPS after resistance exercise followed by adequate protein consumption. The areas under the glucose and insulin curves were 17.5-fold and 5-fold greater, respectively, for PRO + CARB than for PRO. Exercise increased MPS and MPB, but there were no differences between PRO and PRO + CARB in the rested or exercised legs. Phosphorylation of Akt was greater in the PRO + CARB than in the PRO trial; phosphorylations of Akt and acetyl coA carboxylase-beta were greater after exercise than at rest. The concurrent ingestion of 50 g of CHO with 25 g of protein did not stimulate mixed MPS or inhibit MPB more than 25 g of protein alone either at rest or after resistance exercise. The data suggest that insulin is not additive or synergistic to rates of MPS or MPB when CHO is co-ingested with a dose of protein that maximally stimulates rates of MPS.

It was examined the hypothesis that protein co-ingestion with glucose during endurance exercise increases exogenous glucose-oxidation rate, gut comfort, and lowers perceived exertion. In a randomized crossover design, eight male cyclists rode 150-min at 50 percent of peak power on three occasions while ingesting solutions containing either: 8 percent C-enriched glucose and 2 percent milk protein concentrate (protein-glucose); glucose only (glucose), or non-caloric placebo (water). All solutions contained sodium citrate and flavor and were ingested at 150 ml per 15 min. The exogenous-carbohydrate oxidation rate was determined using stable isotope methodology and indirect calorimetry. Protein co-ingestion had no impact on the exogenous-glucose oxidation rate, but increased endogenous-carbohydrate oxidation rate (16 %) relative to glucose. Total carbohydrate and fat oxidation rates were increased (25 %) and decreased (17 %), respectively, by protein co-ingestion relative to water, but the effect relative to glucose was trivial. The plasma glucose concentration relative to glucose was 5.8 percent lower with protein co-ingestion; there were no clear differences in glucose concentration for the remaining comparisons, nor for lactate concentration. Perceived exertion was not altered by protein co-ingestion, however, there was a small decrease in nausea with the protein-glucose solution relative to water, other protein-affected comparisons were without note. Adding protein to a glucose-sodium solution ingested during exercise had neutral impact on exogenous carbohydrate oxidation and perception, and little impact on metabolic measures associated with endurance performance. It was concluded that previously reported effects of protein co-ingestion on endurance capacity were unlikely due to increased exogenous carbohydrate provision.

**Combined CHO-protein ingestion in recovery**

Whereas combined CHO and protein simply lacks efficacy when ingested during exercise, this does not negate any possibility that added protein may be of practical value in other situations (e.g. between exercise bouts). The question under debate, however, is not whether ingesting protein can be efficacious under certain circumstances but whether protein should be included in CHO-based sports supplements. These are conceptually very different questions, with valid justification for the inclusion of protein dependent not only on demonstrable efficacy but also on whether benefits persist even when evaluated within the
context of the habitual diet. This point has greater relevance to recovery than during exercise, as meals are usually a more viable option in recovery. For this reason, consideration of the criteria identified earlier leads inexorably to the conclusion that protein is an unnecessary addition to supplements in a real-world setting. The first of these criteria is easiest to address. Athletes may have a slightly increased daily protein requirement, so dietary protein supplementation might at first seem appropriate for this population. However, the vast majority of athletes in westernized societies exceed their recommended protein intake even without supplementation such that long-term protein requirements are almost always met by diet alone and even the potential short-term benefit of added protein for muscle glycogen synthesis after exercise does not apply to conditions where the existing diet is already adequate. Ingestion of added protein therefore does not supplement the habitual diet in the true sense because there is no chronic protein deficiency to be rectified and also no acute acceleration in the delivery of co-ingested CHO, provided that sufficient is ingested. The only remaining issues to consider are whether protein itself might be more effectively delivered to relevant tissues if ingested in the form of a supplement, particularly at time points so proximal to exercise that meals become an impractical option. Indeed, recent studies on this topic indicate that the temporal proximity and precise types of protein or amino acids ingested can mediate both acute protein synthetic rates and chronic accrual of lean tissue [11389].

Ultimately, rather than adding protein or anything else to sports supplements “just in case,” the approach informing a prevailing view was evidence based, with plausible explanations to reconcile any apparently conflicting findings. In this case, the few documented benefits of added protein for either performance during exercise or recovery thereafter are easily accounted for by the low amounts of CHO ingested in those studies. It therefore remains questionable why anyone would even consider including protein in a sports supplement when the likelihood of attaining a slight performance enhancement is entirely dependent on that supplement already being deficient in another nutrient with far better established ergogenic properties (i.e. CHO). Indeed, co-ingestion of CHO and protein in the immediate postexercise period clearly facilitates processes contributing to both short-term recovery and long-term adaptations (e.g. muscle glycogen and protein synthesis, respectively), with consequent translation into functional/whole-body end points (e.g. improved restoration of performance and training responses). There is no reason why these same outcomes cannot be achieved using nutritionally balanced whole foods as part of a more holistic approach. In fact, studies cited in the challenging view as evidence of improved rehydration with combined CHO-protein ingestion is a prime example of this reasoning. This is because the superior supplement in these studies was actually milk, with the greater fluid retention primarily attributed not to the presence of protein but to milk’s naturally elevated electrolyte content. Athletes, coaches, and supplement manufacturers understandably strive to gain competitive advantage by adopting nutritional strategies in anticipation of future empirical support. However, the scientific data currently available indicate that protein is not an effective addition to CHO-based sports supplements when ingested during exercise, whereas the potential advantages of added protein during recovery can be most comprehensively achieved via whole foods as part of a balanced diet [11389].

Nutritional interventions during recovery from exercise focus on CHO intake to maximize muscle glycogen resynthesis. Studies investigating the effects of adding protein to CHO have very much focused on enhancing muscle glycogen resynthesis during short-term recovery through maximizing the postexercise insulinenic response. Despite some contrasting evidence, the general consensus is similar to that for during exercise; if CHO is provided in adequate quantities then the addition of protein provides no extra benefit. However, reducing the CHO content of a supplement and adding an appropriate source of protein does not seem to have any detrimental effects on muscle glycogen resynthesis or subsequent
performance. Indeed, the addition of protein to a CHO supplement has many other potential benefits, and therefore, in many circumstances, reducing the CHO content and increasing the protein content of a supplement will result in a superior recovery strategy than consuming CHO alone. The majority of studies investigating the performance benefits of adding protein to a CHO recovery supplement have only considered the effect of supplementation on a subsequent exercise bout performed a few hours later. Although some athletes will train twice a day, many more will train hard or compete on consecutive days and therefore require a supplement that will enable them to maintain a high level of performance over multiple days. The addition of amino acids to a CHO drink consumed regularly during a 2-week period has been shown to reduce muscle damage (as evidenced by a decrease in plasma CK), decrease subjective fatigue, and maintain exercise performance after consecutive days of exercise compared with when CHO only was consumed in trained male athletes. Therefore, for those athletes who want to maintain performance over days of heavy training or competition, adding protein to a CHO supplement would be beneficial. Consuming CHO alone in the recovery period after a muscle-damaging exercise seems to have no beneficial effect on attenuating indirect measures of exercise induced muscle damage. The addition of protein to a CHO drink may confer advantages in terms of rehydration during recovery from exercise. There is evidence that consuming solutions containing protein after exercise-induced dehydration improves fluid retention over solutions with no added. Recent interesting data suggest that supplementation with a mixture of CHO and protein after exercise increases plasma albumin content and plasma volume in both older and younger subjects. Postexercise plasma volume increases result in cardiovascular and thermoregulatory adaptation and are therefore an important part of the training process. It has been reported that older individuals have an attenuated postexercise increase in plasma volume potentially due to an insufficient protein intake for albumin synthesis. Although further research is warranted in this area, this provides further evidence that consuming CHO alone during the postexercise period may not be the most appropriate nutritional practice to facilitate all aspects of recovery. In summary, the favorable consequences of consuming CHO–protein mixtures in many cases make them a more effective nutritional strategy than consuming CHO only, particularly over the longer term [11389].

Needs in older, training subjects

The regular performance of resistance exercises and the habitual ingestion of adequate amounts of dietary protein from high-quality sources are two important ways for older persons to slow the progression of and treat sarcopenia, the age-related loss of skeletal muscle mass and function. Resistance training can help older people gain muscle strength, hypertrophy muscle, and increase whole body fat-free mass. It can also help frail elderly people improve balance and physical functioning capabilities. Inadequate protein intake will cause adverse metabolic and physiological accommodation responses that include the loss of fat-free mass and muscle strength and size. Findings from controlled feeding studies show that older persons retain the capacity to metabolically adjust to lower protein intakes by increasing the efficiency of nitrogen retention and amino acid utilization. However, they also suggest that the recommended dietary allowance of 0.8 g protein/kg and day might not be sufficient to prevent subtle accommodations and blunt desired changes in body composition and muscle size with resistance training. Most of the limited research suggests that resistance training-induced improvements in body composition, muscle strength and size, and physical functioning are not enhanced when older people who habitually consume adequate protein (modestly above the recommended dietary allowance, RDA) increase their protein intake by either increasing the ingestion of higher-protein foods or consuming protein-enriched nutritional supplements. The habitual consumption of adequate amounts of high-quality dietary protein and the inclusion of resistance exercise training as part of a physically
active lifestyle are considered important contributors to an adult person's skeletal muscle size and strength, whole body fat-free mass, and health and well-being as they progress through the lifespan. Recent evidence suggests that protein intakes above the RDA might promote resistance-training-induced muscle hypertrophy. If true, this would provide older persons with an effective strategy to counter sarcopenia, the age-related loss of muscle mass, strength, and function that occurs in all persons and contributes to the need for about 15 percent of persons 65 to 75 years of age and 50 percent of persons over age 85 years to require assistance with activities of daily living [07323].

Acute dietary protein intake and resistance exercise
Research primarily done in younger adult men and women provides a strong foundation for how dietary protein and resistance exercise might synergistically work to ultimately promote muscle hypertrophy and increased whole body fat-free mass. Review articles describe these interactions in more detail. However, in general, proteins are constantly being formed (synthesized) and broken down and these processes are influenced by external cues including feeding and physical activity. On a daily to weekly basis the cumulative rates of protein synthesis and breakdown are in equilibrium; consequently, muscle and lean tissue mass are maintained. Measurements of the rates of protein synthesis and breakdown during short-term (acute) experiments have established the following knowledge:

- in a post-absorptive (fasting) state, the rate of protein synthesis is slower than the rate of protein breakdown, which results in a net catabolic state
- in a postprandial (fed) state that includes the ingestion of proteins or mixtures of amino acids, the rate of protein synthesis increases and is faster than the rate of protein breakdown (which is not appreciably changed); a net anabolic state results. As previously stated, these events typically balance each other over time and skeletal muscle and fat-free mass are unchanged
- in a post-absorptive state during the period soon after resistance exercise is performed, the rate of protein synthesis increases modestly more that the rate of protein breakdown increases. While this increases net protein balance, a catabolic state still remains
- the combination of feeding proteins or mixtures of amino acids and resistance exercise result in the greatest net anabolic state. This is achieved via amino acid and exercise-induced increases in protein synthesis and minimal or no change in protein breakdown

The effects of ingesting proteins and resistance exercise on muscle protein synthesis are considered to be independent and additive. Muscle hypertrophy theoretically is achieved from the cumulative periods of positive protein balance that occur after resistance exercise when protein is consumed. While this knowledge has resulted from studies in younger adults, several acute studies in older persons also document that amino acid feeding and resistance exercise increase muscle protein synthesis, findings that support the combined use of dietary protein and resistance training to hypertrophy muscle and offset sarcopenia [07323].

Dietary protein needs of older adults who perform resistance training
Adequate dietary protein is critical to promote the maintenance of skeletal muscle and treat sarcopenia. The RDA for protein is set at 0.8 g protein per day and kg bodyweight for all men and women age 19 years and above, independent of physical activity status. For younger adults, the American College of Sports Medicine, American Dietetic Association and Dietitians of Canada joint position statement on nutrition and athletic performance indicates that athletes might need to consume 50-100 percent more protein for exercise-related energy production, post-exercise muscle damage repair, and muscle hypertrophy. The protein needs of older persons who perform resistance training are not known with confidence. Sarcopenia
and reductions in the intensity and volume of training might modestly reduce the need for protein in older versus younger resistance-trained persons. The influence of resistance training on nitrogen balance and amino acid utilization has been evaluated in a strictly controlled diet study where the 54-78 year-old subjects either remained sedentary or performed lower-body or whole-body resistance exercise 3 days/week for 12 weeks. All of the subjects consumed diets that contained the RDA for protein and sufficient energy for weight maintenance throughout the study. During the first six weeks of intervention, urinary nitrogen excretion decreased and nitrogen balance increased comparably in all three groups, findings that support metabolic adaptation to the constant protein intake and the achievement of increased efficiency of nitrogen retention and amino acid utilization. Over the next six weeks of intervention, the two resistance training groups experienced a modest increase in urinary nitrogen excretion, compared to a continued progressive decline in the sedentary group. While being cautious not to over-interpret these findings, they are consistent with an increased protein requirement for the resistance-training older persons [07323].

Peptides

The production of sport nutrition foods (or parts of foods) and supplements to improve athletic performance, image or health for the consumer is a multibillion dollar industry. Within the broad supplement spectrum are the protein and/or free-form amino acid-based supplements. Research substantiating the efficacy of these supplements is wide-ranging from good evidence to support a reproducible anabolic effect of the ingestion of whey protein for the stimulation of muscle protein accretion, to no evidence for supplements, such as deer antler extracts, which are purported to boost concentrations of the peptide hormone IGF-1. Certainly, peptides have gained unfavourable attention in sports for their use in the (presumed) enhancement of performance, aiming to help athletes/teams to gain a competitive advantage [13357].

Peptides are short chains of amino acids that are produced synthetically, endogenously or derived from food. The longer chains of amino acids are known by most as protein. The peptides derived from dietary proteins by enzymatic hydrolysis are not the concern of the sport authorities involved in the maintenance of the integrity in professional sport. However, certain synthetic peptides can be administered, via a variety of methods (orally, intravenous, intranasal or transdermal cream, etc), that may exert anabolic effects and/or serve as masking agents for other drugs. In particular, the synthetic growth hormone releasing peptides (GHRP), for example, GHRP-6, Hexarelin or CJC-1295, have been demonstrated to stimulate the release of GH from the pituitary gland thereby enhancing its endogenous production. The synthetic GHRPs were specifically developed and intended for patients (not for athletes) with intact or impaired pituitary function to mimic the pulsatile nature of endogenous GH release. In the case of CJC-1295, a single injection of this peptide has been demonstrated to induce an increase in serum GH concentrations that seems to persist for about 2 weeks in healthy adults. GH therapy, however, is often administered in a single daily dose and results in a robust (supraphysiological) increase in systemic concentrations of GH that wanes over time [13357].

Amino acids

It has previously been shown that the aminoacidemia caused by the consumption of a rapidly digested protein after resistance exercise enhances muscle protein synthesis (MPS) more than the amino acid (AA) profile associated with a slowly digested protein. Here, we
investigated whether differential feeding patterns of a whey protein mixture commencing before exercise affect postexercise intracellular signaling and MPS. Twelve resistance-trained males performed leg resistance exercise 45 min after commencing each of three volume-matched nutrition protocols: placebo (PLAC, artificially sweetened water), BOLUS (25 g of whey protein + 5 g of leucine dissolved in artificially sweetened water; 1 × 500 mL), or PULSE (15 × 33 mL aliquots of BOLUS drink every 15 min). The preexercise rise in plasma AA concentration with PULSE was attenuated compared with BOLUS; this effect was reversed after exercise, with two-fold greater leucine concentrations in PULSE compared with BOLUS. One-hour postexercise, phosphorylation of p70 S6K(thr389) and rpS6(ser235/6) was increased above baseline with BOLUS and PULSE, but not PLAC; furthermore, PULSE > BOLUS. MPS throughout 5 h of recovery was higher with protein ingestion compared with PLAC (0.037 ± 0.007), with no differences between BOLUS or PULSE (0.085 ± 0.013 vs 0.095 ± 0.010 % per h, respectively). It was concluded that manipulation of aminoacidemia before resistance exercise via different patterns of intake of protein altered plasma AA profiles and postexercise intracellular signaling. However, there was no difference in the enhancement of the muscle protein synthetic response after exercise. Protein sources producing a slow AA release, when consumed before resistance exercise in sufficient amounts, are as effective as rapidly digested proteins in promoting postexercise MPS [12424].

The authors undertook 2 crossover-designed studies to characterize plasma amino acid (AA) responses to the intake of 20 g of protein. In Study 1, 15 untrained and overnight-fasted subjects consumed 20 g protein from skim milk, soy milk, beefsteak, boiled egg, and a liquid meal supplement. In Study 2, 10 fasted endurance-trained subjects consumed 20 g protein from a protein-rich sports bar at rest and after a 60-min submaximal ride. Plasma AA concentrations were measured immediately before and for 180 min after food ingestion using a gas-chromatography flame-ionization detection technique. A pharmacokinetic analysis was undertaken for profiles of total AAs (TAA), essential AAs, branched-chain AAs (BCAA), and leucine. Although area-under-the-curve values for plasma TAA were similar across protein sources, the pattern of aminoacidemia showed robust differences between foods, with liquid forms of protein achieving peak concentrations twice as quickly after ingestion as solid protein-rich foods (e.g. 50 min vs 100 min) and skim milk achieving a significantly faster peak leucine concentration than all other foods (25 min). Completing exercise before ingesting protein sources did not cause statistically significant changes in the pattern of delivery of key AAs, BCAAs, and leucine apart from a 20-40 percent increase in the rate of elimination. These results may be useful to plan the type and timing of intake of protein-rich foods to maximize the protein synthetic response to various stimuli such as exercise [12421].

Effects of amino acid derivatives on physical, mental and physiological activities
Nutritional ergogenic aids have been in use for a long time to enhance exercise and sports performance. Dietary components that exhibit ergogenic activity are numerous and their consumption is common and popular among athletes. They often come under scrutiny by legal authorities for their claimed benefits and safety concerns. Amino acid derivatives are propagated as being effective aids to enhance physical and mental performance in many ways, even though studies have pointed out that individuals who are deficient are more likely to benefit from dietary supplementation of amino acid derivatives than normal humans. In this review, some of the most common and widely used amino acids derivatives in sports and athletics namely creatine, tyrosine, carnitine, HMB and taurine have been discussed for their effects on exercise performance, mental activity as well as body strength and composition. Creatine, carnitine, HMB and taurine are reported to delay the onset of fatigue, improve exercise performance and body strength. HMB helps in increasing fat free mass and reduce exercise induced muscle injury. Taurine has been found to reduce oxidative stress during exercise and also act as an antihypertensive agent. Although, studies have not been able to
find any favourable effect of tyrosine administration on exercise performance, it has been proved to be very effective in fighting stress, improving mood and cognitive performance particularly in sleep deprived subjects. While available data from published studies and findings are equivocal about the efficacy of creatine, tyrosine and HMB; more comprehensive researches on carnitine and taurine are necessary to provide evidence for the theoretical basis of their ergogenic role in nutritional modification and supplementation [13649].

**Branched-chain amino acid (BCAA)**

The purpose of one study was to determine whether branched-chain amino acid (BCAA) supplementation attenuates indirect indicators of muscle damage during endurance exercise as compared with an isocaloric, carbohydrate (CHO) beverage or a noncaloric placebo (PLAC) beverage. Nine untrained men performed three 90 min cycling bouts at 55 percent VO\(_{2}\text{peak}\). Subjects, blinded to beverage selection, ingested a total of 200 kcal of energy via the CHO or BCAA beverage before and at 60 min of exercise, or they drank the PLAC beverage. Creatine kinase (CK), lactate dehydrogenase (LDH), isokinetic leg-extension and -flexion torque, and muscle soreness were assessed before and immediately, 4 h, 24 h, and 48 h postexercise. The trials were separated by 8 weeks. CK activities were significantly lower after the BCAA trial than in the PLAC trial at 4, 24, and 48 h postexercise, as well as lower than the CHO beverage at 24 h postexercise. CK was lower in the CHO trial at the 24- and 48-h time points than in the PLAC trial. LDH activities were lower in the BCAA trial at 4 h than in the PLAC trial. As compared with the CHO and PLAC trials, ratings of perceived soreness were lower at 24 h postexercise, and leg-flexion torque was higher at the 48-h time point after the BCAA trial. The present data suggest that BCAA supplementation attenuates muscle damage during prolonged endurance exercise in untrained college-age men. CHO ingestion attenuates CK activities at 24 and 48 h postexercise as compared with a placebo beverage [07324].

It was investigated the effect of branched-chain amino acids (BCAA) supplementation on tissue damage during distance running. Eight male distance runners (mean 20 years) participated in a double blinded cross over designed study conducted during training camp. During each intervention period, the subjects were asked to participate in a 25-km run, and the blood BCAA and lactate dehydrogenase (LDH) level, an index of tissue damage, were measured pre- and post-run. Either a drink containing BCAA (0.4 % BCAA in a 4 % carbohydrate solution) or an iso-calorie placebo drink was provided to the subjects 5 times during the run without any restriction in the volume. The total volume of the drink consumed by the subjects did not differ substantially between the trials: 591 ± 188 (2.36 g BCAA) versus 516 ± 169 mL in BCAA and placebo trial, respectively. During the run, the blood BCAA concentration was maintained in the BCAA trial. However, the blood BCAA concentration level tended to decrease in the placebo trial. The extent of the blood LDH increase in the BCAA trial was significantly less than that of the placebo trial (48 % vs 58 %). It was concluded that maintaining the blood BCAA level throughout a long distance run contributes to a reduction in the LDH release and, therefore, the effect of BCAA supplementation is suggested to reduce the degree of muscle damage [07325].

The mechanisms of maintenance of the protein mass of muscle and associated connective tissue and bone are becoming more accessible as a result of the use of a combination of well-established techniques for measurement of protein turnover and measurement of protein expression and phosphorylation state of signalling molecules involved in anabolic and catabolic responses. Amino acids, hormones and physical activity appear to be the major short-term physiological regulators of muscle mass, mainly through their actions on protein
synthesis and breakdown, on a time scale of minutes to hours, with duration of changes in
gene expression up to weeks. Amino acids are the main components in the diet regulating
protein turnover, having marked effects in stimulating muscle protein synthesis and with
almost no effect on muscle protein breakdown. Branched-chain amino acids, and in
particular leucine, simulate protein synthesis via signalling pathways involving mTOR
(mammalian target of rapamycin) in a dose-response manner. Insulin has little effect on
protein synthesis in human muscle, but it has a marked inhibitory effect on protein
breakdown. The amino acid simulation of anabolism is not dependent on the presence of
insulin, IGF-1 (insulin-like growth factor-1) or growth hormone. Exercise not only stimulates
protein synthesis in muscle, but also in tendon; and disuse atrophy is accompanied by
marked decreases of both muscle and tendon collagen protein synthesis. Bone collagen
synthesis appears to be nutritionally regulated by the availability of amino acids, but not lipid
or glucose [07326].

One study was designed to investigate whether a protein hydrolysate enriched in branched
chain amino acids and antioxidants, trace and mineral elements, and vitamins would affect
performance and fatigue. Eighteen sportsmen underwent testing before and after 28 days
supplementation with either treatment in protein hydrolysate or placebo. Testing included
exhaustive aerobic and anaerobic exercises with determination of blood lactate concentration
through exercise and recovery and antioxidant status, but also measurements of maximal
oxidative capacity (VO_{2\text{max}}) and citrate synthase activity from a resting muscle biopsy. Protein
hydrolysate resulted in a significant decrease in fatigue indices, without affecting
performances. A significant increase in enzymatic antioxidant and a decrease in oxidative
damage were observed at rest after treatment but not with a placebo. Decrease in maximal
blood lactate and improvement of blood lactate removal were only observed after protein
hydrolysate treatment. Furthermore, citrate synthase activity increased significantly, whereas
no change was observed in VO_{2\text{max}}. In conclusion, this protein hydrolysate treatment induced
adaptations that may promote a decrease in fatigue during exercises, potentially explained
by changes in parameters used to represent oxidative damage and antioxidant status at rest
and changes in lactate metabolism [07327].

Prolonged endurance exercise stimulates whole-body protein turnover (synthesis and
degradation) but it remains contentious whether this translates into an increased net protein
oxidation or dietary requirement for protein. Skeletal muscle is the major energy consumer
during exercise and the oxidation of branched-chain amino acids (BCAA) is increased
several-fold, suggesting an increased requirement for fuel. Increased BCAA oxidation has
been proposed to impair aerobic energy provision during prolonged exercise, but there is
little evidence to support this theory. Endurance training blunts the acute exercise-induced
increase in whole-body protein turnover and skeletal BCAA oxidation at a given work
intensity. However, training also increases the maximal capacity for skeletal muscle BCAA
oxidation, as evidenced by a higher maximal activity of the rate-determining enzyme
branched-chain oxo acid dehydrogenase. Exercise-induced changes in protein metabolism
are affected by nutritional status, with high carbohydrate availability (as typically practiced by
endurance athletes) generally associated with reduced net protein utilisation. Ingestion of
protein with carbohydrate improves net protein balance during exercise and recovery
compared with carbohydrate alone, but it remains to be determined whether this practice
facilitates the adaptive response to chronic training [07328].

The purpose of one study was to determine whether branched-chain amino acid (BCAA)
supplementation attenuates indirect indicators of muscle damage during endurance exercise
as compared with an isocaloric, carbohydrate (CHO) beverage or a noncaloric placebo
(PLAC) beverage. Nine untrained men performed three 90 min cycling bouts at 55 percent
VO_{2\text{peak}}. Subjects, blinded to beverage selection, ingested a total of 200 kcal of energy via
the CHO or BCAA beverage before and at 60 min of exercise, or they drank the PLAC beverage. Creatine kinase (CK), lactate dehydrogenase (LDH), isokinetic leg-extension and -flexion torque, and muscle soreness were assessed before and immediately, 4 h, 24 h, and 48 h postexercise. The trials were separated by 8 wk. CK activities were significantly lower after the BCAA trial than in the PLAC trial at 4, 24, and 48 h postexercise, as well as lower than the CHO beverage at 24 h postexercise. CK was lower in the CHO trial at the 24- and 48-h time points than in the PLAC trial. LDH activities were lower in the BCAA trial at 4 h than in the PLAC trial. As compared with the CHO and PLAC trials, ratings of perceived soreness were lower at 24 h postexercise, and leg-flexion torque was higher at the 48-h time point after the BCAA trial. The present data suggest that BCAA supplementation attenuates muscle damage during prolonged endurance exercise in untrained college-age men. CHO ingestion attenuates CK activities at 24 and 48 h postexercise as compared with a placebo beverage [07329].

Since the 1980's there has been high interest in branched-chain amino acids (BCAA) by sports nutrition scientists. The metabolism of BCAA is involved in some specific biochemical muscle processes and many studies have been carried out to understand whether sports performance can be enhanced by a BCAA supplementation. However, many of these researches have failed to confirm this hypothesis. Thus, in recent years investigators have changed their research target and focused on the effects of BCAA on the muscle protein matrix and the immune system. Data show that BCAA supplementation before and after exercise has beneficial effects for decreasing exercise-induced muscle damage and promoting muscle-protein synthesis. Muscle damage develops delayed onset muscle soreness: a syndrome that occurs 24-48 h after intensive physical activity that can inhibit athletic performance. Other recent works indicate that BCAA supplementation recovers peripheral blood mononuclear cell proliferation in response to mitogens after a long distance intense exercise, as well as plasma glutamine concentration. The BCAA also modifies the pattern of exercise-related cytokine production, leading to a diversion of the lymphocyte immune response towards a Th1 type. According to these findings, it is possible to consider the BCAA as a useful supplement for muscle recovery and immune regulation for sports events [08387].

The purpose of one study was to determine whether branched-chain amino acid (BCAA) supplementation attenuates indirect indicators of muscle damage during endurance exercise as compared with an isocaloric, carbohydrate (CHO) beverage or a noncaloric placebo beverage. Nine untrained men performed three 90 min cycling bouts at 55 percent of VO2 peak. Subjects, blinded to beverage selection, ingested a total of 200 kcal of energy via the CHO or BCAA beverage before and at 60 min of exercise, or they drank the placebo beverage. Creatine kinase (CK), lactate dehydrogenase (LDH), isokinetic leg-extension and -flexion torque, and muscle soreness were assessed before and immediately, 4 h, 24 h, and 48 h postexercise. The trials were separated by 8 week. Creatine kinase activities were significantly lower after the branched-chain amino acid trial than in the placebo trial at 4, 24, and 48 h postexercise, as well as lower than the carbohydrate beverage at 24 h postexercise. Creatine kinase was lower in the carbohydrate trial at the 24- and 48-h time points than in the placebo trial. LDH activities were lower in the branched-chain amino acid trial at 4 h than in the placebo trial. As compared with the carbohydrate and placebo trials, ratings of perceived soreness were lower at 24 h postexercise, and leg-flexion torque was higher at the 48-h time point after the branched-chain amino acid trial. The presented data suggest that branched-chain amino acid supplementation attenuates muscle damage during prolonged endurance exercise in untrained college-age men. Carbohydrate ingestion attenuates creatine kinase activities at 24 and 48 h postexercise as compared with a placebo beverage [08388].
The three branched chain amino acids (BCAA; leucine, isoleucine and valine) cannot be synthesised by the human body and must therefore be provided in the diet. Food sources containing BCAA are dietary proteins such as meat, poultry, fish, egg, milk and cheese, which contain 15-20 g of BCAA per 100 g of protein. The BCAA are metabolised mainly in skeletal muscle, which means they escape the liver, and ingestion of BCAA causes a rapid increase in the plasma level. The anabolic effect of BCAA on human skeletal muscle was first demonstrated under resting conditions, followed by studies showing similar effects in the recovery period after endurance and resistance exercise. More recent data indicate that the effect of BCAA, particularly leucine, is mediated through activation of regulatory enzymes in the protein synthesis machinery. Increasing the plasma level of BCAA during exercise may reduce the transport of tryptophan into the brain and the synthesis of 5-hydroxytryptamine (5-HT). 5-HT has been suggested to be involved in central fatigue, that is, fatigue emanating from the brain rather than muscle. BCAA supplementation during sustained physical activity has exerted positive effects on cognitive performance and perceived exertion. Under certain conditions, BCAA supplementation can also improve physical performance, although the majority of studies have found no effect of BCAA on performance when supplied together with carbohydrates. The amount of BCAA recommended is 0.03-0.05 g/kg body weight per hour or 2-4 g per hour ingested repeatedly during exercise and recovery, preferably taken as a drink. Large doses (about 30 g per day) are well tolerated; however, they may be detrimental to performance due to increased production of ammonia by the exercising muscle [09344].

The effects of branched-chain amino acid (BCAA) supplementation on the lactate threshold were investigated as an index of endurance exercise capacity. Eight trained male subjects (21 ± 2 y) participated in a double-blind crossover placebo-controlled study. The subjects were randomly assigned to two groups and were provided either a BCAA drink (0.4 % BCAA, 4 % carbohydrate; 1,500 mL/d) or an iso-caloric placebo drink for 6 d. On the 7th day, the subjects performed an incremental loading exercise test with a cycle ergometer until exhaustion in order to measure the lactate threshold. The test drink (500 mL) was ingested 15-min before the test. Oxygen consumption VO$_2$ and the respiratory exchange ratio (RER) during the exercise test were measured with the breath-by-breath method. Blood samples were taken before and during the exercise test to measure the blood lactate and plasma BCAA concentrations. The same exercise test was performed again 1 wk later. BCAA supplementation increased the plasma BCAA concentration during the exercise test, while plasma BCAA concentration decreased in the placebo trial. The RER during the exercise test in the BCAA trial was significantly lower than that in the placebo trial. The VO$_2$ and workload levels at lactate threshold point in the BCAA trial were higher than those in the placebo trial. The VO$_{2\max}$ in the BCAA trial was higher than that in the placebo trial. These results suggest that BCAA supplementation may be effective to increase the endurance exercise capacity [09345].

Skeletal muscle growth is thought to be regulated by the mammalian target of rapamycin (mTOR) pathway, which can be activated by resistance exercise and branched-chain amino acids (BCAA). The major aim of one study was to distinguish between the influence of resistance exercise and BCAA on key enzymes considered to be involved in the regulation of protein synthesis, including p70(S6) kinase (p70(S6k)). Nine healthy subjects (four men and five women) performed unilateral resistance exercise on two occasions separated by 1 month. Subjects were randomly supplied either a mixture of BCAA or flavoured water. Muscle biopsies were taken from both resting and exercising muscle before, after and 1 h after exercise. Phosphorylation of Akt was unaltered by either resistance exercise and/or BCAA supplementation whereas mTOR phosphorylation was significantly enhanced to a similar extent in both exercising and resting muscle following exercise in the absence (70-90 %) and presence of BCAA supplementation (80-130 %). Phosphorylation of p70(S6k) was
unaffected by resistance exercise alone; however, BCAA intake significantly increased this phosphorylation in both legs following exercise. In resting muscle, a 5- and 16-fold increase in p70(S6k) was observed immediately after and 1 h after exercise, respectively, as compared to 11- and 30-fold increases in the exercising muscle. Phosphorylation of eukaryotic elongation factor 2 was attenuated 1 h after exercise in both resting (10-40 %) and exercising muscle (30-50 %) under both conditions. The present findings indicate that resistance exercise and BCAA exert both separate and combined effects on the p70(S6k) phosphorylation in an Akt-independent manner [10496].

The purpose of one study was to investigate whether short-term amino acid supplementation could maintain a short-term net anabolic hormonal profile and decrease muscle cell damage during a period of high-intensity resistance training (overreaching), thereby enhancing recovery and decreasing the risk of injury and illness. Eight previously resistance trained males were randomly assigned to either a high branched chain amino acids (BCAA) or placebo group. Subjects consumed the supplement for 3 weeks before commencing a fourth week of supplementation with concomitant high-intensity total-body resistance training (overreaching) (3 x 6-8 repetitions maximum, 8 exercises). Blood was drawn prior to and after supplementation, then again after 2 and 4 days of training. Serum was analyzed for testosterone, cortisol, and creatine kinase. Serum testosterone levels were significantly higher, and cortisol and creatine kinase levels were significantly lower in the BCAA group during and following resistance training. These findings suggest that short-term amino acid supplementation, which is high in BCAA, may produce a net anabolic hormonal profile while attenuating training-induced increases in muscle tissue damage. Athletes’ nutrient intake, which periodically increases amino acid intake to reflect the increased need for recovery during periods of overreaching, may increase subsequent competitive performance while decreasing the risk of injury or illness [10236].

It has been demonstrated that branched-chain amino acids (BCAA) transaminase activation occurs simultaneously with exercise-induced muscle glycogen reduction, suggesting that BCAA supplementation might play an energetic role in this condition. One study aimed to test whether BCAA supplementation enhances exercise capacity and lipid oxidation in glycogen-depleted subjects. Using a double-blind cross-over design, volunteers (n=7) were randomly assigned to either the BCAA (300 mg/kg/day) or the placebo (maltodextrine) for 3 days. On the second day, subjects were submitted to an exercise-induced glycogen depletion protocol. They then performed an exhaustive exercise test on the third day, after which time to exhaustion, respiratory exchange ratio (RER), plasma glucose, free fatty acids (FFA), blood ketones and lactate were determined. BCAA supplementation promoted a greater resistance to fatigue when compared to the placebo (+17 %). Moreover, subjects supplemented with BCAA showed reduced RER and higher plasma glucose levels during the exhaustive exercise test. In conclusion, BCAA supplementation increases resistance to fatigue and enhances lipid oxidation during exercise in glycogen-depleted subjects [11274].

The purpose of one study was to determine whether branched-chain amino acid (BCAA) supplementation affects aerobic performance, ratings of perceived exertion (RPE), or substrate utilization as compared with an isocaloric, carbohydrate (CHO) beverage or a noncaloric placebo (PLAC) beverage. Nine untrained males performed three 90-minute cycling bouts at 55 percent VO₂ peak followed by 15-minute time trials. Subjects, who were blinded to beverage selection, ingested a total of 200 kcal via the CHO or BCAA beverage before and at 60 minutes of exercise or the PLAC beverage on the same time course. RPE and metabolic measurements were taken every 15 minutes during steady-state exercise, and each of the trials was separated by 8 weeks. Plasma glucose and BCAA concentrations were measured pre- and post-exercise. A significantly greater distance (4.6 ± 0.6 km) was traveled in the time-trial during the CHO trial than the PLAC trial (3.9 ± 0.4 km). There was no
difference between the BCAA (4.4 ± 0.5 km) and PLAC trials. RPE was reduced at the 75-minute and 90-minute mark during the BCAA trial as compared with the PLAC trial. There were no significant differences found for the trial vs. time interaction in regard to respiratory exchange ratio. Thus, CHO supplementation improves performance in a loaded time-trial as compared with a PLAC beverage. BCAA supplementation, although effective at increasing blood concentrations of BCAA, did not influence aerobic performance but did attenuate RPE as compared with a PLAC beverage [11275].

One study investigated the effects of BCAA, arginine and carbohydrate combined beverage (BCAA Drink) on biochemical responses and psychological conditions during recovery after a single bout of exhaustive exercise. Fourteen healthy males were assigned to drink either BCAA Drink (BA trial) or placebo (PL trial) on two sessions separated by 2 weeks. Blood samples of each subject were collected before exercise, 0, 10, 20, 40, 60, 120 min and 24 h after exercise. No significant differences in the levels of lactate, ammonia, creatine kinase and glycerol between the two groups were observed at any of the time points. However, the levels of glucose and insulin were significantly higher in the BA trial as compared to those in the PL trial at the 40 and 60 min recovery points. Furthermore, the testosterone-to-cortisol ratio at the 120 min recovery point was significantly higher in the BA trial as compared to that in the PL trial. The results indicate the occurrence of anabolic response during the recovery period. The benefit of BCAA Drink was also performed by Profile of Mood States to assess the psychological condition. Fatigue score increased immediately at exhaustion in both groups, but the decrease in the fatigue score at 120 min recovery point was significant only in BA trial. These data indicate that a single bout of exhaustive exercise enhanced the feeling of fatigue. The detrimental consequence was reduced by an ingestion of BCAA Drink [11276].

Branched-chain amino acids (BCAAs) especially leucine, were shown in the mid-1970s to be potent regulators of protein turnover. In the mid-1980s BCAAs were also shown to compete with other large neutral amino acids (LNAAs), suggesting that raising the blood concentration of BCAA would limit the formation of false neurotransmitters within the brain. Although these findings would seem to have provided an attractive basis for BCAA supplementation in various physiological or pathological situations, they were not sufficient at that time because the understanding of in vivo metabolism and cell signaling were limited and no clear biomarkers of BCAA status were available. The definition of BCAA requirements in healthy subjects remains a matter of debate, despite the use of accurate modern methods (i.e. 24-h direct amino acid oxidation and balance technique, providing a total of 84 mg/kg/day, which is lower than the 110 mg/kg/day obtained using the short-term indicator amino acid oxidation method). Another important question is how the requirements of a given BCAA influences the requirements of the other two. It has been provided convincing data that varying the intake of one BCAA in the range supplied by normal diets has no effect on the oxidation of the two others. However, these authors also show that this is not the case when a large nonphysiological BCAA intake is given. This is a key factor in the effects (including side effects) of pharmacological administration of a given BCAA or a mixture of BCAA in physiological (e.g. exercise) or pathological (e.g. injury) situations. The rationale behind consuming a BCAA in addition to diet before, during, and/or after exercise is that exercise leads to a considerable increase in BCAA oxidation, especially during endurance exercise, and most of the oxidized BCAA appears to be released into the system by depression of protein synthesis. Thus, the theoretical utility of providing a BCAA supplement is:

- to replace oxidized molecules
- to upregulate protein synthesis

However, this nice story is hampered by a series of facts. First, recent evidence shows that
endurance training does not really increase leucine oxidation and that protein metabolism becomes more efficient (i.e., increased nitrogen balance achieved at a lower rate of oxidation). Second, meeting energy requirements by eating a normal diet limits BCAA oxidation, making BCAA supplementation unwarranted. Nevertheless, a series of elegant studies suggest BCAA supplementation may increase protein synthesis during or after exercise. The molecular targets have been identified: mTOR, p70 S6 kinase, and S6 phosphorylation. Whether the mechanisms underlying the action of BCAAs in resistance and endurance exercise are similar or different remains unclear [06268].

Another action of BCAAs in an exercise context is their action on central fatigue syndrome. Central fatigue is defined as an inability to maintain power output due to events within the central nervous system. One of the various mechanisms that have been proposed to explain central fatigue is an increase in the amount of tryptophan taken up by the brain leading to excessive amounts of the neurotransmitter S-hydroxytryptamine in some neurons. The transport of tryptophan into the brain is regulated by blood concentrations of other LNAAs, in particular the BCAAs that compete with tryptophan for this transport. Hence, it makes sense to provide extra BCAAs to limit brain uptake of tryptophan and consequently central fatigue. The concept has been supported by data obtained during standardized cycle ergometer exercise and in a competitive 30-km cross-country race. Another situation where protein synthesis is depressed is with aging, which leads to sarcopenia, the progressive loss of muscle mass. Given demographic evolution in western countries, this is certainly a major public health concern. The decrease in protein synthesis in the elderly is multifactorial, but amino acids, and especially leucine, could play a major role in this process: first, splanchnic sequestration of leucine following a meal is almost doubled in the elderly compared with young adults; and second, the ability of leucine to stimulate muscle protein synthesis becomes blunted with aging. In other words, more leucine is needed to induce protein synthesis in the elderly to the same extent as in young adults. Resistance to the anabolic action of insulin, energy-dependent–related abnormalities (as gauged by the low stimulation of protein synthesis in the presence of carbohydrates in the meal), blunted leucine transduction signal (at S6K1), and low-grade chronic inflammation could be involved (21). However, to date, and to the best of our knowledge, there is no available clinical interventional study on the effects of BCAA supplementation in preventing or treating sarcopenia [06268].

Fatigue may be defined as the inability to maintain the expected muscle strength, leading to a reduced performance during prolonged exercise. The mechanisms that directly affect the muscles are peripheral and those residing in the brain are central. The following events are considered to be the five major metabolic causes of fatigue:

- muscle phosphocreatinine depletion
- proton accumulation in muscle (acidosis)
- muscle glycogen depletion
- reduced blood glucose concentration
- increased ratio of specific amino acids in plasma (free tryptophan/branched-chain amino acids (TRP/BCAA)).

The first three causes are directly related to peripheral fatigue and the last two are probably related to central fatigue. In addition to these causes, the increased plasma ammonia concentration that occurs during prolonged exercise can be highly toxic and is associated with both peripheral and central fatigue. In contrast to the liver, which can oxidize the twenty amino acids present in the tissue protein of the organism, skeletal muscle can oxidize only six: BCAA, aspartate, asparagine and glutamate. In muscle tissue, BCAA participates in protein synthesis and act as a precursor in the synthesis of other amino acids, in addition to
being used as energy substrates during physical exercise. Of the three BCAAs, leucine is the one primarily responsible for the stimulation of protein synthesis induced by intake of a mixed meal. The stimulatory effect of leucine on protein synthesis is mediated through upregulation of the initiation of mRNA translation. The hypothesis of central fatigue suggests that the increase in serotonin or 5-hydroxytryptamine (5-HT) concentration in the brain during prolonged exercise may be directly related to the development of fatigue and to the consequent reduction in performance. On the other hand, BCAA supplementation may act on the reduction of the plasma TRP/BCAA ratio since these amino acids have the same mechanism of transport through the blood-brain barrier, a fact that may reduce the influx of TRP (a serotonin precursor) in the central nervous system (CNS). This occurrence may cause a reduced 5-HT synthesis in the brain, consequently delaying the symptoms of fatigue. In addition, BCAA may act on glycogen metabolism during prolonged exercise, since supplementation with these amino acids can preserve hepatic and muscle glycogen possibly resulting in increased performance. One study aimed to determine the effects of diets chronically supplemented with branched-chain amino acids (BCAA) on the fatigue mechanisms of trained rats. Thirty-six adult Wistar rats were trained for six weeks. The training protocol consisted of bouts of swimming exercise (one hour a day, five times a week, for six weeks). The animals received a control diet (C) (n=12), a diet supplemented with 3.6 percent BCAA (S1) (n=12), or a diet supplemented with 4.8 percent BCAA (S2) (n=12). On the last day of the training protocol, half the animals in each group were sacrificed after one hour of swimming (1H), and the other half after a swimming exhaustion test (EX). Swimming time until exhaustion was increased by 37 percent in group S1 and reduced by 43 percent in group S2 compared to group C. Results indicate that the S1 diet had a beneficial effect on performance by sparing glycogen in the soleus muscle and by inducing a lower concentration of plasma ammonia, whereas the S2 diet had a negative effect on performance due to hyperammonemia. The hypothalamic concentration of serotonin was not significantly different between the 1H and EX conditions. In conclusion, chronic BCAA supplementation led to increased performance in rats subjected to a swimming test to exhaustion. However, this is a dose-dependent effect, since chronic ingestion of elevated quantities of BCAA led to a reduction in performance [12426].

Previous studies have evaluated the effectiveness of branched-chain amino acid (BCAA) supplementation for preventing delayed onset muscle soreness (DOMS) and muscle damage induced by eccentric exercise, their findings have been inconclusive. Since taurine has anti-inflammatory and anti-oxidative effects, the present study investigated the combined effect of BCAA and taurine on DOMS and muscle damage. Thirty-six untrained male subjects (23 ± 4 years) were assigned to four groups (placebo + placebo [placebo], BCAA + placebo, placebo + taurine, and BCAA + taurine [combined]) and given a combination of 3.2 g BCAA (or placebo) and 2.0 g taurine (or placebo), three times a day, for two weeks prior to and three days after eccentric elbow flexor exercises. DOMS and muscle damage in the biceps brachii were subjectively and objectively evaluated using the visual analogue scale (VAS), upper arm circumference (CIR), and blood parameters (creatine kinase, lactate dehydrogenase, LDH, aldolase, and 8-hydroxydeoxyguanosine, 8-OHdG). In the combined group, VAS and 8-OHdG two days after exercise, CIR two and three days after exercise and LDH from one to three days after exercise were significantly lower than the placebo group. The area under the curve from before exercise to four days later for CIR, LDH, and aldolase was also significantly lower in the combined group than in the placebo group. It was concluded that a combination of 3.2 g BCAA and 2.0 g taurine, three times a day, for two weeks prior to and three days after exercise may be a useful nutritional strategy for attenuating exercise-induced DOMS and muscle damage [13650].

**BCAA plus arginine**
One study aimed at evaluating the effect of a single oral intake of branched-chain amino acids (BCAA) with Arg on skeletal muscle protein metabolism during moderate exercise in young individuals. Eight healthy volunteers (4 males and 4 females) were studied in a randomized double-blind placebo-controlled cross-over trial. The subjects performed 3 bouts of 20-min cycling exercise (5-min break between each bout) at 126 ± 13 W corresponding to 50 percent of the maximal work intensity. A single oral supplement of either a BCAA drink containing 2 g of BCAA and 0.5 g of Arg or an isocaloric placebo drink was given at 10 min of the 1st exercise bout. Both arterial and venous blood samples were simultaneously taken from the radial artery and the femoral vein, respectively. Blood flow in the femoral artery was determined using the ultrasound Doppler technique. The blood sampling and blood flow measurements were performed at rest, every 10 min during each exercise bout. Net balance of BCAA and Phe across the leg muscles were measured by the arteriovenous difference method. The BCAA ingestion resulted in increases in both the plasma BCAA concentration and BCAA uptake into the working leg. The Phe release from the leg during exercise significantly increased as compared to the basal level in the placebo trial. In the BCAA trial, the cumulative Phe release from the leg during the 3rd exercise bout was significantly lower than that in the placebo trial. These results suggest that endurance exercise at moderate intensity enhances proteolysis in working muscles, and a single oral intake of 2 g of BCAA with Arg at onset of exercise effectively suppresses exercise-induced skeletal muscle proteolysis [07330].

Neutral amino acids

One study investigated the effects of high-dose large neutral amino acid (LNAA) supplementation on attenuating fatigue-induced decrements in exercise and motor skill performance in Australian Rules Football players. Fifteen subelite ARF players participated in 3 testing sessions separated by 7 days. Players completed an initial control trial involving a reactive motor skills test (RMST) and a reactive agility test (RAT) carried out before and after fatiguing exercise. In the subsequent experimental trials, players ingested a serotonin-depleting or protein control (PC) LNAA mixture 3 h before testing, allocated in a double-blind randomized cross-over design. Blood samples were taken at presupplementation and pre- and postexercise for analysis of plasma amino acid, insulin, and metabolite concentrations. The effect of the LNAA was established as the difference in the change in the mean RMST and RAT test scores among the depleting, PC, and baseline (BL) trials. Mean overall repetition time of the RAT was moderately improved after ingestion of the serotonin-depleting mixture compared with the BL trial. Serotonin-depleting and PC supplements had a divergent effect on mean repetition time after fatiguing exercise in RMST: depleting serotonin elicited a small improvement in motor skill performance in contrast to a small decrement after ingestion of the PC mixture, when compared to the BL. High-dose serotonin-“depleting” LNAA supplementation given 3 h prior to intermittent high-intensity exercise improved reactive motor skill and agility performance in football players [11528].

Effect on performance

The purpose of one investigation was to examine the effects of amino acid supplementation on muscular performance and resting hormone concentrations during resistance training overreaching. Seventeen resistance-trained men were randomly assigned to either an amino acid (AA) or a placebo (P) group and underwent 4 weeks of total-body resistance training designed to induce a state of overreaching. The protocol consisted of two 2-week phases (phase 1, 3 sets of 8 exercises performed for 8-12 repetitions; phase 2, 5 sets of 5 exercises
performed for 3-5 repetitions). Muscle strength and resting blood samples were determined before (T1) and at the end of each training week (T2-T5). One-repetition maximum squat and bench press decreased at T2 in the P group but not in the AA group; both groups showed similar increases in strength at T3 to T5. Significant elevations in serum creatine kinase and uric acid were observed at T2 in the P group; the elevation in creatine kinase correlated highly to reductions in 1-repetition maximum squat. Significant elevations in serum sex hormone-binding globulin were observed during overreaching in the P group from T2 to T5; this response was abolished in the AA group. Significant reductions in total testosterone were observed in the P group at T4 compared with T1, and total testosterone values were higher for the AA group than for the P group from T2 to T4. Serum 22-kd growth hormone concentrations were elevated at T2 to T5 in P group only. No differences were observed in resting cortisol and insulinlike growth factor 1. Hemoglobin concentrations were significantly reduced at T2 to T5 in the P group. These results indicate that the initial impact of high-volume resistance training is muscle strength reduction and hormonal/biochemical alterations. It appears that amino acid supplementation is effective for attenuating muscle strength loss during initial high-volume stress, possibly by reducing muscle damage by maintaining an anabolic environment [06265].

One study examined 10 weeks of resistance training and the ingestion of supplemental protein and amino acids on muscle performance and markers of muscle anabolism. Nineteen untrained males were randomly assigned to supplement groups containing either 20 g protein (14 g whey and casein protein, 6 g free amino acids) or 20 g dextrose placebo ingested 1 h before and after exercise for a total of 40 g/d. Participants exercised 4 times/week using 3 sets of 6-8 repetitions at 85-90 percent of the one repetition maximum. Data were analyzed with two-way ANOVA. The protein supplement resulted in greater increases in total body mass, fat-free mass, thigh mass, muscle strength, serum IGF-1, IGF-1 mRNA, MHC I and IIa expression, and myofibrillar protein. Ten weeks of resistance training with 20 g protein and amino acids ingested 1 h before and after exercise is more effective than carbohydrate placebo in up-regulating markers of muscle protein synthesis and anabolism along with subsequent improvements in muscle performance [06266].

Effects on muscle soreness

One study investigated the effect of a supplement containing 9 essential and 3 non-essential amino acids on muscle soreness and damage by comparing two endurance exercise bouts of the elbow flexors with amino acid or placebo supplementation in a double blind crossover design. The supplement was ingested 30 min before (10 h post-fasting) and immediately after exercise (Experiment 1), or 30 min before (2-3 h after breakfast), immediately post, and 8 more occasions over 4-day post-exercise (Experiment 2). Changes in muscle soreness and indicators of muscle damage for 4 days following exercise were compared between supplement conditions using two-way ANOVA. No significant differences between conditions were evident for Experiment 1; however, plasma creatine kinase, aldolase, myoglobin, and muscle soreness were significantly lower for the amino acid versus placebo condition in Experiment 2. These results suggest that amino acid supplementation attenuates DOMS and muscle damage when ingested in recovery days [06267].

Arginine

Arginine is a conditionally essential amino acid (C6H14N4O2). It is found in a wide variety of protein-rich foods, including both animal and plant sources. Arginine is an amino acid that serves functions of relevance to athletes. It is not only required for protein synthesis but also
plays a role in regulating protein synthesis. In addition, arginine is a precursor for the production of the messenger molecule nitric oxide, an important vasodilator and also for creatine. Arginine can remove ammonia from the blood, which may be important in recovery from hard training. Finally, the ingestion of arginine can stimulate the secretion of growth hormone and the recovery of muscles after exercise. Supplementation with arginine could be expected to enhance the response to training but may be particularly important to aid recovery from severe training sessions. Under normal conditions blood flow during aerobic exercise is sufficient and arginine supplementation has no beneficial effect. However, blood flow through tendon connective tissue is normally poor and may not provide nutrients sufficiently rapidly for cells to repair stressed or damaged tendons during training. Arginine supplementation increases blood flow due to the provision of nitric oxide. Recovery is dependent upon the activity of the tendon cells, which must be provided with oxygen and nutrients. The ability of arginine to stimulate creatine synthesis and growth hormone secretion could enhance muscle gain from resistance training. Studies have shown varying results, perhaps because the dose of ingested arginine is often insufficient. Effects of arginine supplementation on strength gains during training may be related to the naturally occurring level of arginine. Beneficial effects of arginine supplementation on muscle strength may be minimal in young, healthy men who are already eating plenty of protein. If further research is undertaken, it might be prudent to focus on groups who wish to improve muscle strength without increasing the amount of muscle and therefore body weight (e.g. lightweight rowers, boxers, endurance runners) [09135].

There has been substantial examination of the effect of infusion and ingestion of L-arginine at rest. It has been clearly demonstrated that L-arginine administration improves endothelial function in various disease states. In addition, L-arginine infusion at rest increases plasma insulin, growth hormone, glucagon, catecholamines and prolactin. Such hormonal changes affect metabolism. There has, however, been very little examination of the effect of increases in L-arginine availability during exercise. This is important to study as there is preliminary evidence that L-arginine infusion, probably via increases in nitric oxide (NO), alters skeletal-muscle metabolism during exercise. There is a need for further research, especially to understand the mechanisms of how L-arginine affects exercise metabolism and also to determine whether the hormonal responses that occur in response to L-arginine at rest are also present to some extent during exercise. This line of research may have important therapeutic implications as there are indications that L-arginine augments the effects of exercise training on insulin sensitivity and capillary growth in muscles [07335].

The purpose of one study was to examine the effects of daily oral administration of arginine-based supplements for 4 weeks on the physical working capacity at the fatigue threshold (PWCFT). The PWCFT test is an electromyographic (EMG) procedure for estimating the highest power output that can be maintained without neuromuscular evidence of fatigue. The study used a double-blind, placebo-controlled design. Fifty college-aged men were randomized into 1 of 3 groups: placebo (n=19); 1.5 g arginine (n=14); or 3.0 g arginine (n=17). The placebo was microcrystalline cellulose. The 1.5-g arginine group ingested 1.5 g of arginine and 300 mg of grape seed extract, whereas the 3.0 g arginine group ingested 3.0 g of arginine and 300 mg of grape seed extract. All subjects performed an incremental test to exhaustion on a cycle ergometer to determine their PWCFT before supplementation (PRE) and after 4 weeks of supplementation (POST). Surface EMG signals were recorded from the vastus lateralis using a bipolar electrode arrangement during the incremental tests for the determination of the PRE and POST supplementation PWCFT values. There were significant mean increases (PRE to POST) in PWCFT for the 1.5 g (22 %) and 3.0 g (19 %) supplement groups, but no change for the placebo group (-2 %). These findings supported the use of arginine-based supplements, at the dosages examined in the present investigation, as an ergogenic aid for untrained individuals [10237].
Increasing literature has studied the effects of dietary nitrates and moreover, L-arginine supplementation on tolerance to exercise and O₂ consumption during exercise. However, few studies have investigated the effects of L-arginine on performance measures such as a time trial. It was hypothesised that an acute 3-day L-arginine supplementation would elicit a significant improvement in performance and reduce oxygen consumption during a 20 km time trial. 6 healthy male competitive cyclists (23 ± 5 years) participated in a double-blind crossover study, and consumed either one 500 m placebo (PLA) or L-arginine (ARG) beverage, containing 6 g of L-arginine, over a 2 week testing period. Following a 3-day supplementation, participants completed a ramped incremental test to exhaustion, followed by an hours rest and subsequently a 20 km time trial. Time trial completion time was significantly reduced by 34 s, O₂ consumption during the time trial was also significantly reduced. Furthermore, both systolic and diastolic blood pressure were greatly reduced. However, no differences were seen in participants' VO₂max during the ramped incremental test to exhaustion although Wₚeak was higher during the same test. In conclusion, acute 3-day L-arginine supplementation at a dose of 6 g day⁻¹ increases 20 km time trial performance and reduces O₂ consumption during time trial performance, in addition to reducing systolic and diastolic blood pressure. However, L-arginine appears to have no effect upon VO₂max [11529].

L-arginine may enhance endurance performance mediated by two primary mechanisms including enhanced secretion of endogenous growth hormone (GH) and as a precursor of nitric oxide (NO); however, research in trained participants has been equivocal. The purpose of one study was to investigate the effect of acute L-arginine ingestion on the hormonal and metabolic response during submaximal exercise in trained cyclists. Fifteen aerobically trained men (age: 28 ± 5 years; body mass: 77.4 ± 9.5 kg; height: 180.9 ± 7.9 cm; VO₂max: 59.6 ± 5.9 ml/kg/min) participated in a randomized, double-blind, crossover study. Subjects consumed L-arginine (ARG; 0. 075 g/kg body mass) or a placebo (PLA) before performing an acute bout of submaximal exercise (60 min at 80 % of power output achieved at ventilatory threshold). The ARG condition significantly increased plasma L-arginine concentrations (about 146 %), while no change was detected in the PLA condition. There were no differences between conditions for GH, nonesterified fatty acids (NEFA), lactate, glucose, VO₂, VCO₂, RER, CHO oxidation, and NOx. There was reduced fat oxidation at the start of exercise (ARG 0.36 ± 0.25 vs PLA 0.42 ± 0.23 g/min) and an elevated plasma glycerol concentrations at the 45-min time point (ARG 340 vs PLA: 289 micromol/L) after L-arginine consumption. In conclusion, the acute ingestion of L-arginine did not alter any hormonal, metabolic, or cardio-respiratory responses during submaximal exercise except for a small but significant increase in glycerol at the 45-min time point and a reduction in fat oxidation at the start of exercise [13655].

Combined with ornithine

A placebo-controlled double-blind study was designed to investigate the effect of arginine and ornithine (Arg and Orn) supplementation during 3-week heavy-resistance training on serum growth hormone/insulin-like growth factor-1/insulin-like growth factor-binding protein 3 (GH/IGF-1/IGFBP-3), testosterone, cortisol, and insulin levels in experienced strength-trained athletes. The subjects were randomly divided between a placebo group (n=8) and the L-Arg/L-Orn-supplemented group (n=9), and performed pre and posttraining standard exercise tests with the same absolute load, which consisted of the same exercise schedule as that applied in the training process. Fasting blood samples were obtained at rest, 2 minutes after the cessation of the strength exercise protocol, and after 1 hour of recovery. The resting concentrations of the investigated hormones and IGFBP-3 did not differ significantly between the study groups. In response to exercise test, all the hormones were significantly elevated at

1778
both time points. Significant increases were observed in both GH and IGF-1 serum levels after arg and orn supplementation at both time points, whereas a significant decrease was seen in IGFBP-3 protein during the recovery period [10238].

**Combined with citrulline**

Dietary L-citrulline malate supplements may increase levels of nitric oxide (NO) metabolites, although this response has not been related to an improvement in athletic performance. NO plays an important role in many functions in the body regulating vasodilatation, blood flow, mitochondrial respiration and platelet function. L-Arginine is the main precursor of NO via nitric oxide synthase (NOS) activity. Additionally, L-citrulline has been indicated to be a second NO donor in the NOS-dependent pathway, since it can be converted to L-arginine. The importance of L-citrulline as an ergogenic support derives from the fact that L-citrulline is not subject to pre-systemic elimination and, consequently, could be a more efficient way to elevate extracellular levels of L-arginine by itself. L-Citrulline malate can develop beneficial effects on the elimination of NH₃ in the course of recovery from exhaustive muscular exercise and also as an effective precursor of L-arginine and creatine. Dietary supplementation with L-citrulline alone does not improve exercise performance. The ergogenic response of L-citrulline or L-arginine supplements depends on the training status of the subjects. Studies involving untrained or moderately healthy subjects showed that NO donors could improve tolerance to aerobic and anaerobic exercise. However, when highly-trained subjects were supplemented, no positive effect on performance was indicated [12429].

**Leucine**

Leucine is a key amino acid involved in the regulation of skeletal muscle protein synthesis. It was assessed the effect of the supplementation of a lower-protein mixed macronutrient beverage with varying doses of leucine or a mixture of branched chain amino acids (BCAAs) on myofibrillar protein synthesis (MPS) at rest and after exercise. In a parallel group design, 40 men (21 ± 1 years) completed unilateral knee-extensor resistance exercise before the ingestion of 25 g whey protein (W25) (3.0 g leucine), 6.25 g whey protein (W6) (0.75g leucine), 6.25 g whey protein supplemented with leucine to 3.0 g total leucine (W6+Low-Leu), 6.25 g whey protein supplemented with leucine to 5.0 g total leucine (W6+High-Leu), or 6.25 g whey protein supplemented with leucine, isoleucine, and valine to 5.0 g total leucine. A primed continuous infusion of L-13C6-phenylalanine with serial muscle biopsies was used to measure MPS under baseline fasted and postprandial conditions in both a rested (response to feeding) and exercised (response to combined feeding and resistance exercise) leg. The area under the blood leucine curve was greatest for the W6+High-Leu group compared with the W6 and W6+Low-Leu groups. In the postprandial period, rates of MPS were increased above baseline over 0-1.5 h in all treatments. Over 1.5-4.5 h, MPS remained increased above baseline after all treatments but was greatest after W25 (267 %) and W6+High-Leu (220 %) treatments. It was concluded that a low-protein (6.25 g) mixed macronutrient beverage can be as effective as a high-protein dose (25 g) at stimulating increased MPS rates when supplemented with a high (5.0 g total leucine) amount of leucine. These results have important implications for formulations of protein beverages designed to enhance muscle anabolism [13651].

The aim of one study was to evaluate the effects of the mixture of branched-chain amino acids (BCAAs) supplementation compared with leucine administered orally on muscle biochemical parameters of trained rats submitted to an exercise-induced protocol of glycogen depletion. After 6 weeks of swimming exercise, 8 week-old (250 g, adult) male Wistar rats
were randomly divided into three experimental groups (n=8 per group): the mixture of BCAAs (BCAAs), leucine (LEU), and placebo (PLA). All groups were submitted to swimming exercise for 6 wk and supplemented with either the mixture of BCAAs, leucine, or placebo during the last week of training. At week 7 of the protocol, the rats were submitted to an intermittent, progressive swimming test until exhaustion and sacrificed. Muscle gastrocnemius and liver were depicted to determine total glycogen, tricarboxylic acid cycle (TCA) intermediates, and enzymatic activities. Statistical evaluation was performed by one-way analysis of variance with Tukey post hoc test. Both muscle and liver glycogen degradation ratio were significantly higher in the mixture of BCAAs group compared to the PLA group and the LEU group presented decreased liver glycogen degradation ratio compared with the mixture of BCAAs group. Both muscle and liver glycogen content were significantly spared in the mixture of BCAAs and LEU groups compared to the PLA group. A performance test demonstrated that LEU supplementation enhanced resistance to exhaustion compared to the mixture of BCAAs, however, no difference was found when LEU supplementation was compared to PLA. Muscle citrate content was significantly higher in the mixture of BCAAs group compared with the PLA group. Muscle malate content was significantly elevated in the mixture of BCAAs group compared with both the PLA and LEU groups. BCAT activity was significantly reduced in the mixture of BCAAs supplementation group compared with the LEU group. It was concluded that leucine supplementation improved performance compared with the mixture of BCAAs supplementation, sparing muscle glycogen stores despite the augmentation of some TCA intermediate concentrations on the left side of the TCA cycle [13652].

Prolonged inactivity induces muscle loss due to an activation of proteolysis and decreased protein synthesis; the latter is also involved in the recovery of muscle mass. The aim of the present work was to explore the evolution of muscle mass and protein metabolism during immobilization and recovery and assess the effect of a nutritional strategy for counteracting muscle loss and facilitating recovery. Adult rats (6-8 months) were subjected to unilateral hindlimb casting for 8 days (I0-I8) and then permitted to recover for 10 to 40 days (R10-R40). They were fed a Control or Experimental diet supplemented with antioxidants/polyphenols (AOX) (I0 to I8), AOX and leucine (AOX + LEU) (I8 to R15) and LEU alone (R15 to R40). Muscle mass, absolute protein synthesis rate and proteasome activities were measured in gastrocnemius muscle in casted and non-casted legs in post prandial (PP) and post absorptive (PA) states at each time point. Immobilized gastrocnemius protein content was similarly reduced (-37 %) in both diets compared to the non-casted leg. Muscle mass recovery was accelerated by the AOX and LEU supplementation (+6 % AOX+LEU vs Control, at R40) due to a higher protein synthesis both in PA and PP states (+23 % and 31 % respectively, Experimental vs Control diets, at R40) without difference in trypsin- and chymotrypsin-like activities between diets. Thus, this nutritional supplementation accelerated the recovery of muscle mass via a stimulation of protein synthesis throughout the entire day (in the PP and PA states) and could be a promising strategy to be tested during recovery from bed rest in humans [13653].

Whey protein and leucine ingestion following exercise increases muscle protein synthesis and could influence neutrophil function during recovery from prolonged intense exercise. We examined the effects of whey protein and leucine ingestion post-exercise on neutrophil function and immunomodulators during a period of intense cycling. In a randomized double-blind crossover, 12 male cyclists ingested protein/leucine/carbohydrate/fat (LEUPRO 20/7.5/89/22 g/h, respectively) or isocaloric carbohydrate/fat control (CON 119/22 g/h) beverages for 1-3 h post-exercise during 6 days of high-intensity training. Blood was taken pre- and post-exercise on days 1, 2, 4 and 6 for phorbol myristate acetate (PMA)-stimulated neutrophil superoxide (O_2^-) production, immune cell counts, amino acid and lipid metabolism via metabolomics, hormones (cortisol, testosterone) and cytokines (interleukin-6, interleukin-
During recovery on day 1, LEUPRO ingestion increased mean concentrations of plasma amino acids (glycine, arginine, glutamine, leucine) and myristic acid metabolites (acylcarnitines C14, myristoylcarnitine; and C14:1-OH, hydroxymyristoylcarnitine) with neutrophil priming capacity, and reduced neutrophil O$_2^-$ production (15-17 mmol O$_2^-$/cell ± 90 % confidence limits 20 mmol O$_2^-$/cell). On day 2, LEUPRO increased pre-exercise plasma volume (6.6 ± 3.8 %) but haematological effects were trivial. LEUPRO supplementation did not substantially alter neutrophil elastase, testosterone, or cytokine concentrations. By day 6, however, LEUPRO reduced pre-exercise cortisol 21 percent (±15 %) and acylcarnitine C16 (palmitoylcarnitine) during exercise, and increased post-exercise neutrophil O$_2^-$ (33 ± 20 mmol O$_2^-$/cell) relative to control. Altered plasma amino acid and acylcarnitine concentrations with protein-leucine feeding might partly explain the acute post-exercise reduction in neutrophil function and increased exercise-stimulated neutrophil oxidative burst on day 6, which could impact neutrophil-dependent processes during recovery from intense training [13654].

One study aimed to determine the effect of postexercise protein-leucine coingestion with CHO-lipid on subsequent high-intensity endurance performance and to investigate candidate mechanisms using stable isotope methods and metabolomics. In the double-blind, randomized, crossover study, 12 male cyclists ingested a leucine/protein/CHO/fat supplement (LEUPRO 7.5/20/89/22 g/h), respectively) or isocaloric CHO/fat control (119/22 g/h) 1-3 h after exercise during a 6-d training block (intense intervals, recovery, repeated-sprint performance rides). Daily protein intake was clamped at 1.9 g/kg/d (LEUPRO) and 1.5 g/kg/d. Stable isotope infusions (1-$^{13}$C-leucine and 6,6-$^{2}$H$_2$-glucose), mass spectrometry-based metabolomics, and nitrogen balance methods were used to determine the effects of LEUPRO on whole-body branched-chain amino acid (BCAA) and glucose metabolism and protein turnover. After exercise, LEUPRO increased BCAA levels in plasma (2.6-fold) and urine (2.8-fold) and increased products of BCAA metabolism plasma acylcarnitine C5 (3.0-fold) and urinary leucine (3.6-fold) and beta-aminoisobutyrate (3.4-fold), indicating that ingesting about 10 g leucine per hour during recovery exceeds the capacity to metabolize BCAA. Furthermore, LEUPRO increased leucine oxidation (5.6-fold) and nonoxidative disposal (4.8-fold) and left leucine balance positive relative to control. With the exception of day 1 subsequent (days 2-5) nitrogen balance was positive for both conditions. Compared with control feeding, LEUPRO lowered the serum creatine kinase concentration by 21-25 percent but the effect on sprint power was trivial. Thus, postexercise protein-leucine supplementation saturates BCAA metabolism and attenuates tissue damage, but effects on subsequent intense endurance performance may be inconsequential under conditions of positive daily nitrogen balance [12428].

One study determined the effect of nutritional supplementation throughout endurance exercise on whole-body leucine kinetics (leucine rate of appearance [Ra], oxidation [Ox], and nonoxidative leucine disposal [NOLD]) during recovery. Five trained men underwent a 2-h run at 65% VO$_{2\text{max}}$, during which a carbohydrate (CHO), mixed protein-carbohydrate (milk), or placebo (PLA) drink was consumed. Leucine kinetics were assessed during recovery using a primed, continuous infusion of 1-$^{13}$C leucine. Leucine Ra and NOLD were lower for milk than for PLA, Ox was higher after milk-supplemented exercise than after CHO or PLA. Although consuming milk during the run affected whole-body leucine kinetics, the benefits of such a practice for athletes remain unclear. Additional studies are needed to determine whether protein supplementation during exercise can optimize protein utilization during recovery [07340].

Leucine has been suggested to have the potential to modulate muscle protein metabolism by increasing muscle protein synthesis. The objective of one study was to investigate the surplus value of the co-ingestion of free leucine with protein hydrolysate and carbohydrate
following physical activity in elderly men. Eight elderly men (mean age 73 ± 1 years) were randomly assigned to two cross-over treatments consuming either carbohydrate and protein hydrolysate (CHO+PRO) or carbohydrate, protein hydrolysate with additional leucine (CHO+PRO+leu) after performing 30 min of standardized physical activity. Primed, continuous infusions with L-(ring-\(^{13}\)C\(_6\))phenylalanine and L-(ring-\(^2\)H\(_2\))tyrosine were applied, and blood and muscle samples were collected to assess whole-body protein turnover as well as protein fractional synthetic rate in the vastus lateralis muscle over a 6 h period. Whole-body protein breakdown and synthesis rates were not different between treatments. Phenylalanine oxidation rates were significantly lower in the CHO+PRO+leu versus CHO+PRO treatment. As a result, whole-body protein balance was significantly greater in the CHO+PRO+leu compared to the CHO+PRO treatment. Mixed muscle fractional synthetic rate averaged 0.081 and 0.082 percent/h in the CHO+PRO+leu and CHO+PRO treatment, respectively. Thus, co-ingestion of leucine with carbohydrate and protein following physical activity does not further elevate muscle protein fractional synthetic rate in elderly men when ample protein is ingested [07341].

One study determined the effect of nutritional supplementation throughout endurance exercise on whole-body leucine kinetics (leucine rate of appearance [Ra], oxidation [Ox], and nonoxidative leucine disposal, NOLD, during recovery. Five trained men underwent a 2-h run at 65 percent VO\(_{2}\)max, during which a carbohydrate (CHO), mixed protein-carbohydrate (milk), or placebo (PLA) drink was consumed. Leucine kinetics were assessed during recovery using a primed, continuous infusion of 1-\(^{13}\)C leucine. Leucine Ra and NOLD were lower for milk than for PLA. Ox was higher after milk-supplemented exercise than after CHO or PLA. Although consuming milk during the run affected whole-body leucine kinetics, the benefits of such a practice for athletes remain unclear. Additional studies are needed to determine whether protein supplementation during exercise can optimize protein utilization during recovery [07342].

Essential amino acids (EAA) stimulate skeletal muscle protein synthesis (MPS) in humans. Leucine may have a greater stimulatory effect on MPS than other EAA and/or decrease muscle protein breakdown (MPB). To determine the effect of 2 different leucine concentrations on muscle protein turnover and associated signaling, young men (n=6) and women (n=8) ingested 10 g EAA in 1 of 2 groups: composition typical of high quality proteins (CTRL; 1.8 g leucine) or increased leucine concentration (LEU; 3.5 g leucine). Participants were studied for 180 min postingestion. Fractional synthetic rate and leg phenylalanine and leucine kinetics were assessed on muscle biopsies using stable isotopic techniques. Signaling was determined by immunoblotting. Arterial leucine concentration and delivery to the leg increased in both groups and was significantly higher in LEU than in CTRL; however, transport into the muscle and intracellular availability did not differ between groups. MPS increased similarly in both groups 60 min postingestion. MPB decreased at 60 min only in LEU, but net muscle protein balance improved similarly. Components of mammalian target of rapamycin (mTOR) signaling were improved in LEU, but no changes were observed in ubiquitin-proteasome system signaling. Changes in light chain 3 and mTOR association with Unc-51-like kinase 1 indicate autophagy decreased more in LEU. We conclude that in 10 g of EAA, the leucine content typical of high quality proteins (1.8 g) is sufficient to induce a maximal skeletal muscle protein anabolic response in young adults, but leucine may play a role in autophagy regulation [10497].

To investigate the effects of daily oral L-leucine ingestion on strength, bone mineral-free lean tissue mass (LTM) and fat mass (FM) of free living humans during a 12-wk resistance-training program 26 initially untrained men (n=13 per group) ingested either 4 g/d of L-leucine (leucine group: age 29 ± 8 years, body mass index 25 ± 4 kg/m\(^2\)) or a corresponding amount of lactose (placebo group: age 28 ± 7 years, body mass index 25 ± 4 kg/m\(^2\)). All participants
trained under supervision twice per week following a prescribed resistance training program using eight standard exercise machines. Testing took place at baseline and at the end of the supplementation period. Strength on each exercise was assessed by five repetition maximum (5-RM), and body composition was assessed by dual energy X-ray absorptiometry (DXA). The leucine group demonstrated significantly higher gains in total 5-RM strength (sum of 5-RM in eight exercises) and 5-RM strength in five out of the eight exercises. Significant differences did not exist between groups in either total percentage LTM gains or total percentage FM losses. These results suggest that 4 g/d of L-leucine supplementation may be used as a nutritional supplement to enhance strength performance during a 12-week resistance training program of initially untrained male participants [11277].

Growing evidence supports the conclusion that consumption of protein in close temporal proximity to the performance of resistance exercise promotes greater muscular hypertrophy. It can also be stated with good certainty that merely consuming energy, as carbohydrate for example, is also not sufficient to maximise muscle protein synthesis leading to anabolism and net new muscle protein accretion. Recent work also indicates that certain types of proteins, particular those that are rapidly digested and high in leucine content (i.e. whey protein), appear to be more efficient at stimulating muscle protein synthesis. Continued practice of consumption of these types or proteins after exercise should lead to greater hypertrophy. Reviews of numerous training studies indicate that studies in which milk proteins and principally whey protein show an advantage of these proteins over and above isoenergetic carbohydrate and soya protein in promoting hypertrophy. Thus, the combined evidence suggests a strategic advantage of practising early post-exercise consumption of whey protein or dairy-based protein to promote muscle protein synthesis, net muscle protein accretion and ultimately hypertrophy [11278].

Leucine is one of the three BCAAs and is an essential amino acid (EAA) that has to be provided in the diet. Besides serving as building blocks for protein synthesis, leucine can also regulate the rate of protein synthesis via a stimulatory effect on enzymes involved in the translation of specific mRNAs. Direct stimulation by leucine of the rate of protein synthesis in muscle tissue was first demonstrated in various preparations from experimental animals and, more recently, in the intact animal after oral administration. Infusion of leucine in human subjects was shown to improve the net protein balance and to increase phosphorylation/activation of the regulatory enzyme 70-kD ribosomal protein S6 kinase (p70S6K), indicating a stimulatory effect of leucine on protein synthesis also on human muscle after oral ingestion, although this remains to be confirmed. When leucine is ingested together with the other EAA as after resistance exercise, the rate of protein synthesis increases to a larger extent than without nutritional supply and a positive net protein balance is achieved during the period after exercise. However, adding extra leucine to a protein hydrolysate or an EAA mixture has no effect, or only minor additional effect, on the rate of protein synthesis in young subjects. On the other hand, recent data show that excluding leucine from the EAA mixture eliminates the stimulatory effect on protein synthesis and on phosphorylation of p70S6K. There is some evidence from animal studies that leucine can also inhibit muscle protein breakdown, however, this has not yet been confirmed in human muscle. Intake of leucine together with the other EAAs is recommended in connection with exercise. Available data indicate that the amount of leucine required to reach an optimal effect is 1.5-2.5 g in young individuals, which is the normal content in about 20 g of high-quality protein. In elderly individuals, the amount can be increased [11279].

The purpose of one study was to determine whether a practical leucine-protein, high-carbohydrate postexercise feeding regimen could improve recovery, as measured by subsequent cycling performance and mechanistic markers, relative to control feeding. In a crossover, 10 male cyclists performed 2- to 2.5-h interval training bouts on 3 consecutive
evenings, ingesting either leucine-protein, high-carbohydrate nutrition (0.1/0.4/1.2/0.2 g/kg/h; leucine, protein, carbohydrate, fat, respectively) or isocaloric control (0.06/1.6/0.2 g/kg/h; protein, carbohydrate, fat, respectively) nutrition for 1.5 h postexercise. Throughout the experimental period diet was controlled, energy and macronutrient intake balanced, and protein intake clamped at 1.6 g/kg/day. The alternate supplement was provided the next morning, thereby isolating the postexercise nutrition effect. Following 39 h of recovery, cyclists performed a repeat-sprint performance test. Postexercise leucine-protein ingestion significantly improved mean sprint power by 2.5 percent and reduced perceived overall tiredness during the sprints by 13 percent, but perceptions of leg tiredness and soreness were unaffected. Before exercise, creatine-kinase concentration was lowered by 19 percent, but lactate dehydrogenase and pressure-pain threshold were unaltered. There was a small reduction in anger, but other moods were unchanged. Plasma leucine (3-fold) and essential amino acid (47 %) concentrations were elevated postexercise. Net nitrogen balance trended mildly negative in both conditions. The ingestion of a leucine-protein supplement along with other high-carbohydrate food following intense training on consecutive days enhances subsequent high-intensity endurance performance and may attenuate muscle membrane disruption in well-trained male cyclists [11280].

To determine the effect of post-exercise protein-leucine co-ingestion with carbohydrate-lipid on subsequent high-intensity endurance performance, and to investigate candidate mechanisms using stable isotope methods and metabolomics in a double-blind randomized crossover, 12 male cyclists ingested a leucine/protein/carbohydrate/fat supplement (LEUPRO, respectively 7.5/20/89/22 g/h) or isocaloric carbohydrate/fat control (119/22 g/h) 1-3 h post-exercise during a 6-day training block (intense intervals, recovery, repeated-sprint performance rides). Daily protein intake was clamped at 1.9 (LEUPRO) and 1.5 g/kg/d (control). Stable isotope infusions (1-C-leucine and 6,6-H2-glucose), mass-spectrometry based metabolomics, and nitrogen balance methodology were used to determine effects of LEUPRO on whole-body branch-chain amino acid (BCAA) and glucose metabolism, and protein turnover. Following exercise, LEUPRO increased BCAAs in plasma (2.6-fold) and urine (2.8-fold), and products of BCAA metabolism plasma acylcarnitine C5 (3.0-fold) and urinary leucine (3.6-fold) and beta-aminoisobutyrate (3.4-fold), indicating ingesting about 10 g leucine/h during recovery exceeds the capacity to metabolise BCAA. Furthermore, LEUPRO increased leucine oxidation (5.6-fold), non-oxidative disposal (4.8-fold), and left leucine balance positive, relative to control. With the exception of day-1 (LEUPRO 17 ± 20 mg N/kg; control 90 ± 44 mg N/kg), subsequent (day 2-5) nitrogen balance was positive for both conditions (LEUPRO 130 ± 110 mg N/kg; control 111 ± 86 mg N/kg). Compared to control feeding, LEUPRO lowered the serum creatine kinase concentration by 21-25 percent, but the impact on sprint power was trivial. It was concluded that post-exercise leucine-protein supplementation saturates BCAA metabolism and attenuates tissue damage, but effects on subsequent intense endurance performance may be inconsequential under conditions of positive daily nitrogen balance [11281].

Leucine is one of the three BCAAs and is an essential amino acid (EAA) that has to be provided in the diet. Besides serving as building blocks for protein synthesis, leucine can also regulate the rate of protein synthesis via a stimulatory effect on enzymes involved in the translation of specific mRNAs. Direct stimulation by leucine of the rate of protein synthesis in muscle tissue was first demonstrated in various preparations from experimental animals and, more recently, in the intact animal after oral administration. Infusion of leucine in human subjects was shown to improve the net protein balance and to increase phosphorylation/activation of the regulatory enzyme 70-kD ribosomal protein S6 kinase (p7OS6k), indicating a stimulatory effect of leucine on protein synthesis also on human muscle after oral ingestion, although this remains to be confirmed. When leucine is ingested together with the other EAAs after resistance exercise, the rate of protein synthesis increases to a larger extent than
without nutritional supply and a positive net protein balance is achieved during the period after exercise. However, adding extra leucine to a protein hydrolysate or an EAA mixture has no effect, or only minor additional effect, on the rate of protein synthesis in young subjects. On the other hand, recent data show that excluding leucine from the EAA mixture eliminates the stimulatory effect on protein synthesis and on phosphorylation of p70S6k. In elderly subjects, in whom the required amount of leucine to reach optimal stimulation on protein synthesis may be enhanced, leucine-enriched diet improves muscle protein synthesis acutely, whereas no effect on muscle mass is observed during long-term supplementation. There is some evidence from animal studies that leucine can also inhibit muscle protein breakdown, however, this has not yet been confirmed in human muscle. Intake of leucine together with the other EAA's is recommended in connection with exercise. Available data indicate that the amount of leucine required to reach an optimal effect is 1.5-2.5 g in young individuals, which is the normal content in about 20 g of high-quality protein. In elderly individuals, the amount can be increased [11414].

The effects of essential amino acid (EAA) supplementation during moderate steady state (ie, endurance) exercise on postexercise skeletal muscle metabolism are not well described, and the potential role of supplemental leucine on muscle protein synthesis (MPS) and associated molecular responses remains to be elucidated. One randomized crossover study examined whether EAA supplementation with 2 different concentrations of leucine affected post-steady state exercise MPS, whole-body protein turnover, and mammalian target of rapamycin 1 (mTORC1) intracellular signaling. Eight adults completed 2 separate bouts of cycle ergometry (60 min, 60 % VO_2 peak, peak oxygen uptake). Isonitrogenous (10 g EAA) drinks with different leucine contents (leucine-enriched (L)-EAA, 3.5 g leucine; EAA, 1.87 g leucine) were consumed during exercise. MPS and whole-body protein turnover were determined by using primed continuous infusions of (2H_5)phenylalanine and 1_13C-leucine. Multiplex and immunoblot analyses were used to quantify mTORC1 signaling. MPS was 33 percent greater after consumption of L-EAA than after consumption of EAA. Whole-body protein breakdown and synthesis were lower and oxidation was greater after consumption of L-EAA than after consumption of EAA. Regardless of dietary treatment, multiplex analysis indicated that Akt and mammalian target of rapamycin phosphorylation were increased 30 min after exercise. Immunoblot analysis indicated that phosphorylation of ribosomal protein S6 and extracellular-signal regulated protein kinase increased and phosphorylation of eukaryotic elongation factor 2 decreased after exercise but was not affected by dietary treatment. These findings suggest that increasing the concentration of leucine in an EAA supplement consumed during steady state exercise elicits a greater MPS response during recovery [11389].

Branched chain amino acids (BCAA), particularly leucine, have been suggested to be ergogenic for both endurance and strength/power performance. One study investigated the effects of dietary leucine supplementation on the exercise performance of outrigger canoeists. Thirteen (ten female, three male) competitive outrigger canoeists underwent testing before and after 6-week supplementation with either capsulated L-leucine (45 mg/kg/day; n=6) or placebo (cornflour; n=7). Testing included anthropometry, 10 s upper body power and work and a row to exhaustion at 70-75 percent maximal aerobic power where perceived exertion (RPE), heart rate (HR) and plasma BCAA and tryptophan concentrations were assessed. Leucine supplementation resulted in significant increases in plasma leucine and total BCAA concentrations. Upper body power and work significantly increased in both groups after supplementation but power was significantly greater after leucine supplementation compared to the placebo. Rowing time significantly increased and average RPE significantly decreased with leucine supplementation while these variables were unchanged with the placebo. Leucine supplementation had no effect on the plasma tryptophan to BCAA ratio, HR or anthropometric variables. Six weeks' dietary leucine supplementation significantly improved endurance performance and upper body power in
outrigger canoeists without significant change in the plasma ratio of tryptophan to BCAA [06276].

4-hydroxyisoleucine

The purpose of this study was to determine the effect of adding fenugreek extract (FG) to post-exercise carbohydrate feeding on glycogen resynthesis and subsequent exercise performance in normoglycemic male endurance athletes. A muscle biopsy sample was obtained from the vastus lateralis from subjects prior to exercise for 5 h at 50 percent of peak cycling power (VO$_{2peak}$). A second muscle biopsy sample was obtained immediately after exercise. Immediately after and 2 h after the second biopsy subjects ingested either an oral dose of dextrose (GLU) or GLU with FG containing 1.99 ± 0.20 mg/kg 4-hydroxyisoleucine (GLU + FG) in a randomized, cross-over, double blind design. At 4 h post-exercise a third biopsy was taken and subjects received a standardised meal along with FG or a placebo capsule. At 15 h post-exercise subjects underwent their final muscle biopsy before completing a simulated 40 km cycling time trial. There was no difference in muscle glycogen at any time between GLU and GLU + FG. Additionally, 40 km time trial performance was similar for average power output and for time to completion for the GLU and GLU + FG, respectively. Despite earlier data to the contrary, the present results do not support an effect of fenugreek supplementation on glycogen resynthesis, even though this may have been the result of differences in experimental protocol [07343].

L-tryptophan

Physical exercise is often terminated not due to muscle fatigue but because of inadequate neural drive in the serotonergic system. Modifications in activity levels of the serotonergic system, induced by variations in the availability of L-tryptophan (a serotonin precursor) may alter neural drive. It was examined the effect of L-tryptophan supplementation on physical performance by combining aerobic work with brief periods of supramaximal intensity that closely mimics the activity typical of team sports. Twenty healthy young sportsmen performed a submaximal exercise on a cycle ergometer, with a workload corresponding to 50 percent of their respective VO$_{2max}$ for 10 min, followed by a maximal intensity exercise for 30 s. This sequence was repeated three times and, after the fourth series, each participant continued to exercise at the highest speed that he could sustain for 20 min. This protocol was performed twice: once with and finally without supplementation of L-tryptophan, in random order and double-blind. Peak power output, average anaerobic power output, and power output during the last 20 min of the trial were higher on the trials performed with L-tryptophan supplementation than on those performed with placebo. The distance covered during the last 20 min of the trial was 11,959 ± 1,753 m on placebo and 12,526 ± 1,617 m on L-tryptophan, which was a significant difference. In conclusion, in some types of exercises, modification of the serotonergic system may improve the physical performance [10239].

Taurine

Taurine is a sulphur-containing beta-amino acid (2-aminoethanesulphonic acid) that is conditionally classified as an indispensable amino acid. It is the most abundant free amino acid in the heart, brain, leukocytes and skeletal muscle but is not incorporated into protein within skeletal muscle. It has been suggested to be involved in a wide range of cellular processes including regulation of cell volume, Ca$^{2+}$-dependent excitation-contraction processes, antioxidant defence from stress responses, modulation of nerve excitement.
potential, and several metabolic effects related to improved glucose tolerance, insulin sensitivity and substrate uptake, storage and oxidation in skeletal and cardiac muscle. Rodent studies have shown that taurine supplementation increases muscle taurine content and prolonged exercise causes a decline in skeletal muscle taurine content. In addition, changes in taurine concentrations have been correlated with muscle function: when muscle taurine was decreased, contractile ability declined and, when it was increased, muscle force production improved. Lastly, the knock-out of taurine transporters has been associated with low muscle taurine levels and severe impairment of skeletal muscle exercise capacity. However, none of these effects has been demonstrated in human subjects. Taurine is currently claimed to be a functional ingredient (1000-2000 mg taurine per serving) in several commercialised energy drinks, with many manufacturers claiming that taurine is ergogenic for many types of exercise. However, scientific evidence to support these claims does not exist, as most studies have examined the metabolic, cognitive and performance effects of taurine supplements in combination with many additional ingredients that have been shown to be ergogenic (eg, caffeine and carbohydrate) or have not utilised appropriate placebo control beverages. Therefore, a role for taurine alone to improve exercise performance and/or alter metabolism in humans has not been demonstrated. Recent studies have examined the plasma taurine kinetics following a single acute dose of taurine, as well as the chronic effects of 7 days of taurine supplementation on skeletal muscle taurine content and substrate metabolism during 2 h of submaximal cycling. Acute supplementation with about 1.7 g taurine caused a 13-fold increase in plasma taurine at 2 h and remained elevated for at least 4 h, but 7 days of taurine supplementation (5 g/day) did not increase the taurine content before exercise or alter skeletal muscle taurine content, or substrate utilisation during 2 h of submaximal exercise. The effects of an acute dose of taurine (1.7 g) given 1 h before a 90 min of submaximal cycle and subsequent endurance time-trial (TT) performance and whole-body metabolism in well-trained cyclists was also studied. Acute taurine ingestion had no effect on TT performance compared to the control trial. In a third trial, telling the subjects they were receiving taurine in their preexercise drink, when they did not (placebo), was also not ergogenic. Taurine also had no effect on the normal physiological and mental responses to exercise such as heart rate and rating of perceived exertion as compared with control and placebo trials. Acute taurine ingestion did produce a significant 16 percent increase in total whole-body fat oxidation during 90 min of submaximal exercise prior to the time trial, although this was not evident in the previous study. In conclusion, despite large increases in plasma taurine following the acute ingestion of a large dose of taurine, it does not enhance endurance performance in well-trained athletes and does not accumulate in skeletal muscles when consumed chronically (5 g/day for 7 days). These data suggest that supplemental taurine is not ergogenic in human subjects [12300].

One study examined the plasma taurine response to acute oral taurine supplementation and the effects of 7 days of taurin on muscle amino acid content and substrate metabolism during 2 h of cycling at approximately 60 percent peak oxygen consumption (VO2peak). There was no difference in muscle glycogen or other muscle metabolites between conditions, but there were notable interaction effects for muscle valine, isoleucine, leucine, cysteine, glutamate, alanine, and arginine amino acid content following exercise after taurin. These data indicate that acute taurin produces a 13-fold increase in plasma taurine concentration. Despite the ability to significantly elevate plasma taurine for extended periods throughout the day, 7 days of taurin does not alter skeletal muscle taurine content or carbohydrate and fat oxidation during exercise. However, taurin appears to have some impact on muscle amino acid response to exercise [08415].

Although the effect of taurine on the heart and liver is well studied, there has been no direct observation concerning the effect of taurine on spatial learning and memory at the behavior level. In one study, it was tested the effect of subacute taurine supplementation with

1787
evaluation by the Morris water maze method. Although swim distance to find the platform of taurine-supplemented rats was significantly longer than that of control rats due to increase of swimming velocity, escape latency and the efficacy of learning and memory was comparable in both groups. These results suggest that taurine supplemented orally does not affect the learning and memory function [09346].

Cystein and cystine

Cysteine is a non-essential amino acid and, together with glycine and glutamate, is an important precursor of the tripeptide glutathione. Glycine and glutamate are readily available within the body and it is thought that the limiting step in the synthesis of glutathione is the availability of cysteine. Glutathione is one of the key antioxidants within the body and is an essential component of immune function. Reid et al intravenously infused 150 mg/kg body weight of N-acetylcysteine (NAC) before electrical stimulation of the tibialis anterior to fatigue at either 10 or 40 Hz. NAC infusion increased force output during electrical stimulation at 10 Hz by 15 percent, but failed to affect performance at 40 Hz. More recent research has investigated the effect of cystine, a dipeptide of cysteine, supplemented in conjunction with theanine, on the immune response to intense exercise training. Initial results suggest that immune function improved with oral cystine/theanine supplementation during intense training periods. In conclusion, more work needs to be carried out in this area, particularly looking at oral cysteine ingestion and exercise performance and the effect of cystine supplementation on the immune response to training [10347].

The aim of one study was to ascertain the influence of cysteine derivatives on pro-antioxidant equilibrium and to compare the antioxidant effectiveness of N-acetylcysteine, alpha-lipoic acid, and taurine by using Loverro’s coefficient (pro-antioxidant ratio) in healthy men exposed to intensity-resistance exercise. Fifty-five men were randomly assigned to one of four groups: control (CON, placebo), N-acetylcysteine (NAC 1.8 g/day, 3 days), alpha-lipoic acid (LIP 1.2 g/day, 3 days), or taurine (TAU 3 g/day, 3 days). The erythrocyte superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) activities, lipid peroxidation products (TBARS), and plasma protein thiol concentrations were evaluated. The P/A ratio was determined from the mean values of TBARS, SOD, GPx, and CAT. The applied exercise at maximal intensity induced the significant changes in pro-antioxidant equilibrium toward peroxidation, which was proved by a 25 percent increase in TBARS concentration in the CON group. The peroxidation was significantly diminished by NAC (-14 %) and LIP (-16 %), whereas TAU had no effect on the TBARS concentration. Cysteine derivatives administration prevented exercise-induced decline in SOD activity and increased in GPx activity during exercise. CAT activity changed only in the LIP group. The estimation of P/A ratio showed the lowest level of pro-antioxidant equilibrium after LIP administration. In the CON group, P/A ratio was directly correlated with the protein thiols level. These data confirm the antioxidant action of tested cysteine derivatives, particularly lipoic acid, and demonstrate the practical application of P/A ratio to evaluate the effectiveness of antioxidants in athletes [07344].

Cystine/theanine

Athletes become increasingly susceptible to infection with intense training that results in immune suppression. The immune state was investigated after administering cystine/theanine, which has been reported to have an immune reinforcement effect, to athletes before training involving a prolonged period of intense exercise. Fifteen long-distance runners were each allocated to the cystine/theanine or placebo group, and the test
food was ingested for 10 d prior to the start of training. Clinical examinations were performed before and after the training. The results indicate a significant increase in the high-sensitivity C-reactive protein (hs-CRP) and neutrophil count in the blood, as well as a decreasing tendency for lymphocytes in the placebo group, but not the cystine/theanine group. These observations suggest that the ingestion of cystine/theanine contributed to suppressing the change in inflammatory response, prevented a decrease in the immune function, and prevented infection and reduced symptoms when infected associated with continuous intense exercise [09347].

Glutathione and glutamate

Glutathione (gamma-glutamyl-cysteinyl-glycine; GSH) is the predominant low molecular weight thiol (0.5–10 mmol/L) in mammalian cells. Most GSH (85-90 %) is cytosolic, with the remainder located in organelles (including mitochondria, nuclear matrix and peroxisomes). This tripeptide is a key antioxidant within cells, critical to regulating the reactive oxygen species (ROS) concentration. Reduced glutathione may be used to remove damaging ROS such as H$_2$O$_2$ and convert it to harmless H$_2$O, generating oxidised glutathione (GSSG) via glutathione peroxidase. Disulphide formation and glutationylation are reversible forms of protein covalent modification dependent on glutathione and can provide mechanisms for regulation of metabolic, signalling and transcriptional processes, including skeletal muscle adaptation to exercise and training. The cellular redox state is crucial for molecular signalling, and glutathione is a key regulator/sensor for redox status; thus strategies aiming at increasing GSH synthesis should be beneficial to exercise performance. Exercise stimulates ROS and reactive nitrogen species (RNS) production, dependent on exercise type, duration and intensity, culminating in changes in skeletal muscle redox state. Excessive ROS and RNS production is associated with deleterious effects in many diseases including diabetes. Antioxidant supplementation strategies have been assessed for their ability to decrease ROS levels and the deleterious effects of oxidative/nitrosative damage. While excessive ROS and RNS can exert harmful effects within skeletal muscle during exercise, lower levels are crucial for adaptation of metabolic and signalling pathways in response to exercise. For example, redox changes are essential for the production and release of myokines such as interleukin 6 (IL-6), which, in part, optimises fuel provision for sustained activity. Although antioxidant supplementation may at first be considered as beneficial, the consequent reduction of ROS/RNS could have negative effects. Muscle redox state may be best improved by providing skeletal muscle cells with key natural precursors for GSH synthesis and allowing the cells to synthesise what they actually require. Exercise-induced free radical production in skeletal muscle is not detrimental to human health; thus endogenous antioxidants may be sufficient to protect against exercise-induced oxidative damage. The synthesis of GSH from glutamate, cysteine and glycine is catalysed sequentially by two key cytosolic enzymes, gamma-glutamylcysteine synthetase (GCS) and GSH synthetase. The availability of these amino acids is essential for GSH synthesis. Supplementation with cysteine precursors, such as N-acetylcysteine, increases glutathione levels. However, de novo GSH synthesis depends on glutamate, which is a constituent of the GSH molecule. It also acts as an amino acid donor in serine synthesis, which can subsequently be converted to glycine. GSH is a non-allosteric feedback inhibitor of GCS but competes with glutamate; thus high intracellular glutamate concentrations will enhance GSH synthesis. In conclusion, amino acid supplementation that increases intracellular glutamate and cysteine could improve muscle GSH synthesis. Future studies need to determine which amino acids can increase intracellular glutamate and GSH synthesis in skeletal muscle without the risk of blunting essential redox changes required for exercise adaptation. Potential amino acid candidates include branched chain amino acids (BCAA), arginine and glutamine [11283].
The major thiol-disulfide couple of reduced glutathione (GSH) and oxidized glutathione is a key regulator of major transcriptional pathways regulating aseptic inflammation and recovery of skeletal muscle after aseptic injury. Antioxidant supplementation may hamper exercise-induced cellular adaptations. The objective of one study was to examine how thiol-based antioxidant supplementation affects skeletal muscle's performance and redox-sensitive signaling during the inflammatory and repair phases associated with exercise-induced microtrauma. In a double-blind, crossover design, 10 men received placebo or N-acetylcysteine (NAC; 20 mg/kg/d) after muscle-damaging exercise (300 eccentric contractions). In each trial, muscle performance was measured at baseline, after exercise, 2 h after exercise, and daily for 8 consecutive days. Muscle biopsy samples from vastus lateralis and blood samples were collected before exercise and 2 h, 2 d, and 8 d after exercise. NAC attenuated the elevation of inflammatory markers of muscle damage (creatine kinase activity, C-reactive protein, proinflammatory cytokines), nuclear factor κB phosphorylation, and the decrease in strength during the first 2 d of recovery. NAC also blunted the increase in phosphorylation of protein kinase B, mammalian target of rapamycin, p70 ribosomal S6 kinase, ribosomal protein S6, and mitogen activated protein kinase p38 at 2 and 8 d after exercise. NAC also abolished the increase in myogenic determination factor and reduced tumor necrosis factor-α 8 d after exercise. Performance was completely recovered only in the placebo group. It was concluded that although thiol-based antioxidant supplementation enhances GSH availability in skeletal muscle, it disrupts the skeletal muscle inflammatory response and repair capability, potentially because of a blunted activation of redox-sensitive signaling pathways [13733].

Glutamine

Glutamine is a popular dietary supplement consumed for purported ergogenic benefits of increased strength, quicker recovery, decreased frequency of respiratory infections, and prevention of overtraining. From a biochemical standpoint, glutamine does play a physiologic role in each of these areas, but it remains only one of a host of factors involved. There is demonstrated a lack of evidence for definitive positive ergogenic benefits as a result of glutamine supplementation [08416].

Blood ammonia concentration increases during endurance exercise and has been proposed as a cause for both peripheral and central fatigue. It was examined the impact of glutamine and/or carbohydrate supplementation on ammonemia in high-level runners. Fifteen men in pre-competitive training ran 120 min (approximately 34 km) outdoors on 4 occasions. On the first day, the 15 athletes ran without the use of supplements and blood samples were taken every 30 min. After that, each day for 4 d before the next 3 exercise trials, it was supplemented the athletes' normal diets in bolus with carbohydrate (1 g/kg per day), glutamine (70 mg/kg per day) or a combination of both in a double-blind study. Blood ammonia level was determined before the run and every 30 min during the run. During the control trial ammonia increased progressively to approximately 70 percent above rest concentration. Following supplementation, independent of treatment, ammonia was not different for the first 60 min, but for the second hour it was lower than in the control. Supplementation in high-level, endurance athletes reduced the accumulation of blood ammonia during prolonged, strenuous exercise in a field situation [07331].

High-intensity and prolonged exercise significantly enhances the levels of plasma ammonia, a metabolite with toxic effects on the central nervous system. The main purpose of one study
was to evaluate the metabolic response of athletes to glutamine (Gln) and alanine (Ala) supplementation, since these amino acids have a significant influence on both anaplerosis and gluconeogenesis. Professional football players were assigned to groups receiving either Gln or Ala supplementation (100 mg/kg body weight); this supplementation was either short-term or long-term and was given immediately before exercise. The players were evaluated using two exercise protocols, one with intervals (n=18) and the other with continuous intensity (n=12). Both types of exercises increased ammonia, urate, urea and creatinine in blood. Chronic Gln supplementation partially protected against hyperammonemia after a football match (intermittent exercise: Gln -140 vs Ala -240 %) and after continuous exercise at 80 percent of the maximum heart rate (Gln -481 % vs placebo -778 %). Urate increased by 10-20 percent in all groups, independently of supplementation. Glutamine once a day supplementation induced a greater elevation in urate as compared to alanine at the end of the game; however, long-term supplementation provoked a lesser increment in urate. Exercise induced similar increases in creatinine as compared to their respective controls in either acute or chronic glutamine administration. Taken together, the results suggest that chronically supplemented Gln protects against exercise-induced hyperammonemia depending on exercise intensity and supplementation duration [07332].

Glutamine is a popular dietary supplement consumed for purported ergogenic benefits of increased strength, quicker recovery, decreased frequency of respiratory infections, and prevention of overtraining. From a biochemical standpoint, glutamine does play a physiologic role in each of these areas, but it remains only one of a host of factors involved. One review examines the effects of glutamine on exercise and demonstrates a lack of evidence for definitive positive ergogenic benefits as a result of glutamine supplementation [07333].

The distinction between positive and negative training adaptation is an important prerequisite in the identification of any marker for monitoring training in athletes. To investigate the glutamine responses to progressive endurance training, twenty healthy males were randomly assigned to a training group or a non-exercising control group. The training group performed a progressive (3 to 6 x 90 minute sessions per week at 70 % VO2max) six-week endurance training programme on a cycle ergometer, while the control group did not participate in any exercise during this period. Performance assessments VO2max and time to exhaustion) and resting blood samples (for hemoglobin concentration, hematocrit, cortisol, ferritin, creatine kinase, glutamine, uric acid and urea analysis) were obtained prior to the commencement of training (Pre) and at the end of week 2, week 4 and week 6. The training group showed significant improvements in time to exhaustion, and VO2max at all time points (except week 2 for VO2max while the control group performance measures did not change. In the training group, hemoglobin concentration and hematocrit were significantly lower than pretraining values at week 2 and 4, as percentage changes in plasma volume indicated a significant hemodilution (+ 6 to 9 %) was present at week 2, 4 and 6. No changes were seen in the control group. In the training group, plasma glutamine (week 2, 4 and 6), creatine kinase (week 2 and 4), uric acid (week 2 and 4) and urea (week 2 and 4) all increased significantly from pretraining levels. No changes in cortisol or ferritin were found in the training group and no changes in any blood variables were present in the control group. Plasma glutamine was the only blood variable to remain significantly above pretraining (966 ± 32 micromol) levels at week 6 (1176 ± 24 micromol) The elevation seen here in glutamine levels, after 6 weeks of progressive endurance training, is in contrast to previous reports of decreased glutamine concentrations in overtrained athletes. In conclusion, 6 weeks of progressive endurance training steadily increased plasma glutamine levels, which may prove useful in the monitoring of training responses [07334].

Some athletes can have high intakes of l-glutamine because of their high energy and protein intakes and also because they consume protein supplements, protein hydrolysates, and free
amino acids. Prolonged exercise and periods of heavy training are associated with a decrease in the plasma glutamine concentration and this has been suggested to be a potential cause of the exercise-induced immune impairment and increased susceptibility to infection in athletes. However, several recent glutamine feeding intervention studies indicate that although the plasma glutamine concentration can be kept constant during and after prolonged strenuous exercise, the glutamine supplementation does not prevent the postexercise changes in several aspects of immune function. Although glutamine is essential for lymphocyte proliferation, the plasma glutamine concentration does not fall sufficiently low after exercise to compromise the rate of proliferation. Acute intakes of glutamine of approximately 20-30 g seem to be without ill effect in healthy adult humans and no harm was reported in a study in which athletes consumed glutamine every day for 14 d. Doses of up to 0.65 g/kg body mass of glutamine (in solution or as a suspension) have been reported to be tolerated by patients and did not result in abnormal plasma ammonia levels. However, the suggested reasons for taking glutamine supplements (support for immune system, increased glycogen synthesis, anticatabolic effect) have received little support from well-controlled scientific studies in healthy, well-nourished humans.

Blood ammonia concentration increases during endurance exercise and has been proposed as a cause for both peripheral and central fatigue. It was examined the impact of glutamine and (or) carbohydrate supplementation on ammonemia in high-level runners. Fifteen men in pre-competitive training ran 120 min (approximately 34 km) outdoors on 4 occasions. On the first day, the 15 athletes ran without the use of supplements and blood samples were taken every 30 min. After that, each day for 4 d before the next 3 exercise trials, it was supplemented the athletes' normal diets in bolus with carbohydrate (1 g/kg and day), glutamine (70 mg/kg and day), or a combination of both in a double-blind study. Blood ammonia level was determined before the run and every 30 min during the run. During the control trial ammonia increased progressively to approximately 70 percent above rest concentration. Following supplementation, independent of treatment, ammonia was not different for the first 60 min, but for the second hour it was lower than in the control. Supplementation in high-level, endurance athletes reduced the accumulation of blood ammonia during prolonged, strenuous exercise in a field situation.

Different dietary proteins affect whole body protein anabolism and accretion and therefore, have the potential to influence results obtained from resistance training. One study examined the effects of supplementation with two proteins, hydrolyzed whey isolate and casein, on strength, body composition, and plasma glutamine levels during a 10 wk, supervised resistance training program. In a double-blind protocol, 13 male, recreational bodybuilders supplemented their normal diet with either whey or casein (1.5 g/kg body wt/d) for the duration of the program. Strength was assessed by 1-RM in three exercises (barbell bench press, squat, and cable pull-down). Body composition was assessed by dual energy X-ray absorptiometry. Plasma glutamine levels were determined by the enzymatic method with spectrophotometric detection. All assessments occurred in the week before and the week following 10 wk of training. Plasma glutamine levels did not change in either supplement group following the intervention.

High-intensity and prolonged exercise significantly enhances the levels of plasma ammonia, a metabolite with toxic effects on the central nervous system. The main purpose of the present study was to evaluate the metabolic response of athletes to glutamine and alanine supplementation, since these amino acids have a significant influence on both anaplerosis and gluconeogenesis. Professional football players were assigned to groups receiving either glutamine and alanine supplementation (100 mg/kg body weight); this supplementation was either short-term or long-term and was given immediately before exercise. The players were evaluated using two exercise protocols, one with intervals (n=18) and the other with...
continuous intensity (n=12). Both types of exercises increased ammonia, urate, urea and creatinine in blood. Chronic glutamine supplementation partially protected against hyperammonemia after a football match and after continuous exercise at 80 percent of the maximum heart rate. Urate increased by 10-20 percent in all groups, independently of supplementation. Glutamine once a day supplementation induced a greater elevation in urate as compared to alanine at the end of the game; however, long-term supplementation provoked a lesser increment in urate. Exercise induced similar increases in creatinine as compared to their respective controls in either acute or chronic glutamine administration. Taken together, the results suggest that chronically supplemented glutamine protects against exercise-induced hyperammonemia depending on exercise intensity and supplementation duration [08420].

Glutamine (Gln), the most abundant amino acid in the body, has recently become regarded as conditionally essential rather than non-essential. Glutamine is synthesised, stored and released predominantly by skeletal muscle: it is taken up by intestinal cells, such as enterocytes and colonocytes, by the liver and kidney and by some key immune cells. In clinical studies, the plasma concentration of glutamine is decreased in trauma and starvation: glutamine provision has been reported to have a beneficial effect on gut function, morbidity and mortality, and immune cell function. Clinical evidence suggests that glutamine provision helps recovery from surgery and maintains muscle protein mass. The normal resting, fasting plasma glutamine is 500-700 micromol/l and is often higher in athletes: the muscle concentration can reach 20 mM (60 % of the intramuscular pool). During short-term strenuous exercise, the concentration is usually markedly increased, probably due to the release of glutamine into the circulation from skeletal muscle. However, glutamine is often substantially reduced by prolonged, exhaustive exercise: this decrease often occurs concomitantly with relatively transient immunodepression. Decreased concentrations may contribute to overtraining. Glutamine supplementation after exercise reduced the self-reported incidence of illness in endurance athletes. However, when glutamine was given to athletes to combat exercise-induced depletion of circulating glutamine, no effects were observed on the immune parameters studied, apart from reduced neutrocytosis and increased circulating IL-6. It remains to be determined which other aspects of exercise-induced immunodepression might be altered by glutamine supplementation. Although the main focus of the series and this article is the ergogenic effects of supplementation, immunodepression must also be taken into account, since its elimination will allow more effective training and thus better performance. Glutamine supplementation has been well studied in both clinical and exercise situations, particularly in terms of its effects on immune function. After endurance exercise, muscle glycogen repletion is an important factor in recovery and subsequent performance. Post-exercise intake of carbohydrate provides a substrate for glycogen synthesis and also stimulates insulin secretion, which subsequently activates exercise and the glycogen synthase enzyme in muscle. Sports drinks containing glutamine as a free amino acid or part of a dipeptide are widely available but the low levels recommended are unlikely to help improve immune or muscle function. Glutamine supplementation (L-alanyl-L-glutamine dipeptide, at 0.05 and 0.2 g/kg) led to a significant ergogenic benefit by increasing time to exhaustion during a mild hydration stress. This ergogenic effect was thought likely to be mediated by an enhanced fluid and electrolyte uptake. There is also evidence of a role for glutamine, versus alanine, in protecting footballers against an exercise-induced increase in blood ammonia, which would have an impact on fatigue. The first product of glutamine metabolism, catalysed by the enzyme glutaminase, is the excitatory neurotransmitter glutamate. The latter has sometimes been used for supplementation. This may seem rather surprising, since its appearance in plasma at a high concentration correlates neurotoxicity and sometimes with clinical problems. Glutathione, for which glutamine is a precursor via glutamate, is a powerful antioxidant and, in its reduced form, is a good marker of antioxidant capacity, while an increase in its oxidised
form is a good marker of oxidative stress. Overall, there is no consensus or unifying concept to explain the efficacy of exogenous provision of glutamine alone on performance in athletes, although in combination with carbohydrate or other amino acids, significant improvements have been reported. Although there is some evidence that glutamine is effective in decreasing the self-reported incidence of upper respiratory tract illness, it has been difficult to obtain evidence of an effect on any specific aspect of the immune system. There is no doubt that it is important for the athlete to combat immunodepression, and glutamine would be particularly advantageous if it could be proved useful in this way, since it is not a banned substance. Its effects on performance per se are not convincing and, although space precludes citing every study, it is clear that more studies are needed to back up the small amount of evidence already reported [11283].

The aim of one study was to investigate whether supplementation of carbohydrate together with peptide glutamine would prevent anaerobic power decrease during repeated competitions. Twenty-eight physical education male students voluntarily participated in the study. Subjects were randomly divided on a maximal power (Max power) output value basis into four groups: 1) G group (oral ingestion of glutamine at the dose of 0.25 g/kg body mass in 250 ml of water), 2) M group (a single carbohydrate at a concentration of 50g of maltodextrin in 250 ml of water), 3) GM group (carbohydrate at a concentration of 50g of maltodextrin + glutamine at the dose of 0.25 g/kg body mass in 250 ml of water) and, 4) P group (just 250 ml of water and 30 gram sweetener). Each subject performed three times Running-based Anaerobic Sprint Test (RAST) with intervals of 1 hour. Max power, Minimal power (Min power) and fatigue were calculated for each participant. There was a significant decrease in Max and Min power in P group in time series. Furthermore, regarding the Max and Min power, there was significant difference between P and GM group in third bout indicating stronger influence of combination of maltodextrin and glutamine in comparison with pure consumption of glutamine and maltodextrin. It was concluded that it seems acute supplementation of glutamine and maltodextrin combination, 2 hours before exercise is more efficient in prevention of anaerobic power decrease than consumption of a pure carbohydrate or glutamine in repeated bouts of RAST protocol. Thus, supplementation with both carbohydrate and peptide glutamine improved the physical performance of athletes during repeated competitions [13656].

Glutamine has had its 15 min of fame, but it will not go away. In a 2012 survey of fitness and bodybuilding Internet sites, glutamine was one of the six most commonly touted supplements. These Internet sites tout glutamine as “essential for serious athletes” to “increase growth hormone” and “enhance muscle metabolism.” They claim glutamine is vital for optimal immune function. They claim you cannot get enough glutamine from food or make enough when you are training hard. Your best bet is to buy it from them. The drumbeat for glutamine that began 15 to 20 years ago was based on intriguing hypotheses. Glutamine is the most abundant free amino acid in muscle and plasma. Skeletal muscle is a major site for glutamine synthesis, and it releases glutamine into plasma at a high rate. Glutamine in muscle may buffer acids and help build glycogen and protein. Glutamine also fuels lymphocytes and macrophages. So glutamine is seen as possibly anabolic and immunity boosting. Plasma glutamine levels tend to be decreased for a few hours after prolonged exercise. A popular early hypothesis was that this decrease contributes to an “open window” of impaired immunity after marathons. So the myth was born: Glutamine supplements are vital for strong muscles and immunity [13657].

Studies in intensive care units

In ICU observational studies, low plasma glutamine levels, compared to moderate levels, have been tied to a higher mortality rate. The hypothesis was that loss of muscle led to
glutamine deficiency that weakened immune and other host defenses. But observational studies are tricky; one pitfall was that those few with the very highest glutamine levels (most had acute liver failure) also had a higher mortality rate. Self-correction is now occurring in glutamine research. The ICU observational studies that tied lower glutamine to higher mortality led to clinical trials of glutamine supplementation in critically ill ICU patients. Some trials suggested improved survival on glutamine, but the two largest ICU trials found no clear benefit from glutamine. Now comes the blockbuster study, a randomized controlled trial in a leading journal. More than 1,200 critically ill adults in 40 ICUs were randomized to receive glutamine, antioxidants, both, or placebo for up to 28 d. Glutamine had no effect on infections and did not improve clinical outcome. In fact, glutamine was tied to a slight increase in risk of death, statistically significant at 6 months. The authors concluded that glutamine “was harmful”, and an editorialist said this study allows one to reject with confidence the hypothesis that glutamine supplementation in very ill patients in the ICU improves outcome. It seems glutamine has no role in the ICU [13657].

**Glutamine, athletes, and immunity**

Glutamine has no role in sports, either, because the early hypotheses have not held up. Early studies found that plasma glutamine typically fell transiently after prolonged exercise, such as cycling nearly 4 h or running a marathon. One study suggested that two small doses of glutamine (vs placebo) after a marathon could reduce “infections,” self-reported on questionnaires, over the next week. However, such questionnaires are validated for classical viral upper respiratory infection (URI) and are less reliable for diagnosing URI in the face of allergies or stress reactions from the marathon. In other words, the pitfall is that not all “infections” after marathons are true infections. Other studies refute the hypothesis that low plasma glutamine after exercise increases the risk of infection. In a 4-week study of intensified training of swimmers, glutamine levels tended to increase, not fall, and no difference in glutamine levels was seen in swimmers who reported URI versus those who did not. In laboratory studies of athletes, glutamine supplements during exercise did not prevent the typical postexercise “immunodepression.” In other words, even though plasma glutamine stayed normal, these exercisers still developed transient decrements in lymphocyte counts and functions, in activity of natural killer cells, and in salivary immunoglobulin A levels. Experts now see no causal link between low plasma glutamine, impaired immune function, and increased risk of URI in athletes. Scientists and sports medicine physicians have known for a decade that plasma glutamine is not linked to any putative “exercise-induced immunodepression”, but glutamine sellers remain willfully ignorant on their way to the bank [13657].

**Glutamine, muscle function, and athletic performance**

The claim that glutamine can spur release of growth hormone seems to be based on one study of nine middle-aged volunteers. The mean value of plasma growth hormone, 90 min after a 2-g oral dose of glutamine, was 4-fold higher than the control (placebo) value, but sharp rises in growth hormone level after glutamine occurred in only 4 subjects, and the results were not statistically significant. It should be noted that 1 h of strenuous exercise could increase plasma growth hormone level 20-fold, so no reason exists for athletes in training to take glutamine to spur growth hormone release. Nor is there evidence that extra glutamine is anabolic. After glycogen-depleting exercise, glutamine cannot improve on glucose alone at restoring muscle glycogen. Adding glutamine to essential amino acids and carbohydrate does not enhance muscle glycogen or protein synthesis after glycogen-depleting endurance exercise. Finally, there is no good evidence that glutamine supplements improve athletic performance. When 10 trained men performed five maximal
cycling bouts, acute ingestion of glutamine did not enhance performance, and in a randomized, double-blind study of daily glutamine (vs placebo) during 6 weeks of strength training in 31 young adults, the glutamine added nothing: Gains in lean muscle mass and strength were the same on placebo as on glutamine. The bottom line is therefore still that there is no reason for athletes to buy or use glutamine supplements. Dr. George Phillips, writing 6 years ago, was right to conclude that the glutamine fad is another sad example of how marketing can trump science [13657].

Alanine

High-intensity exercise results in reduced substrate levels and accumulation of metabolites in the skeletal muscle. The accumulation of these metabolites (e.g. ADP, Pi and H+) can have deleterious effects on skeletal muscle function and force generation, thus contributing to fatigue. Clearly this is a challenge to sport and exercise performance and, as such, any intervention capable of reducing the negative impact of these metabolites would be of use. Carnosine (beta-alanyl-L-histidine) is a cytoplasmic dipeptide found in high concentrations in the skeletal muscle of both vertebrates and non-vertebrates and is formed by bonding histidine and beta-alanine in a reaction catalysed by carnosine synthase. Due to the pKa of its imidazole ring (6.83) and its location within skeletal muscle, carnosine has a key role to play in intracellular pH buffering over the physiological pH range, although other physiological roles for carnosine have also been suggested. The concentration of histidine in muscle and plasma is high relative to its K_m with muscle carnosine synthase, whereas beta-alanine exists in low concentration in muscle and has a higher K_m with muscle carnosine synthase, which indicates that it is the availability of beta-alanine that is limiting to the synthesis of carnosine in skeletal muscle. Thus, the elevation of muscle carnosine concentrations through the dietary intake of carnosine, or chemically related dipeptides that release beta-alanine on absorption, or supplementation with beta-alanine directly could provide a method of increasing intracellular buffering capacity during exercise, which could provide a means of increasing high-intensity exercise capacity and performance. One paper reviewed the available evidence relating to the effects of beta-alanine supplementation on muscle carnosine synthesis and the subsequent effects on exercise performance. In addition, the effects of training, with or without beta-alanine supplementation, on muscle carnosine concentrations were also reviewed [10411].

The influence of alanine on plasma amino acid concentrations and fuel substrates as well as cycling performance was examined. Four solutions (6 % alanine, ALA; 6 % sucrose, CHO; 6 % alanine and 6 % sucrose, ALA-CHO; and an artificially sweetened placebo, PLC) were tested using a double-blind, randomised, cross-over design. During each trial, ten cyclists ingested 500 mL of test solution 30 min before exercise and 250 mL after 15, 30, and 45 min of exercise. Participants cycled for 45 min at 75 percent VO_{2max} followed by a 15-min performance trial. Blood was collected before beverage consumption and prior to the performance trial. Alanine concentration was increased by approximately tenfold for ALA and ALA-CHO and less than twofold for CHO and PLC. Alanine ingestion increased concentrations of most gluconeogenic amino acids. Overall, alanine supplementation tended to produce favourable metabolic effects, but did not influence performance [09342].

It was examined the effect of beta-alanine supplementation plus sodium bicarbonate on high-intensity cycling capacity. Twenty males (age = 25 ± 5 years, height = 1.79 ± 0.06 m, body mass = 80.0 ± 10.3 kg) were assigned to either a placebo (P) or a beta-alanine (BA; 6.4 g/day) for 4 weeks) group based on power max, completing four cycling capacity tests at 110 percent of power max (CCT110%) to determine time to exhaustion (TTE) and total work done. A CCT110) was performed twice (habituation and baseline) before supplementation
(with maltodextrin) and twice after supplementation (with maltodextrin and with sodium bicarbonate), using a crossover design with 2 days of rest between trials, creating four study conditions (PMD, PSB, BAMD, and BASB). Blood pH, Lactate, bicarbonate and base excess were determined at baseline, before exercise, immediately after exercise, and 5 min after exercise. Data were analyzed using repeated-measures ANOVA. TTE was increased in all conditions after supplementation (+1.6 % PMD, +6.5 % PSB, +12.1 % BAMD, and +16.2 % BASB). Both BAMD and BASB resulted in significantly improved TTE compared with that before supplementation. Although further increases in TTE (4.1 %) were shown in BASB compared with BAMD, these differences were not significant. Differences in total work done were similar to those of TTE. Blood bicarbonate concentrations were significantly elevated before exercise in PSB and BASB but not in PMD or BAMD. Blood lactate concentrations were significantly elevated after exercise, remaining elevated after 5 min of recovery and were highest in PSB and BASB. It was concluded that the results show that beta-alanine improved high-intensity cycling capacity. However, despite a 6-s (about 4 %) increase in TTE with the addition of SB, this did not reach statistical significance, but magnitude-based inferences suggested a about 70 percent probability of a meaningful positive difference [11530].

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**L-alanine**

Dietary supplements containing L-arginine, a semi-essential amino acid, are one of the latest ergogenic aids intended to enhance strength, power and muscle recovery associated with both aerobic and resistance exercise. L-arginine is claimed to promote vasodilation by increasing nitric oxide (NO) production in the active muscle during exercise, improving strength, power and muscular recovery through increased substrate utilization and metabolite removal, such as lactate and ammonia. Research on L-arginine has recently tested this hypothesis, under the assumption that it may be the active compound associated with the vasodilator effects of NO. There were only five acute studies retrieved from the literature that evaluated exercise performance after L-arginine supplementation, three of which reported significant improvements. Regarding studies on chronic effects, eight studies
were encountered: four reported enhancements in exercise performance, whilst four reports showed no changes. Whether these improvements in exercise performance - regardless of the aerobic or anaerobic nature of the exercise - can be associated with increases in NO production, has yet to be demonstrated in future studies. Low oral doses (≤20 g) are well tolerated and clinical side effects are rare in healthy subjects. In summary, it is still premature to recommend dietary supplements containing L-arginine as an ergogenic aid for healthy physically active subjects [11284].

The ergogenic effect of L-arginine on an endurance-trained population is not well studied. The few studies that have investigated L-arginine on this population have not been conducted in a laboratory setting or measured aerobic variables. The purpose of one study was to determine if 28 days of L-arginine supplementation in trained male cyclists affects $V\text{O}_2\text{max}$ and ventilatory threshold (VT). Eighteen endurance-trained male cyclists performed a graded exercise test before and after 28 days of supplementation with L-arginine (ARG; 2 × 6 g·d) or placebo (PLA; cornstarch). The GXT was conducted on the subject’s own bicycle. $V\text{O}_2$ was continuously recorded, and VT was established by plotting the ventilatory equivalent for $O_2$ (VE/$V\text{O}_2$) and the ventilatory equivalent for $CO_2$ (VE/$V\text{CO}_2$) and identifying the point at which VE/$V\text{O}_2$ increases with no substantial changes in VE/$V\text{CO}_2$. L-arginine supplementation had no effect from initial $V\text{O}_2\text{max}$ to postsupplement $V\text{O}_2\text{max}$. Also, no effect was seen from initial VT to postsupplement VT. These results indicate that L-arginine does not impact $V\text{O}_2\text{max}$ or VT in trained male cyclists [11285].

**beta-Alanine**

Muscle carnosine synthesis is limited by the availability of beta-alanine. Thirteen male subjects were supplemented with beta-alanine for 4 weeks, 8 of these for 10 weeks. A biopsy of the vastus lateralis was obtained from 6 of the 8 at 0, 4 and 10 wks. Subjects undertook a cycle capacity test to determine total work done (TWD) at 110 percent (CCT(110%)) of their maximum power ($W_{max}$). Twelve matched subjects received a placebo. Eleven of these completed the CCT(110%) at 0 and 4 weeks, and 8, 10 weeks. Muscle biopsies were obtained from 5 of the 8 and one additional subject. Muscle carnosine was significantly increased by 59 percent and 80 percent after 4 and 10 weeks beta-alanine supplementation. Carnosine, initially 1.71 times higher in type IIa fibres, increased equally in both type I and IIa fibres. No increase was seen in control subjects. Taurine was unchanged by 10 weeks of supplementation. Four weeks of beta-alanine supplementation resulted in a significant increase in TWD (+13 %); with a further 3 percent increase at 10 weeks. TWD was unchanged at 4 and 10 weeks in the control subjects. The increase in TWD with supplementation followed the increase in muscle carnosine [07336].

beta-Alanine seems to be without adverse effects if the dose of 10 mg/kg body weight per 2-h interval, which can lead to paraesthesia, is not exceeded. It was also shown that carnosine loading leads to better performance during high-intensity exercise (cycling at 110 % of $W_{max}$ to exhaustion). With the availability of more recent studies, it becomes evident that mostly high-intensity efforts that last several minutes experience a positive influence of beta-alanine supplementation. Indeed, beta-alanine was not ergogenic for repeated sprint performance (10×5 s sprint), while both a simulated 2000-m rowing race (± 6 min) and the sprint at the end of 2-h simulated cycling race were improved by beta-alanine. It is plausible that high-intensity performance is affected in long-term vegetarians, as they are characterised with lower muscle carnosine levels (~26 %). The ergogenic mechanism of beta-alanine may reside in the attenuation of acidosis through the proton-buffering capacity of carnosine, whereas other physiological properties of carnosine (antioxidant, calcium sensitisation) cannot be excluded at present [11241].
Beta-alanine in blood-plasma when administered as A) histidine dipeptides (equivalent to 40 mg/kg bwt of beta-alanine) in chicken broth, or B) 10, C) 20 and D) 40 mg/kg bwt beta-alanine (CarnoSyn, NAI, USA), peaked at 428, 47, 374, and 833 microM. Concentrations regained baseline at 2 h. Carnosine was not detected in plasma with A) although traces of this and anserine were found in urine. Loss of beta-alanine in urine with B) to D) was <5 percent. Plasma taurine was increased by beta-alanine ingestion but this did not result in any increased loss via urine. Pharmacodynamics were further investigated with 3 x B) per day given for 15 d. Dietary supplementation with I) 3.2 and II) 6.4 g . d(-1) beta-alanine (as multiple doses of 400 or 800 mg) or III) L-carnosine (isomolar to II) for 4 w resulted in significant increases in muscle carnosine estimated at 42, 64 and 66 percent [06277].

Among the newer dietary supplements popular with athletes and fitness enthusiasts is beta-alanine, an amino acid that has a myriad of purported health and exercise benefits. Recent reviews on this topic. Beta-alanine is an amino acid that, when combined with histidine, forms the dipeptide carnosine within skeletal muscle. Carnosine and beta-alanine each have multiple purposes within the human body; one review focuses on their roles as ergogenic aids to exercise performance and suggests how to best quantify the former's merits as a buffer. Carnosine normally makes a small contribution to a cell's total buffer capacity; yet beta-alanine supplementation raises intracellular carnosine concentrations that in turn improve a muscle's ability to buffer protons. Numerous studies assessed the impact of oral beta-alanine intake on muscle carnosine levels and exercise performance. Beta-alanine may best act as an ergogenic aid when metabolic acidosis is the primary factor for compromised exercise performance. Blood lactate kinetics, whereby the concentration of the metabolite is measured as it enters and leaves the vasculature over time, affords the best opportunity to assess the merits of beta-alanine supplementation's ergogenic effect. Optimal beta-alanine dosages have not been determined for persons of different ages, genders and nutritional/health conditions. A recent meta-analysis shows a heightened exercise capacity, yet no significant improvement in performance, from oral beta-alanine supplementation. Meta-analysis results included a median beta-alanine intake of 179 g per intervention led to a median improvement of less than 3 percent. The disparity in results from these earlier reviews clearly indicate the types of studies and exercise bouts previously employed have not allowed oral beta-alanine supplementation to be optimally examined and utilized for its ergogenic properties. It is clear from examinations of cellular biochemistry that exercise which evokes intracellular H+ accrual may benefit most from oral beta-alanine intake. Yet some reviews offered limited information on exercise-induced cellular conditions (metabolic acidosis) that led to increased beta-alanine and carnosine research, as well as little quantitative evidence of the latter's prowess to continue to act as a potent buffer in the face of successively larger intramuscular pH decrements. Far from a mere recitation and review of prior study outcomes, one paper offered insights as to how beta-alanine may best be used, as well as how its ergogenic properties can be objectively and accurately quantified through continued exercise-based research. Paraesthesia is to date the only side effect from oral beta-alanine ingestion. The severity and duration of paraesthesia episodes are dose-dependent. It may be unwise for persons with a history of paraesthesia to ingest beta-alanine. As for any supplement, caution should be exercised with beta-alanine supplementation [12431].

The benefits associated with histidine dipeptides and muscle buffering capacity has been known for more than 20 years. However, the ergogenic role of beta-alanine when combined with histidine became popular before the 2008 Olympic games. Several studies published during the early part of the past decade suggested that beta-alanine ingestion by itself, or when combined with creatine, may significantly enhance anaerobic athletic performance (e.g. resistance exercise, repeated sprints) to a greater magnitude than a placebo or to creatine by
itself. These studies have indicated that beta-alanine can enhance the quality of a workout and sport performance by delaying skeletal muscle fatigue. This has resulted in a surge in its popularity over the last years. Recently, the benefits of beta-alanine supplementation on nonathletic and/or clinical populations has become of interest within the medical community. Its potential therapeutic role, especially in the aging population, may prove to be valuable. This article will examine the physiology of beta-alanine. The popularity of beta-alanine stems from its unique ability to enhance intramuscular buffering capacity and thereby attenuating fatigue. One review provided an overview of the physiology that underlies the mechanisms of action behind beta-alanine, examine dosing schemes, and examine the studies that have been conducted on the efficacy of this supplement. The ergogenic benefits of beta-alanine supplementation thus are most evident in activities that elicit a strong intramuscular acidotic condition (intense exercise between 60 and 240 s) by delaying the onset of skeletal muscle fatigue. A delay in fatigue during repeated or prolonged sprinting and an enhanced volume of resistance training (greater number of repetitions performed) are common responses observed following beta-alanine ingestion [12432].

It was examined the isolated and combined effects of beta-alanine (BA) and sodium bicarbonate (SB) on high-intensity intermittent upper-body performance in judo and jiu-jitsu competitors. Thirty-seven athletes were assigned to one of four groups: (1) placebo (PL)+PL; (2) BA+PL; (3) PL+SB or (4) BA+SB. BA or dextrose (placebo) (6.4 g/day) was ingested for 4 weeks and 500 mg7kg BM of SB or calcium carbonate (placebo) was ingested for 7 days during the 4th week. Before and after 4 weeks of supplementation, the athletes completed four 30-s upper-body Wingate tests, separated by 3 min. Blood lactate was determined at rest, immediately after and 5 min after the 4th exercise bout, with perceived exertion reported immediately after the 4th bout. BA and SB alone increased the total work done in 7 and 8 percent, respectively. The co-ingestion resulted in an additive effect (+14). BA alone significantly improved mean power in the 2nd and 3rd bouts and tended to improve the 4th bout. SB alone significantly improved mean power in the 4th bout and tended to improve in the 2nd and 3rd bouts. BA+SB enhanced mean power in all four bouts. PL+PL did not elicit any alteration on mean and peak power. Post-exercise blood lactate increased with all treatments except with PL+PL. Only BA+SB resulted in lower ratings of perceived exertion.. Chronic BA and SB supplementation alone equally enhanced high-intensity intermittent upper-body performance in well-trained athletes. Combined BA and SB promoted a clear additive ergogenic effect [13657].

Beta-alanine may benefit short duration, high-intensity exercise performance. The aim of one randomised, double-blind, placebo controlled study was to examine the effects of beta-alanine supplementation on aspects of muscular performance in highly-trained cyclists. Sixteen highly-trained cyclists (mean±SD; age 24 ± 7 years) supplemented with either beta-alanine (n=8, 65 mg/kg BM) or a placebo (n=8, dextrose monohydrate) over four weeks. Pre- and post-supplementation cyclists performed: 1) a four-minute maximal cycling test to measure average power and 2) 30 reciprocal maximal isokinetic knee contractions at a fixed angular velocity of 180°·sec⁻¹ to measure average power/repetition, total work done (TWD) and fatigue index (%). Blood pH, lactate (La⁻) and bicarbonate concentrations were measured pre- and post-isokinetic testing at baseline and following the supplementation period. β-alanine supplementation was 44% likely to increase average power output during the four-minute cycling time-trial when compared to the placebo, although this was not statistically significant. Isokinetic average power/repetition was significantly increased post-beta-alanine supplementation, while fatigue index was significantly reduced. TWD was 89 percent likely to be improved following beta-alanine supplementation, however this was not statistically significant. There were no significant differences in blood pH, lactate and HCO₃⁻ between groups. Four weeks of beta-alanine supplementation resulted in worthwhile changes in time-trial performance and short-duration muscular force production in highly-
trained cyclists [13659].

Beta-alanine supplementation has been shown to improve exercise performance in short-term, high-intensity efforts. The aim of one study was to assess if beta-alanine supplementation could improve 800 m track running performance in male recreational club runners (n=18). Participants completed duplicate trials (2 pre-supplementation, 2 post-supplementation) of an 800 m race, separated by 28 days of either beta-alanine (n=9; 80 mg/kg BM/day) or placebo (n=9) supplementation (single blind design). Using ANCOVA (pre-supplementation times as covariate), post-supplementation race times were significantly faster following beta-alanine, with post- versus pre-supplementation race times being faster after beta-alanine but not placebo. These improvements were supported by a moderate effect size and a “very likely” (99 %) benefit in the beta-alanine group after supplementation. Split times (ANCOVA) at 400 m were significantly faster post-supplementation in the beta-alanine group, compared with placebo. This was supported by large effect sizes and a “very likely” (99 %) benefit at the 400 and 800 m splits when comparing pre- to post-supplementation with beta-alanine. Additionally, the first and second halves of the race were faster post- compared to pre-supplementation following beta-alanine. No significant differences between groups or pre- and post-supplementation were observed for post-race blood lactate and pH. Thus, overall, 28 days of beta-alanine supplementation improved 800 m track performance in recreational club runners [13660].

Beta-alanine (BA) is a popular ergogenic supplement because it can induce muscle carnosine loading. It was hypothesize that, by analogy with creatine supplementation, an inverse relationship between urinary excretion and muscle loading is present, and the latter is stimulated by carbohydrate- and protein-induced insulin action. In study A, the effect of a 5-week slow-release BA (SRBA) supplementation (4.8 g/day) on whole body BA retention was determined in seven men. It was further determined whether the coingestion of carbohydrates and proteins with SRBA would improve retention. In study B (34 subjects), it was explored the effect of meal timing on muscle carnosine loading (3.2 g/day) during 6-7 weeks). One group received pure BA (PBA) in between the meals; the other received PBA at the start of the meals, to explore the effect of meal-induced insulin release. Further, it was compared with a third group receiving SRBA at the start of the meals. Orally ingested SRBA has a very high whole body retention (97 %-98 %) that is not declining throughout the 5-week supplementation period, nor is it influenced by the coingestion of macronutrients. Thus, a very small portion (1 %-2 %) is lost through urinary excretion, and equally only a small portion is incorporated into muscle carnosine (about 3 %), indicating that most ingested BA is metabolized (possibly through oxidation). Second, in soleus muscles, the efficiency of carnosine loading is significantly higher when PBA is coingested with a meal (+64 %) compared with in between the meals (+41 %), suggesting that insulin stimulates muscle carnosine loading. Finally, the chronic supplementation of SRBA versus PBA seems equally effective [13658].

To critically review the methodological quality and synthesize information from systematic reviews and high quality studies on the effects of beta alanine (BA) on exercise and athletic performance a search strategy was developed in accordance with the standards for the reporting of scientific literature via systematic reviews. Five databases were thoroughly searched from inception to November 2012. Inclusion criteria were English language, human studies, used BA to increase exercise or athletic performance, systematic reviews or randomized controlled trials and were published in a peer-reviewed journal. Included studies were systematically graded for their methodological quality by rotating pairs of reviewers and the results were qualitatively synthesized. One systematic review and nineteen randomized trials were included in this review. There is one systematic review with several methodological weaknesses that limit the confidence in its results. There are moderate to
high quality studies that appear to support that BA may increase power output and working capacity, decrease the feeling of fatigue and exhaustion, and have of positive effect on body composition and carnosine content. The reporting of side effects from BA supplementation in the athletic population was generally under-reported. It was concluded that there appears to be some evidence from this review that supplementation with BA may increase athletic performance. However, there is insufficient evidence examining the safety of BA supplementation and its side effects. It is therefore recommended to err on the side of caution in using BA as an ergogenic aid until there is sufficient evidence confirming its safety [13659].

beta-Alanine supplementation has become a common practice among competitive athletes participating in a range of different sports. Although the mechanism by which chronic beta-alanine supplementation could have an ergogenic effect is widely debated, the popular view is that beta-alanine supplementation augments intramuscular carnosine content, leading to an increase in muscle buffer capacity, a delay in the onset of muscular fatigue and a facilitated recovery during repeated bouts of high-intensity exercise. beta-Alanine supplementation appears to be most effective for exercise tasks that rely heavily on ATP synthesis from anaerobic glycolysis. However, research investigating its efficacy as an ergogenic aid remains equivocal, making it difficult to draw conclusions as to its effectiveness for training and competition. The aim of one review was to update, summarise and critically evaluate the findings associated with beta-alanine supplementation and exercise performance with the most recent research available to allow the development of practical recommendations for coaches and athletes. A critical review of the literature reveals that when significant ergogenic effects have been found, they have been generally shown in untrained individuals performing exercise bouts under laboratory conditions. The body of scientific data available concerning highly-trained athletes performing single competition-like exercise tasks indicates that this type of population receives modest but potentially worthwhile performance benefits from beta-alanine supplementation. Recent data indicate that athletes may not only be using beta-alanine supplementation to enhance sports performance, but also as a training aid to augment bouts of high-intensity training. beta-Alanine supplementation has also been shown to increase resistance training performance and training volume in team-sport athletes, which may allow for greater overload and superior adaptations compared to training alone. The ergogenic potential of beta-alanine supplementation for elite athletes performing repeated high-intensity exercise bouts, either during training or during competition in sports which require repeated maximal efforts (e.g. rugby and soccer) needs scientific confirmation [13660].

Beta-alanine (BA) is a non-essential amino acid that combines with L-histidine, to form the dipeptide carnosine. BA is thought to be the rate-limiting step in the synthesis of carnosine. Carnosine acts as an intracellular buffer during high-intensity exercise, and elevations in muscle carnosine concentration have been demonstrated to enhance cycling capacity, ventilatory threshold, and delay fatigue. A meta-analysis has shown a significant ergogenic effect of BA supplementation during high intensity exercise lasting 60–240 s in duration. However, the efficacy of BA supplementation during single exercise durations shorter than 60 s durations is not clear. Although the efficacy of BA on repeated sprint performance is not very well known, studies examining BA and resistance training performance have indicated significant increases in training volume, suggesting that BA ingestion would be beneficial for repetitive high intensity exercise activities [13608].

It was aimed to manipulate physiological determinants of severe exercise performance. We hypothesized that beta-alanine supplementation would increase intramuscular carnosine and buffering capacity and dampen acidosis during severe cycling, that high-intensity interval training (HIT) would enhance aerobic energy contribution during severe cycling, and that HIT
preceded by beta-alanine supplementation would have greater benefits. Sixteen active men performed incremental cycling tests and 90-s severe (110 % peak power) cycling tests at three time points: before and after oral supplementation with either beta-alanine or placebo, and after an 11-days HIT block (9 sessions, 4 × 4 min), which followed supplementation. Carnosine was assessed via MR spectroscopy. Energy contribution during 90-s severe cycling was estimated from the O₂ deficit. Biopsies from m. vastus lateralis were taken before and after the test. Beta-alanine increased leg muscle carnosine. Buffering capacity and incremental cycling were unaffected, but during 90-s severe cycling, beta-alanine increased aerobic energy contribution concurrent with reduced O₂ deficit and muscle lactate accumulation while having no effect on pH. beta-Alanine also enhanced motivation and perceived state during the HIT block. There were no between-group differences in adaptations to the training block, namely increased buffering capacity and glycogen storage. It was concluded that beta-alanine did not affect buffering considerably, but has beneficial effects on severe exercise metabolism as well as psychological parameters during intense training phases [13661].

Carnosine (beta-alanyl-l-histidine) is present in high concentrations in human skeletal muscle. The ingestion of beta-alanine, the rate-limiting precursor of carnosine, has been shown to elevate the muscle carnosine content. It was aimed to investigate, using proton magnetic resonance spectroscopy (proton MRS), whether oral supplementation with beta-alanine during 4 weeks would elevate the calf muscle carnosine content and affect exercise performance in 400-m sprint-trained competitive athletes. Fifteen male athletes participated in a placebo-controlled, double-blind study and were supplemented orally for 4 weeks with either 4.8 g/day beta-alanine or placebo. Muscle carnosine concentration was quantified in soleus and gastrocnemius by proton MRS. Performance was evaluated by isokinetic testing during five bouts of 30 maximal voluntary knee extensions, by endurance during isometric contraction at 45 percent maximal voluntary contraction, and by the indoor 400-m running time. beta-Alanine supplementation significantly increased the carnosine content in both the soleus (+47 %) and gastrocnemius (+37 %). In placebo, carnosine remained stable in soleus, while a small and significant increase of +16 percent occurred in gastrocnemius. Dynamic knee extension torque during the fourth and fifth bout was significantly improved with beta-alanine but not with placebo. Isometric endurance and 400-m race time were not affected by treatment. In conclusion, proton MRS can be used to noninvasively quantify human muscle carnosine content; muscle carnosine is increased by oral beta-alanine supplementation in sprint-trained athletes; carnosine loading slightly but significantly attenuated fatigue in repeated bouts of exhaustive dynamic contractions; and the increase in muscle carnosine did not improve isometric endurance or 400-m race time [07337].

One study examined the effects of 28 days of beta-alanine supplementation on the physical working capacity at fatigue threshold (PWCFT), ventilatory threshold (VT), maximal oxygen consumption (VO₂max), and time-to-exhaustion (TTE) in women. Twenty-two women (age 27 ± 6 years) participated and were randomly assigned to either the beta-alanine or Placebo (PL) group. Before (pre) and after (post) the supplementation period, participants performed a continuous, incremental cycle ergometry test to exhaustion to determine the PWCFT, VT, VO₂max, and TTE. There was a 14, 13 and 3 percent increase in VT, PWCFT, and TTE, respectively, for the beta-alanine group, with no changes in the PL. There were no changes for VO₂max in either group. Results of this study indicate that beta-alanine supplementation delays the onset of neuromuscular fatigue and the ventilatory threshold at submaximal workloads, and increase in TTE during maximal cycle ergometry performance. However, beta-alanine supplementation did not affect maximal aerobic power (VO₂max). In conclusion, beta-alanine supplementation appears to improve submaximal cycle ergometry performance and TTE in young women, perhaps as a result of an increased buffering capacity due to elevated muscle carnosine concentrations [07338].
**Physiology of beta-alanine**

beta-Alanine is a nonessential nonproteogenic amino acid that is synthesized in the liver. It will also be consumed in many of our foods, such as beef, chicken, and turkey. When beta-alanine is ingested as part of a meal, it is generally in the form of a histidine-containing dipeptide such as anserine, balenine, or carnosine. It is carnosine that is predominant in the human skeletal muscle. The hydrolysis of these dipeptides yields beta-alanine, which is then absorbed into the skeletal tissue for the resynthesis of carnosine. The ergogenic properties of beta-alanine by itself appear to be very limited, but when combined with histidine to form carnosine in the skeletal muscle, it does appear to have ergogenic effects. The primary role of carnosine is in the maintenance of acid-base homeostasis through enhanced intramuscular hydrogen ion (H⁺) buffering capacity. Increasing intramuscular carnosine concentration through beta-alanine supplementation has demonstrated an ergogenic potential for endurance, strength, and power athletes. In a recent meta-analysis, beta-alanine proved to be ergogenic for maximal exercise lasting 60 to 240 s [12432].

**Efficacy of beta-alanine supplementation**

The mechanism of action emanating from beta-alanine ingestion suggests that it would be most effective in performances involving high intensity activity. The investigations that have focused on this aspect of athletic performance have consistently reported positive results in the ergogenic benefit of A-alanine supplementation. However, the lack of significant strength improvement is consistent with other studies that have failed to show significant improvements in strength following beta-alanine supplement durations lasting between 4 and 10 weeks. These results are not surprising considering the physiological changes that are influenced by elevations in muscle carnosine concentrations. An improved intramuscular buffering system has its greatest effect on fatiguing-type exercises by extending the duration of exercise but does not appear to have a direct effect on strength development during these relatively short-duration training protocols. Such training durations may not be of sufficient length to stimulate significant strength improvements, especially in experienced resistance-trained. The benefits of beta-alanine ingestion and endurance exercise appear to be inconclusive. While there is limited evidence to suggest an ergogenic effect of beta-alanine supplementation on endurance performance, the use of beta-alanine supplementation may improve cardiovascular fitness and increase total work done in recreationally active individuals. Decreases in muscle carnosine concentrations may pose potential health risks. Carnosine acts as an antioxidant, whereby it may scavenge reactive oxygen species, aldehydes, ketones, and inflammatory cells. A deficiency of muscle carnosine concentrations may increase oxidative stress-induced lipid and protein oxidation, increase inflammation, and possibly impair insulin signaling in skeletal muscle [12432].

**Side effects associated with beta-alanine supplementation**

The only known adverse effect associated with A-alanine supplementation is flushing (also sometimes experienced as paresthesia). Paresthesia is a sensation of numbing or tingling in the skin and often appears when high doses of beta-alanine are ingested. It generally disappears within 1 h following ingestion. If A-alanine is mixed with a carbohydrate and electrolyte drink, the appearance of this adverse effect seems negligible [12432].

**beta-Alanine dosing scheme**

It was examined the effect of three different dosing regimens (10, 20, and 40 mg/kg body weight). The two highest doses yielded the greatest increase in plasma beta-alanine concentrations, but these doses also were associated with uncomfortable adverse effects (e.g. paresthesias as a tingling-like sensation felt in the skin) that prohibit those dosages from being used. The kinetics of the A-alanine response to the low dosing scheme was a time to peak in plasma A-alanine concentration of 30 to 40 min following ingestion, a
concentration half-life (time at which there is a 50% reduction in peak concentration) of 25 min, and a return-to-baseline concentration by 3 h after ingestion. According to this kinetic profile, the appropriate dosing regimen should be 0.8 g of A-alanine taken every 3 to 4 h. This would provide a daily dosing regimen of 4.8 to 6.4 g/day. Recent studies have examined the use of time-release capsule technology and have demonstrated that dosages of 1.6 g per ingestion four times per day also can be consumed without any adverse effects and result in a 40 percent increase in muscle carnosine concentrations. This latter method appears to be a more practical dosing pattern that has slower absorption kinetics, improved whole-body retention, and sensory side effects that cannot be differentiated from placebo. On the basis of the available evidence, it is not clear whether a ceiling effect exists regarding increases in muscle carnosine content and A-alanine supplementation. Limitations may be more related to the adverse effects associated with higher dosing regimens. With the use of slow-release capsules, the rate of A-alanine release into circulation is slowed, allowing less excretion through the urine, resulting in a greater percentage of A-alanine retained for carnosine synthesis in the muscle [12432].

Just like any dietary supplement, optimal beta-alanine dosages are based on factors such as a person’s age, gender and nutritional/health practices. Unfortunately most studies have used young- or college-age men. Results suggest ergogenic effects are more likely at higher dosages. In contrast upon cessation of beta-alanine intake carnosine levels decline in a linear fashion at a relatively slow rate of 2 percent per-week until pre-supplementation values of the dipeptide are restored. High initial intramuscular carnosine levels do not apparently limit a cell’s ability to accrue further increases of the dipeptide. Some believe persons with naturally lower absolute carnosine concentrations (those with high percentages of type I muscle fibers, women, vegetarians, elderly) respond best to oral beta-alanine ingestion. A single 400 mg beta-alanine dose produced favorable changes in blood-based markers, but did not improve exercise performance as compared to a placebo intervention. Such a dose might represent first pass liver metabolism that must be overcome before deposition into muscle cells occurs. For 30-day interventions devoid of physical activity a 4.8 g/day, but not a 1.2 g/day, beta-alanine dosage had an ergogenic effect on resistive exercise performance. Thus the minimal β-alanine dose needed for an ergogenic effect may reside between 1.2 and 4.8 g/day in healthy men. Dosages higher than 6.4 g/day have yet to be studied [12431].

Precautions

Just like any dietary supplement, beta-alanine may be abused if not consumed in a proper fashion; in addition the manufacturers do not typically specify the production purity and presence of trace contaminants, which has received little prior attention. Beta-alanine precautions relate to its potential to induce paraesthesia, characterized by heightened sensitivity of nociceptive neurons that transmit neuropathic pain, which lead to flushing and prickly sensations on the skin. The severity of paraesthesia episodes is dose-dependent but generally last 60 min after ingestion. In some cases beta-alanine intake was curtailed or terminated due to the severity of the paraesthesia. When beta-alanine was consumed as part of a chicken broth elixir at the 40 mg/kg body mass dose no side effects occurred, yet when that same dosage was given as an oral dietary supplement some subjects incurred paraesthesia. Given this finding, it appears in addition to the peak dosage given, the occurrence of beta-alanine-induced paraesthesia episodes may relate to the manner of its administration. It was suggested individual beta-alanine doses should mimic those incurred from a normal diet to limit the risk of paraesthesia. The same project also assessed chronic beta-alanine administration in healthy men. Several dosing strategies were examined; one of which entailed intake of a 10 mg/kg body mass dose three times per day for two weeks. The dosing protocol elicited few side effects that included occasional mild flushing. Two other strategies entailed 3.2 and 6.4 g/day given to healthy men for four weeks in 400–800 mg doses. The four-week protocols produced a few cases of mild paraesthesia and a sore throat.
in one subject. Thus it appears paraesthesia-like symptoms begin at dosages above 10 mg/kg body mass. The prevalence and severity of paraesthesia relate to peak beta-alanine levels in the bloodstream which led to the demand for time-release versions of the supplement. Despite recent studies, unanswered questions still remain. While numerous trials examined its role as an ergogenic aid, the potential of oral beta-alanine intake to improve buffer capacity has yet to be fully quantified. It is thus important to measure, from both in-vitro and in-vivo trials, for a given oral beta-alanine dosage the amount of carnosine accretion, and magnitude of increase in intracellular buffering capacity. Since lactate offers an indication of the degree of H+ accrual and acidosis, measurements of blood lactate concentrations over time foretells concurrent intracellular changes in response to periods of exercise and recovery. In theory beta-alanine intake should permit higher work capacities before acidosis-induced exercise cessation, despite attainment of greater peak blood lactate levels. New trials should compare changes to blood lactate values from supramaximal exercise bouts performed with and without prior beta-alanine supplementation. Until such studies are conducted on physical activity paradigms that entail successive bouts of supramaximal exercise, the true extent of ergogenic effects from beta-alanine will remain unresolved. Future recommendations also include determination of optimal beta-alanine doses for persons based upon their age, gender and nutritional/health practices. The occurrence of paraesthesia in a variety of human subjects, from both standard and time-released versions of the supplement, should continue to be assessed [12431].

**Combined with bicarbonate**

Despite the large variety of so-called ergogenic supplements used by the sporting community, only few of them are effectively supported by scientific proof. One of the recent evidence-based supplements that entered the market is beta-alanine. Beta-alanine is the rate-limiting precursor for the synthesis of the dipeptide carnosine (beta-alanyl-L-histidine) in human muscle. The chronic daily ingestion of beta-alanine can markedly elevate muscle carnosine content, which results in improved exercise capacity, especially in sports that include high-intensity exercise episodes. The use of beta-alanine is exponentially growing in recent years. One chapter aimed to discuss the scientific basis and physiological background of beta-alanine and its synthesis product carnosine, and translate these scientific findings to practical applications in sports [13663].

Enhanced carnosine levels have been shown to be ergogenic for high-intensity exercise performances, although the role of carnosine in the control of muscle function is poorly understood. Therefore, the aim of this study was to investigate the effect of long-term supplementation with increasing doses of carnosine and beta-alanine on muscle carnosine, anserine, and taurine levels and on in vitro contractility and fatigue in mice. Male Naval Medical Research Institute mice (n= 66) were control fed or supplemented with either carnosine (0.1 %, 0.5 %, or 1.8 %) or beta-alanine (0.6 or 1.2 %) in their drinking water for 8-12 wk. Soleus and extensor digitorum longus (EDL) were tested for in vitro contractile properties, and carnosine, anserine, and taurine content were measured in EDL and tibialis anterior by high-performance liquid chromatography. Only supplementation with 1.8 percent carnosine and 1.2 percent beta-alanine resulted in markedly higher carnosine (up to +160 %) and anserine levels (up to +46 %) compared with control mice. Beta-alanine supplementation (1.2 %) resulted in increased fatigue resistance in the beginning of the fatigue protocol in soleus (+2 %-% 4 %) and a marked leftward shift of the force-frequency relation in EDL (10 %-% 31 % higher relative forces). It was concluded that comparable with humans, beta-alanine availability seems to be the rate-limiting step for synthesis of muscle histidine-containing dipeptides in mice. Moreover, muscle histidine-containing dipeptides loading in mice moderately and muscle dependently affects excitation-contraction coupling and fatigue [13664].
One study aimed to investigate if combining beta alanine (BA) and sodium bicarbonate (NaHCO₃) supplementation could lead to enhanced repeated-sprint performance in team-sport athletes, beyond what is possible with either supplement alone. Participants (n=24) completed duplicate trials of a repeated-sprint test (3 sets; 6 × 20 m departing every 25 seconds, 4 minutes active recovery between sets) and were then allocated into 4 groups as follows: BA only (n=6; 28 days BA, acute sodium chloride placebo); NaHCO₃ only (n=6; 28 days glucose placebo, acute NaHCO₃); BA/NaHCO₃ (n=6; 28 days BA, acute NaHCO₃); placebo only (n=6; 28 days glucose placebo, acute sodium chloride placebo), then completed duplicate trials postsupplementation. Sodium bicarbonate alone resulted in moderate effect size and "likely" and "very likely" benefit for overall total sprint times (TST) and for each individual set and for first sprint (sets 2 and 3) and best sprint time (sets 2 and 3). Combining BA and NaHCO₃ resulted in "possible" to "likely" benefits for overall TST and for sets 2 and 3. First sprint (set 3) and best sprint time (sets 2 and 3) also showed "likely" benefit after this trial. The BA and placebo groups showed no differences in performance after supplementation. In conclusion, these results indicate that supplementation with acute NaHCO₃ improved repeated-sprint performance more than either a combination of NaHCO₃ and BA or BA alone [13665].

Lack of effect on sprint
Recent research has shown that beta-alanine (BA) supplementation can increase intramuscular carnosine levels. Carnosine is an intramuscular buffer, and it has been linked to improvements in performance, specifically during bouts of high-intensity exercise that are likely limited by muscle acidosis. Therefore, the purpose of one study was to examine the effect of BA supplementation on sprint endurance at 2 different supramaximal intensities. Twenty-one anaerobically trained (4 rugby players, 11 wrestlers, and 60 recreationally strength trained athletes) college-aged men participated in a double-blind, placebo controlled study. The subjects performed an incremental VO₂max test and 2 sprint to exhaustion tests set at 115 and 140 percent of their VO₂max on a motorized treadmill before (PRE) and after (POST) a 5-week supplementation period. During this time, the subjects ingested either a BA supplement or placebo (PLA) with meals. The subjects ingested 4 g/day of BA or PLA during the first week and 6 g/day the following 4 weeks. Capillary blood samples were taken before and after each sprint to determine blood lactate response to the sprint exercise. No significant group (BA, PLA) × intensity), group by time, or group × intensity × time interactions were observed for time to exhaustion. In addition, similar nonsignificant observations were made for lactate response to the sprint group. From the results of this study, it was concluded that beta-alanine supplementation did not have a significant effect on sprint endurance at supramaximal intensities [13666].

Running
Beta-alanine supplementation has been shown to improve exercise performance in short-term, high-intensity efforts. The aim of one study was to assess if beta-alanine supplementation could improve 800 m track running performance in male recreational club runners (n=18). Participants completed duplicate trials (2 presupplementation, 2 postsupplementation) of an 800 m race, separated by 28 days of either beta-alanine (n=9; 80 mg/kg BM/day) or placebo (n=9) supplementation. Using ANCOVA (presupplementation times as covariate), postsupplementation race times were significantly faster following beta-alanine, with post- versus presupplementation race times being faster after beta-alanine but not placebo. These improvements were supported by a moderate effect size and a very likely (99 %) benefit in the beta-alanine group after supplementation. Split times (ANCOVA) at 400 m were significantly faster postsupplementation in the beta-alanine group, compared with placebo. This was supported by large effect sizes and a very likely (99 %) benefit at the 400 and 800 m splits when comparing pre- to postsupplementation with beta-alanine. In addition, the first and second halves of the race were faster post- compared with presupplementation.
following beta-alanine (1st half likely 78% chance of benefit; 2nd half very likely 98% chance of benefit). No significant differences between groups or pre- and postsupplementation were observed for postrace blood lactate and pH. It was concluded that overall, 28 days of beta-alanine supplementation (80 mg/kg BM/day) improved 800 m track performance in recreational club runners [13667].

**Biking**

Beta-alanine may benefit short-duration, high-intensity exercise performance. The aim of one randomized double-blind placebo-controlled study was to examine the effects of beta-alanine supplementation on aspects of muscular performance in highly trained cyclists. Sixteen highly trained cyclists (age 24 ± 7 years) supplemented with either beta-alanine (n=8, 65 mg/kg BM) or a placebo (n=8; dextrose monohydrate) over 4 weeks. Pre- and postsupplementation cyclists performed a 4-minute maximal cycling test to measure average power and 30 reciprocal maximal isokinetic knee contractions at a fixed angular velocity of 180°-sec-1 to measure average power/repetition, total work done (TWD), and fatigue index (%). Blood pH, lactate (La-) and bicarbonate (HCO₃⁻) concentrations were measured pre- and postsisokinetic testing at baseline and following the supplementation period. Beta-alanine supplementation was 44 percent likely to increase average power output during the 4-minute cycling time trial when compared with the placebo, although this was not statistically significant. Isokinetic average power/repetition was significantly increased post beta-alanine supplementation compared with placebo (85% likely benefit), while fatigue index was significantly reduced (95% likely benefit). TWD was 89 percent likely to be improved following beta-alanine supplementation; however, this was not statistically significant. There were no significant differences in blood pH, lactate, and bicarbonate between groups. Four weeks of beta-alanine supplementation resulted in worthwhile changes in time-trial performance and short-duration muscular force production in highly trained cyclists [13668].

**Alanine plus creatine**

It has been examined the combination of both creatine and beta-alanine supplements. The hypothesis was that this combination of supplements would provide a significant benefit for strength/power athletes. Results of one study demonstrated that this combination significantly improved the quality of the workout more so than creatine alone. Specifically, improvements in training volume were found to be associated with significantly greater gains in lean body mass and decreases in fat mass [12432].

The effect of beta-alanine (beta-Ala) alone or in combination with creatine monohydrate (Cr) on aerobic exercise performance is unknown. The purpose of this study was to examine the effects of 4 weeks of beta-Ala and Cr supplementation on indices of endurance performance. Fifty-five men (25 years) participated in a double-blind, placebo-controlled study and randomly assigned to one of 4 groups; placebo (PL, n=13), creatine (Cr, n=12), beta-alanine (beta-Ala, n=14), or beta-alanine plus creatine (CrBA, n=16). Prior to and following supplementation, participants performed a graded exercise test on a cycle ergometer to determine VO₂peak, time to exhaustion (TTE), and power output, VO₂, and percent VO₂peak associated with VT and LT. No significant group effects were found. However, within groups, a significant time effect was observed for CrBA on 5 of the 8 parameters measured. These data suggest that CrBA may potentially enhance endurance performance [07339].

The effects of creatine and creatine plus beta-alanine on strength, power, body composition, and endocrine changes were examined during a 10-wk resistance training program in collegiate football players. Thirty-three male subjects were randomly assigned to either a placebo (P), creatine (C), or creatine plus beta-alanine (CA) group. During each testing session subjects were assessed for strength (maximum bench press and squat), power
Wingate anaerobic power test, 20-jump test), and body composition. Resting blood samples were analyzed for total testosterone, cortisol, growth hormone, IGF-1, and sex hormone binding globulin. Changes in lean body mass and percent body fat were greater in CA compared to C or P. Significantly greater strength improvements were seen in CA and C compared to P. Resting testosterone concentrations were elevated in C, however, no other significant endocrine changes were noted. Results of this study demonstrate the efficacy of creatine and creatine plus beta-alanine on strength performance. Creatine plus beta-alanine supplementation appeared to have the greatest effect on lean tissue accretion and body fat composition [06278].

The purpose of one study was to examine the effects of 28 days of beta-alanine (b-Ala) and creatine monohydrate (CrM) supplementation on the onset of neuromuscular fatigue by using the physical working capacity at neuromuscular fatigue threshold (PWCFT) test in untrained men. Fifty-one men (mean age 25) volunteered to participate in this 28-day, double-blind, placebo-controlled study and were randomly assigned to 1 of 4 groups: placebo (PLA; 34 g dextrose; n=13), CrM (5.25 g CrM plus 34 g dextrose; n=12), b-Ala (1.6 g b-Ala plus 34 g of dextrose; n=12), or b-Ala plus CrM (CrBA; 5.25 g CrM plus 1.6 g b-Ala plus 34 g dextrose; n=14). The supplement was ingested 4 times per day for 6 consecutive days, then twice per day for 22 days before posttesting. Before and after the supplementation, subjects performed a continuous incremental cycle ergometry test while a surface electromyographic signal was recorded from the vastus lateralis muscle to determine PWCFT. The adjusted mean posttest PWCFT values (covaried for pretest PWCFT values) for the b-Ala and CrBA groups were greater than those for the PLA group. However, there were no differences between the CrM versus PLA, CrBA versus b-Ala, CrM versus b-Ala, or CrM versus CrBA groups. These findings suggested that b-Ala supplementation may delay the onset of neuromuscular fatigue. Furthermore, there appeared to be no additive or unique effects of CrM versus b-Ala alone on PWCFT [06279].

The effect of beta-alanine (beta-Ala) alone or in combination with creatine monohydrate (Cr) on aerobic exercise performance is unknown. The purpose of one study was to examine the effects of 4 weeks of beta-Ala and Cr supplementation on indices of endurance performance. Fifty-five men (25 years) participated in a double-blind, placebo-controlled study and randomly assigned to one of 4 groups; placebo (PL, n=13), creatine (Cr, n=12), beta-alanine (beta-Ala, n=14), or beta-alanine plus creatine (CrBA, n=16). Prior to and following supplementation, participants performed a graded exercise test on a cycle ergometer to determine VO2peak, time to exhaustion (TTE), and power output, VO2, and percent VO2peak associated with VT and LT. No significant group effects were found. However, within groups, a significant time effect was observed for CrBA on 5 of the 8 parameters measured. These data suggest that CrBA may potentially enhance endurance performance [06280].

Methionine

Methionine is an indispensable amino acid that acts as a substrate, much like the other indispensable amino acids, for building functional muscle proteins. It is clear, however, that methionine also holds a unique role, when compared against the other indispensable amino acids, as it serves as a methyl group donor for DNA/RNA intermediates and for the synthesis of cysteine. Daily methionine requirement, reported as a constituent of the requirement for total sulphur amino acids, is 13-15 mg/kg/day with the risk for toxicity manifesting only at levels exceeding >10-fold excess in humans. Daily requirements of methionine are easily obtained in the diet due to its widespread abundance in meats, eggs, cheeses, fruits and vegetables. However, vegan athletes may need increased self-awareness of daily methionine intake. Supplemental methionine have very little effect for enhancement of the
performance, provided the athlete is healthy and consuming a mixed diet containing an adequate amount of energy to meet training needs. Indeed, methionine is fundamental, together with arginine and glycine, for the endogenous synthesis of creatine. However, there is no evidence to suggest that additional crystalline methionine to normal dietary intake would be more beneficial or even helpful, than oral intake of creatine monohydrate; a supplement that has been shown to increase muscle strength and hypertrophy after resistance training [11523].

**Threonine**

Threonine is one of the nine essential amino acids required by humans. As such, threonine cannot be synthesised endogenously, and thus threonine must be obtained through diet or nutritional supplementation. Foods high in threonine include cottage cheese, poultry, fish, meat, lentils and sesame seeds. In animals, threonine is a primary component of intestinal mucin protein and plasma γ-globulin required for maintaining gut function. Threonine supplementation in chickens, pigs and mice has been shown to elevate serum antibodies against various viruses, providing support for a role of dietary threonine in modulating immune function. In rats, threonine deficiency has been found to depress the synthetic rate of phospholipid and nucleoprotein phosphorus fractions in the liver, resulting in an increase in the deposition of liver fat.3 As far as the author is aware, there is no published research to support threonine supplementation in healthy humans, and as such, there is currently no evidence to suggest that additional threonine can improve athletic performance. However, based on the aforementioned animal research, there may be some potential for threonine supplementation to support immune function, which may be beneficial for athletes, particularly during periods of strenuous training when immune function has been shown to be depressed [12427].

**Tryptophan**

Tryptophan (C\textsubscript{11}H\textsubscript{12}N\textsubscript{2}O\textsubscript{2}) is an essential amino acid which contains an indole functional group and, uniquely among amino acids, binds to albumin in the blood. Mobilisation of fatty acids during exercise leads to an increase in non-esterified fatty acids (NEFA), which compete for the same binding site. The increase in NEFA results in tryptophan becoming unbound. This leads to an increase in the plasma concentration of free tryptophan and thus to an increase in the plasma concentration ratio of free tryptophan : large neutral amino acids, particularly BCAA.5 Tryptophan is a precursor of the neurotransmitter 5-hydroxytryptamine (5-HT, also known as serotonin) which is involved in sleep, mood changes and fatigue. There is evidence that an increase in free and total tryptophan across the blood–brain barrier leads to an increased rate of synthesis of 5-HT and kynurenic acid.6 Central fatigue (emanating from the brain) is an important aspect of exercise-induced fatigue in addition to muscle (peripheral) fatigue. The suppression of voluntary movement due to fatigue is a result of modulation of motor-neuron pathways in both the central and peripheral nervous systems. It has been well documented that this is triggered by tryptophan. Tryptophan supplementation has been prescribed for many years in the general community to alleviate depression. It has also been investigated in relation to sports performance. Two studies have claimed a beneficial effect of acute tryptophan supplementation on exercise capacity, hypothesising that its mood-enhancing effect might reduce the perception of pain or discomfort during exercise. However, other studies have failed to show this effect.10 In fact, given the known role of tryptophan as a key substance in the initial reactions leading to fatigue and perception of fatigue, it seems more likely that tryptophan supplementation and a
consequent increase in the plasma concentration of tryptophan might lead to premature fatigue in, for example, endurance events. Indeed, this has been shown in rats where suppression of tryptophan uptake in the brain, via pretreatment with 2-amino-2-norbornanecarboxylic acid, has been seen to diminish central fatigue and to improve running performance. The availability of tryptophan has been observed to be an important factor in immune system function; however, this has not been studied in the context of exercise. In addition, the potential role of tryptophan as a sleep agent to promote recovery after exercise is a current topic of interest but this has not has not been studied specifically in athletes. Finally, it is important to be aware that, in the late 1980s, cases of eosinophilia-myalgia syndrome (EMS) occurred, including some deaths, in many people using tryptophan supplements. This was generally, but not unanimously, attributed to contamination during synthesis by one manufacturer. As a result, the sale of tryptophan as an isolated over-the-counter (OTC) supplement was banned in most countries. However, OTC tryptophan was reintroduced in many countries in the mid-2000s. In summary, there is currently no evidence that tryptophan supplementation has any beneficial effects on athletes. Indeed tryptophan is known to be a key substance in the initial reactions leading to fatigue and perception of fatigue [12427].

**Tyrosine**

Tyrosine (4-hydroxyphenylalanine) is an amino acid that can be synthesised in the body from phenylalanine. Tyrosine shares a common transport molecule with large neutral amino acids at the blood–brain barrier; therefore an increase in the tyrosine ratio causes an increase in brain tyrosine that would lead to an increase in brain dopamine (DA) and norepinephrine (NE) concentration. Since both DA and NE play a key role in a variety of stress-related behaviors, it is not surprising that tyrosine has been the focus of considerable military interest for its cognitive antistress effects. A series of preclinical, animal studies clearly indicate that tyrosine reduces many of the adverse effects of acute stress on cognitive performance in a wide variety of stressful environments. Although it has been difficult to demonstrate conclusively that tyrosine has beneficial effects in humans, in part due to ethical concerns, most of the evidence suggests that tyrosine has utility as an acute treatment to prevent stress-related declines in cognitive function. Tyrosine affects the same neurotransmitter systems as the amphetamines and related drugs, which are potent performance-enhancing compounds, although they have many side effects. Studies examining acute tyrosine supplementation could not show benefits either on prolonged exercise capacity or performance in temperate conditions. A recent study showed that supplementing tyrosine 1 h pre-exercise is associated with increased exercise capacity in the heat [12427].

**Valine**

Valine is an EAA which, together with leucine and isoleucine, is classified as a BCAA. The investigation of valine, in isolation from other BCAA, has received little attention. Valine is involved as a precursor for anaplerosis of the succinyl-CoA in the tricarboxylic acid (TCA) cycle but it has been shown, in combination with other BCAA, that it does not alter the TCA cycle pool expansion during exercise. This limits the use of valine in delaying the decrease of the TCA cycle flux during exercise and thus of local muscle fatigue. Alternatively, BCAA have been shown to be effective in stimulating muscle protein synthesis; however, in isolation, valine has been shown to induce an inferior anabolic signalling response in vitro when compared with leucine and other EAA. As previously reviewed, BCAA may also have a role in reducing central fatigue. Valine alone has been shown to support the central fatigue hypothesis by attenuating the exercise-induced rise in 5-hydroxytryptamine in the
hippocampus of rats. At present, despite marketing claims, there is little evidence to support the use of valine as an ergogenic aid in isolation from other BCAA and EAA [13676].

**CBEX**

Besides beta-alanine supplementation, CBEX is also used, mainly in Japan, to elevate muscle carnosine stores. CBEX, obtained via hot water extraction of chicken breast, is a rich source of HCD, like anserine (1.4 g/100 ml) and carnosine (0.6 g/100 ml). An increase in the muscle carnosine content is likely to occur as a result of chronic CBEX supplementation. Long-term CBEX supplementation enhanced the time to exhaustion at the last spurt of a relatively high-intensity endurance performance. It was shown that the acute supplementation with CBEX (0.4 g carnosine + 1.1 g anserine) 30 min before 10×5 s repeated sprints, decreased the bicarbonate buffering in blood but did not affect performance. The effect of acute supplementation with histidine-containing dipeptides on blood buffering and high-intensity exercise performance needs further investigation [11241].

**Carnosine (beta-alanyl-l-histidine)**

Chronic oral beta-alanine supplementation can elevate muscle carnosine (beta-alanyl-L-histidine) content and improve high-intensity exercise performance. However, the regulation of muscle carnosine levels is poorly understood. The uptake of the rate-limiting precursor beta-alanine and the enzyme catalyzing the dipeptide synthesis are thought to be key steps. The aims of this study were to investigate the expression of possible carnosine-related enzymes and transporters in both human and mouse skeletal muscle in response to carnosine-altering stimuli. Human gastrocnemius lateralis and mouse tibialis anterior muscle samples were subjected to HPLC and qPCR analysis. Mice were subjected to chronic oral supplementation of beta-alanine and carnosine or to orchidectomy (7 and 30 days, with or without testosterone replacement), stimuli known to, respectively, increase and decrease muscle carnosine and anserine. The following carnosine-related enzymes and transporters were expressed in human and/or mouse muscles: carnosine synthase (CARN5), carnosinase-2 (CNDP2), the carnosine/histidine transporters PHT1 and PHT2, the beta-alanine transporters TauT and PAT1, beta-alanine transaminase (ABAT) and histidine decarboxylase (HDC). Six of these genes showed altered expression in the investigated interventions. Orchidectomy led to decreased muscle carnosine content, which was paralleled with decreased TauT expression, whereas CARN5 expression was surprisingly increased. Beta-alanine supplementation increased both muscle carnosine content and TauT, CARN5 and ABAT expression, suggesting that muscles increase beta-alanine utilization through both dipeptide synthesis (CARN5) and deamination (ABAT) and further oxidation, in conditions of excess availability. Collectively, these data show that muscle carnosine homeostasis is regulated by nutritional and hormonal stimuli in a complex interplay between related transporters and enzymes [12430].

The effects of a submaximum single physical load with a mixed aerobic-anaerobic character (combined rowing test) on the intensity of lipid peroxidation (LPO) processes, antioxidant state of the organism, and rheological properties of blood have been studied in a group of athletes. The administration of natural antioxidants significantly decreased the LPO stress induced by the physical load, reduced the suppression of the antioxidant system of the organism, and normalized the LPO-disturbed hemorheological parameters. Antioxidants such as carnosine, cytamine, and apilac can be used as non-doping means for the accelerated recovery and increase in the physical work capacity in athletes [07357].
Carnosine is an abundant dipeptide in human skeletal muscle with proton buffering capacity. There is controversy as to whether training can increase muscle carnosine and thereby provide a mechanism for increased buffering capacity. This study investigated the effects of 5 weeks sprint training combined with a vegetarian or mixed diet on muscle carnosine, carnosine synthase mRNA expression and muscle buffering capacity. Twenty omnivorous subjects participated in a 5 week sprint training intervention (2-3 times per week). They were randomized into a vegetarian and mixed diet group. Measurements (before and after the intervention period) included carnosine content in soleus, gastrocnemius lateralis and tibialis anterior by proton magnetic resonance spectroscopy ($^1$H-MRS), true-cut biopsy of the gastrocnemius lateralis to determine in vitro non-bicarbonate muscle buffering capacity, carnosine content (HPLC method) and carnosine synthase (CARN) mRNA expression and 6 × 6 s repeated sprint ability (RSA) test. There was a significant diet × training interaction in soleus carnosine content, which was non-significantly increased (+11 %) with mixed diet and non-significantly decreased (-9 %) with vegetarian diet. Carnosine content in other muscles and gastrocnemius buffer capacity were not influenced by training. CARN mRNA expression was independent of training, but decreased significantly in the vegetarian group. The performance during the RSA test improved by training, without difference between groups. It was found a positive correlation between an invasive and non-invasive method for muscle carnosine quantification. In conclusion, the study shows that 5 weeks sprint training has no effect on the muscle carnosine content and carnosine synthase mRNA.

The dipeptide carnosine ($\beta$-alanyl-$l$-histidine) is one of a number of histidine containing dipeptides (HCDs) including anserine ($\beta$-alanyl-$l$-$1$-methylhistidine) and balanine ($\beta$-alanyl-$l$-$3$-methylhistidine). Carnosine is abundant in human muscle tissue: around 20-25 mmol/kg dry muscle was previously considered normal for human skeletal muscle. However, the concentration of carnosine in type II muscle fibres is 1.5-2 times higher than type I. The histidine located imidazole ring in the carnosine molecule has a $pK_a$ of 6.83. As this lies within the transit pH range between rest and exercise of skeletal muscle, carnosine is therefore an effective intracellular buffer. Carnosine is synthesised in situ in muscle by carnosine synthase from beta-alanine and histidine, and degraded by an extracellular dipeptidase, carnosinase. Synthesis in muscle is limited by the availability of beta-alanine produced from uracil degradation in the liver, augmented by ingestion of beta-alanine containing HCDs found in meat. Deprived of a dietary source, vegetarian subjects have lower muscle carnosine concentrations, 10-14 mmol/kg. It has been found that 4 weeks’ dietary supplementation of beta-alanine increased carnosine skeletal muscle concentrations by 40-60 percent, and shown an 80 percent increase after 10 weeks with values exceeding 40 mmol/kg. The increase occurred equally in types I and II muscle fibres, despite initially higher levels in type II. When beta-alanine supplementation was stopped, the muscle carnosine concentration declined slowly back towards the basal level with a half-life of approximately 9 weeks. While strength-trained athletes appear to have higher muscle carnosine concentrations, training alone of up to 12 weeks’ duration had no effect. In addition, acute training had no effect on the increase in muscle carnosine seen with beta-alanine supplementation. The capacity to undertake strenuous bicycle exercise increases with the increase in muscle carnosine following supplementation. Total work done in a cycle exercise test performed at 110 percent power max (expected duration approximately 2.5 min) increased 13 percent after 4 weeks (mean increase in muscle carnosine, 59 %) and 16 percent after 10 weeks (mean increase in muscle carnosine, 80 %). It was suggested that this was the result of the increase in muscle buffering capacity. Further evidence for this comes from a report that beta-alanine supplementation attenuates the fall in blood pH during high-intensity exercise, without affecting blood lactate or bicarbonate concentration. beta-Alanine supplementation has already become an ergogenic aid widely used by athletes at the highest level of international competition. Supplementation doses are typically based on
levels obtained from the ingestion of meats, such as turkey and chicken breast, which are rich in HCDs containing beta-alanine [09343].

Intramuscular carnosine buffers protons (H) in skeletal muscle. It was examined the effects of supplementation with chicken breast meat extract (CBEX) containing carnosine and anserine on hormonal responses to resistance exercise. Twenty-two men were assigned to a CBEX drink group (CBEX containing total 2 g of carnosine and anserine) (n=14) or a placebo drink group (n=8). The subjects ingested the prescribed drink (100 mL) twice daily for 30 days without physical training. Before and after the supplementation period, the subjects completed 5 sets of bilateral knee extension exercises (with a 90-s rest between sets). The magnitude of the increase in exercise-induced free testosterone did not change significantly after supplementation in either group. The blood lactate response to exercise was significantly attenuated after supplementation in both groups. In the CBEX group, the plasma epinephrine and norepinephrine concentrations after exercise were significantly lower after supplementation. The serum growth hormone response to exercise was also significantly reduced in the CBEX group after supplementation. No significant differences in exercise-induced strength reduction (fatigue index) were observed in the 2 groups after supplementation. These results suggest that short-term supplementation with CBEX attenuates the exercise-induced epinephrine, norepinephrine, and growth hormone responses [10245].

Carnosine is a dipeptide with a high concentration in mammalian skeletal muscle. It is synthesized by carnosine synthase from the amino acids L-histidine and beta-alanine, of which the latter is the rate-limiting precursor, and degraded by carnosinase. Recent studies have shown that the chronic oral ingestion of beta-alanine can substantially elevate (up to 80%) the carnosine content of human skeletal muscle. Interestingly, muscle carnosine loading leads to improved performance in high-intensity exercise in both untrained and trained individuals. Although carnosine is not involved in the classic adenosine triphosphate-generating metabolic pathways, this suggests an important role of the dipeptide in the homeostasis of contracting muscle cells, especially during high rates of anaerobic energy delivery. Carnosine may attenuate acidosis by acting as a pH buffer, but improved contractile performance may also be obtained by improved excitation-contraction coupling and defence against reactive oxygen species. High carnosine concentrations are found in individuals with a high proportion of fast-twitch fibres, because these fibres are enriched with the dipeptide. Muscle carnosine content is lower in women, declines with age and is probably lower in vegetarians, whose diets are deprived of beta-alanine. Sprint-trained athletes display markedly high muscular carnosine, but the acute effect of several weeks of training on muscle carnosine is limited. High carnosine levels in elite sprinters are therefore either an important genetically determined talent selection criterion or a result of slow adaptation to years of training. Beta-Alanine is rapidly developing as a popular ergogenic nutritional supplement for athletes worldwide, and the currently available scientific literature suggests that its use is evidence based. However, many aspects of the supplement, such as the potential side effects and the mechanism of action, require additional and thorough investigation by the sports science community [10246].

In this narrative review, it was presented and discussed the current knowledge available on carnosine and beta-alanine metabolism as well as the effects of beta-alanine supplementation on exercise performance. Intramuscular acidosis has been attributed to be one of the main causes of fatigue during intense exercise. Carnosine has been shown to play a significant role in muscle pH regulation. Carnosine is synthesized in skeletal muscle from the amino acids L-histidine and beta-alanine. The rate-limiting factor of carnosine synthesis is beta-alanine availability. Supplementation with beta-alanine has been shown to increase muscle carnosine content and therefore total muscle buffer capacity, with the potential to
elicit improvements in physical performance during high-intensity exercise. Studies on beta-alanine supplementation and exercise performance have demonstrated improvements in performance during multiple bouts of high-intensity exercise and in single bouts of exercise lasting more than 60 s. Similarly, beta-alanine supplementation has been shown to delay the onset of neuromuscular fatigue. Although beta-alanine does not improve maximal strength or VO2max, some aspects of endurance performance, such as anaerobic threshold and time to exhaustion, can be enhanced. Symptoms of paresthesia may be observed if a single dose higher than 800 mg is ingested. The symptoms, however, are transient and related to the increase in plasma concentration. They can be prevented by using controlled release capsules and smaller dosing strategies. No important side effect was related to the use of this amino acid so far. In conclusion, beta-alanine supplementation seems to be a safe nutritional strategy capable of improving high-intensity anaerobic performance [10247].

Carnosine is found in high concentrations in skeletal muscles, where it is involved in several physiological functions. The muscle carnosine content measured within a population can vary by a factor 4. The aim of one study was to further characterize suggested determinants of the muscle carnosine content (diet, gender and age) and to identify new determinants (plasma carnosinase activity and testosterone). It was investigated a group of 149 healthy subjects, which consisted of 94 men (12 vegetarians) and 55 women. Muscle carnosine was quantified in M. soleus, gastrocnemius and tibialis anterior using magnetic resonance proton spectroscopy and blood samples were collected to determine CNDP1 genotype, plasma carnosinase activity and testosterone concentrations. Compared to women, men have 36, 28 and 82% higher carnosine concentrations in M. soleus, gastrocnemius and tibialis anterior muscle, respectively, whereas circulating testosterone concentrations were unrelated to muscle carnosine levels in healthy men. The carnosine content of the M. soleus is negatively related to the subjects’ age. Vegetarians have a lower carnosine content of 26% in gastrocnemius compared to omnivores. In contrast, there is no difference in muscle carnosine content between omnivores with a high or low ingestion of β-alanine. Muscle carnosine levels are not related to the polymorphism of the CNDP1 gene or to the enzymatic activity of the plasma carnosinase. In conclusion, neither CNDP1 genotype nor the normal variation in circulating testosterone levels affects the muscular carnosine content, whereas vegetarianism, female gender and increasing age are the factors associated with reduced muscle carnosine stores [11282].

Carnosine (beta-alanyl-L-histidine) and its methylated analogues anserine (beta-alanyl-N1-methylhistidine) and balenine (beta-alanyl-N3-methylhistidine) are histidine-containing dipeptides (HCDs) present abundantly in mammalian skeletal muscles. Carnosine is the only HCD found in human skeletal muscles, whereas all three HCDs are present in the omnivorous human diet. In an attempt to understand the role of carnosine in human muscle, it was searched for a carnosine loading protocol. They showed that beta-alanine supplementation (4-6 g/day) for several weeks (4-10 weeks) resulted in elevated muscle carnosine levels (up to 80 %) [11241].

Because carnosine is located in other excitable tissues other than skeletal muscle, such as the brain and heart, it appears to have additional physiological roles. Some studies have suggested that carnosine may serve as a neuroprotector, possibly aiding in the treatment and prevention of neurodegenerative disorders induced by oxidative stress and antiaging activity. Carnosine is composed of the amino acids beta-alanine and histidine. However, its ability to be absorbed from the circulation by muscle is limited; therefore, it needs to be synthesized within the skeletal muscle. Because the skeletal muscle has a relatively low concentration of beta-alanine, but a high concentration of histidine and carnosine synthetase (the enzyme responsible for carnosine synthesis), beta-alanine becomes the rate-limiting step in carnosine synthesis. Supplementing with beta-alanine (3.2 to 6 g/day) for 4 weeks
has resulted in increases of carnosine concentrations within the skeletal muscle between 37 and 64 percent). Recently, it was demonstrated that 1.6 g/day was sufficient to increase muscle carnosine over a 2-week period. It was concluded that the increase in carnosine is dependent on the total beta-alanine consumed over time and is not dependent on baseline muscle carnosine, the muscle fiber type, or daily amount of supplemented beta-alanine. Carnosine is found primarily in human fast-twitch (type II) skeletal muscle and is estimated to contribute up to 40 percent of the skeletal muscle’s buffering capacity of H^+ that are produced during a high-intensity exercise. The increase in Ca^{2+} sensitivity from greater carnosine concentrations within the skeletal muscle has been demonstrated to enhance the excitation-contraction coupling, potentially reducing the rate of fatigue during muscle performance [12432].

Carnosine is a dipeptide of beta-alanine and L-histidine found in high concentrations in skeletal muscle. Combined with beta-alanine, the pKa of the histidine imidazole ring is raised to 6.8, placing it within the muscle intracellular pH high-intensity exercise transit range. Combination with beta-alanine renders the dipeptide inert to intracellular enzymic hydrolysis and blocks the histidinyl residue from participation in proteogenesis, thus making it an ideal, stable intracellular buffer. For vegetarians, synthesis is limited by beta-alanine availability; for meat-eaters, hepatic synthesis is supplemented with beta-alanine from the hydrolysis of dietary carnosine. Direct oral beta-alanine supplementation will compensate for low meat and fish intake, significantly raising the muscle carnosine concentration. This is best achieved with a sustained-release formulation of beta-alanine to avoid paresthesia symptoms and decreasing urinary spillover. In humans, increased levels of carnosine through beta-alanine supplementation have been shown to increase exercise capacity and performance of several types, particularly where the high-intensity exercise range is 1-4 min. Beta-alanine supplementation is used by athletes competing in high-intensity track and field cycling, rowing, swimming events and other competitions [13669].

Carnosine was first discovered in skeletal muscle, where its concentration is higher than in any other tissue. This, along with an understanding of its role as an intracellular pH buffer has made it a dipeptide of interest for the athletic population with its potential to increase high-intensity exercise performance and capacity. The ability to increase muscle carnosine levels via beta-alanine supplementation has spawned a new area of research into its use as an ergogenic aid. The current evidence base relating to the use of beta-alanine as an ergogenic aid is reviewed here, alongside our current thoughts on the potential mechanism(s) to support any effect. There is also some emerging evidence for a potential therapeutic role for carnosine, with this potential being, at least theoretically, shown in ageing, neurological diseases, diabetes and cancer. The currently available evidence to support this potential therapeutic role is also reviewed here, as are the potential limitations of its use for these purposes, which mainly focusses on issues surrounding carnosine bioavailability [13670].

Little research has been done on the physiological and performance effects of altitude training on team-sport athletes. Therefore, this study examined changes in 2000-m time-trial running performance (TT), hemoglobin mass (Hbmass), and intramuscular carnosine content of elite Australian Football (AF) players after a preseason altitude camp. Thirty elite AF players completed 19 days of living and training at either moderate altitude (about 2130 m; ALT, n=21) or sea level (CON, n=9). TT performance and Hbmass were assessed preintervention (PRE) and postintervention (POST1) in both groups and at 4 weeks after returning to sea level (POST2) in ALT only. Improvement in TT performance after altitude was likely 1.5 percent (± 4.8-90 % CL) greater in ALT than in CON, with an individual responsiveness of 0.8 percent. Improvements in TT were maintained at POST2 in ALT. Hbmass after altitude was very likely increased in ALT compared with CON (2.8 % ± 3.5 %),
with an individual responsiveness of 1.3 percent. Hbmass returned to baseline at POST2. Intramuscular carnosine did not change in either gastrocnemius or soleus from PRE to POST1. A preseason altitude camp improved TT performance and Hbmass in elite AF players to a magnitude similar to that demonstrated by elite endurance athletes undertaking altitude training. The individual responsiveness of both TT and Hbmass was approximately half the group mean effect, indicating that most players gained benefit. The maintenance of running performance for 4 weeks, despite Hbmass returning to baseline, suggests that altitude training is a valuable preparation for AF players leading into the competitive season [13671].

Carnosine occurs in high concentrations in human skeletal muscle and assists working capacity during high-intensity exercise. Chronic beta-alanine (BA) supplementation has consistently been shown to augment muscle carnosine concentration, but the effect of training on the carnosine loading efficiency is poorly understood. The aim of one study was to compare muscle carnosine loading between trained and untrained arm and leg muscles. In a first study (n=17), reliability of carnosine quantification by proton magnetic resonance spectroscopy (1H-MRS) was evaluated in deltoid and triceps brachii muscles. In a second study, participants (n=35; 10 nonathletes, 10 cyclists, 10 swimmers, and 5 kayakers) were supplemented with 6.4 g/day of slow-release BA for 23 days. Carnosine content was evaluated in soleus, gastrocnemius medialis, and deltoid muscles by 1H-MRS. All the results are reported as arbitrary units. In the nonathletes, BA supplementation increased carnosine content by 47 percent in the arm and 33 percent in the leg muscles (not significant). In kayakers, the increase was more pronounced in arm (deltoid) vs. leg (soleus + gastrocnemius) muscles whereas the reverse pattern was observed in cyclists. Swimmers had significantly higher increase in carnosine in both deltoid and gastrocnemius muscle compared with nonathletes. It was shown that carnosine content can be reliably measured by 1H-MRS in deltoid muscle, carnosine loading is equally effective in arm versus leg muscles of nonathletes, and carnosine loading is more pronounced in trained versus untrained muscles [13672].

Carnosine (beta-alanyl-L-histidine) was discovered in 1900 as an abundant non-protein nitrogen-containing compound of meat. The dipeptide is not only found in skeletal muscle, but also in other excitable tissues. Most animals, except humans, also possess a methylated variant of carnosine, either anserine or ophidine/balenine, collectively called the histidine-containing dipeptides. One review aimed to decipher the physiological roles of carnosine, based on its biochemical properties. The latter include pH-buffering, metal-ion chelation, and antioxidant capacity as well as the capacity to protect against formation of advanced glycation and lipoxidation end-products. For these reasons, the therapeutic potential of carnosine supplementation has been tested in numerous diseases in which ischemic or oxidative stress are involved. For several pathologies, such as diabetes and its complications, ocular disease, aging, and neurological disorders, promising preclinical and clinical results have been obtained. Also the pathophysiological relevance of serum carnosinase, the enzyme actively degrading carnosine into L-histidine and beta-alanine, is discussed. The carnosine system has evolved as a pluripotent solution to a number of homeostatic challenges. L-Histidine, and more specifically its imidazole moiety, appears to be the prime bioactive component, whereas beta-alanine is mainly regulating the synthesis of the dipeptide. This paper summarizes a century of scientific exploration on the (patho)physiological role of carnosine and related compounds. However, far more experiments in the fields of physiology and related disciplines (biology, pharmacology, genetics, molecular biology, etc.) are required to gain a full understanding of the function and applications of this intriguing molecule [13673].
Beta-alanine (BA) supplementation has been shown to delay neuromuscular fatigue as a result of increased muscle carnosine concentrations. Carnosine has also been found in brain and cardiac tissue. The physical working capacity test at heart rate threshold (PWC_{HRT}) is a global estimate of the onset of fatigue during exercise, influenced by central and peripheral factors. The purpose of this study was to determine the effects of 28 days of BA supplementation on the PWC_{HRT}.

Thirty subjects (age 21 ± 2 years) were randomly assigned to BA (n=15) or placebo (PL, n=15) groups. Testing included eight to nine total visits: an enrolment day, physical screening, peak oxygen consumption (VO_{2peak}) and two PWC_{HRT} assessments over 4 days. Significant differences existed between BA and PL for PWC_{HRT}, but not for VO_{2peak}, time to exhaustion (TTE) or ventilatory threshold (VT). Results suggest that BA may increase heart rate training threshold. These results, in combination with one previous study reporting a potential effect of BA on HR, suggest that future studies should evaluate both central and peripheral aspects of fatigue with BA intake [13674].

**With and without sodium bicarbonate**

To examine the effect of beta-alanine only and beta-alanine with sodium bicarbonate supplementation on 2,000-m rowing performance 20 well-trained rowers (age 23 ± 4 years) were assigned to either a placebo or beta-alanine (6.4 g/d for 4 weeks) group. A 2,000-m rowing time trial (TT) was performed before supplementation (Baseline) and after 28 and 30 days of supplementation. The post supplementation trials involved supplementation with either maltodextrin or sodium bicarbonate in a double-blind, crossover design, creating four study conditions (placebo with maltodextrin; placebo with sodium bicarbonate; beta-alanine with maltodextrin; beta-alanine with sodium bicarbonate). Blood lactate, pH, bicarbonate, and base excess were measured pre-TT, immediately post-TT and at TT+5 min. Performance data were analyzed using magnitude based inferences. Beta-alanine supplementation was very likely to be beneficial to 2,000-m rowing performance, with the effect of sodium bicarbonate having a likely benefit. There was a small but possibly beneficial additional effect when combining chronic beta-alanine supplementation with acute sodium bicarbonate supplementation compared with chronic beta-alanine supplementation alone. Sodium bicarbonate ingestion led to increases in plasma pH, base excess, bicarbonate, and lactate concentrations. It was concluded that chronic beta-alanine and acute sodium bicarbonate supplementation alone had positive effects on 2,000-m rowing performance. The addition of acute sodium bicarbonate to chronic beta-alanine supplementation may further enhance rowing performance [13675].

**Effect of training**

Chronic training does appear to have a profound effect on muscle carnosine concentrations. One of the primary physiological adaptations from anaerobic conditioning programs is an enhanced buffering capacity. It has been demonstrated that highly trained anaerobic athletes have a greater buffering capacity and a significantly greater skeletal muscle concentration of carnosine than endurance trained athletes and untrained subjects. Sprinters also have been reported to have muscle carnosine concentrations ranging between 17 and 25 mmol/kg of dry muscle, which is significantly higher than that typically found in endurance athletes, untrained individuals, and the elderly. There is a positive relationship is reported between muscle carnosine concentrations and mean power during a 30-s sprint. Considering that most of the available evidence to date indicates that neither short-term nor long-term high-intensity training regimens can elevate muscle carnosine concentrations, the method that appears to best increase muscle carnosine content is supplementing with beta-alanine [12432].
Citrulline

Citrulline is a non-essential alpha-amino acid (C_6H_13N_3O_3) found in protein-rich foods of both animal and plant origin. The body uses it to synthesise arginine, a precursor of nitric oxide (NO) production. In fact, citrulline supplementation appears to be more effective in increasing plasma arginine concentration than the ingestion of arginine itself. Citrulline is also an intermediate in the urea cycle, an important pathway that removes ammonia from muscle and liver cells. Despite some reported benefits of citrulline supplementation in clinical populations, few studies have investigated its potential benefits for athletic performance. Surprisingly, one study found a potentially blunted NO production and a reduction in treadmill time to exhaustion after ingestion by healthy volunteers of 3 and 9 g L-citrulline over 24 h before testing. However, a field study showed that ingesting 6 g citrulline malate before a cycling event increased post-race NO concentration in polymorphonuclear neutrophils compared with a control group, potentially reducing exercise-induced immunodepression. Currently, there is no conclusive evidence that citrulline ingestion enhances athletic performance [10251].

Low calorie diets are designed to reduce body weight and fat mass, but they also lead to a detrimental loss of lean body mass, which is an important problem for overweight people trying to lose weight. In this context, a specific dietary intervention that preserves muscle mass in people following a slimming regime would be of great benefit. Leucine (LEU) and Citrulline (CIT) are known to stimulate muscle protein synthesis (MPS) in post-prandial and post-absorptive state, respectively. This makes them interesting bioactive components to test in the context of dietary restriction. It was tested the concept of combining LEU and CIT in adult female rats. It was postulated that the sequential administration of LEU (mixed in chow) and CIT (given in drinking water before a rest period) could be beneficial for preservation of muscle function during food restriction. Sixty female rats (22 weeks old) were randomized into six groups: one group fed ad libitum with a standard diet (C) and five food-restricted groups (60 % of spontaneous intake for 2 weeks) receiving a standard diet (R group), a CIT-supplemented diet (0.2 or 1 g/kg/day, CIT0.2 group and CIT1 group, respectively), a LEU-supplemented diet (1.0 g/kg/day) or a CIT + LEU-supplemented diet (CIT + LEU 1.0 g/kg/day each). At the end of the experiment, body composition, muscle contractile properties and muscle protein synthesis (MPS) rate were studied in the tibialis anterior muscle. Dietary restriction tended to decrease MPS and decrease muscle strength. Only CIT administration (1 g/kg) was able to restore MPS and increase muscle maximum tetanic force and muscle strength LEU had no effect and CIT + LEU supplementation had few effects, limited to adipose mass and fatigue force. The results of this study highlight the ability of CIT alone to preserve muscle function during dietary restriction. Surprisingly, LEU antagonized some effects of CIT. The mechanisms involved in this antagonistic effect warrant further study [13680].

Aspartate and asparagine

Aspartate is a non-essential amino acid (C_4H_7NO_4). The normal daily requirement of L-aspartate in humans is approximately 2 g. It is found mainly in meat, fish, seafood (0.6-2.6 g/100 g), cheese (1.2-2.9 g/100 g) and eggs (1.3-1.5 g/100 g); less in plant sources. The main purported ergogenic effects of aspartate supplementation are increased exercise endurance and attenuation of exercise-induced hyperammonaemia. Six rat studies showed increased endurance time from 15 percent to 111 percent; six rat and one dog studies showed no positive effect on endurance exercise. In humans, the first supplementation study
showed 51 percent increased time to exhaustion. Two studies reported increased endurance time during cycling exercise (15 %, 16 %) versus five studies with no effects on endurance exercise. No correlation has been found between the dosage of aspartate and increments of exercise time. Claims of glycogen-sparing, reduced hyperammonaemia or a higher rate of free fatty acid oxidation are not confirmed by the aspartate literature. Aspartate has not been shown to increase muscle endurance or strength. When combined with asparagine (C₆H₁₀N₂O₃), aspartate supplementation resulted in a significantly longer time to exhaustion in rats with lower blood lactate levels and skeletal muscle and liver glycogen degradation. However, a recent study performed in 15 triathletes did not confirm the results obtained in rats [09135].

**Aspartame**

Aspartame (C₁₄H₁₈N₂O₅) is an example of an intense or non-nutritive sweetener and an ingredient of many thousands of drink and food products consumed worldwide. It is a methyl ester of a dipeptide composed of the amino acids aspartic acid and phenylalanine, which are constituents of all protein-containing foods. Aspartame is about 180 times sweeter than sucrose with, for most individuals, minimal bitterness and a good quality of sweet taste. Being composed of amino acids, it has an energy value of 4 kcal/g; however, at the concentrations needed to sweeten foods and drinks, its nutritive value is negligible. In products it may be blended with one or more other intense sweeteners or with sugars, including sucrose, fructose and glucose. The safety of aspartame has been the subject of much, often ill-informed, debate. After ingestion, aspartame is broken down to its constituent amino acids and methanol, and some further minor products. Even at high dietary intakes of aspartame, the amount of methanol produced is too small to be harmful. Because high intakes of phenylalanine are undesirable for those born with phenylketonuria, products with aspartame may contain information such as “Contains a source of phenylalanine.” By replacing sugars in products, intense sweeteners can potentially aid control of energy intake and weight, but the extent of any benefit would appear to be dependent on the context of use. Additionally, aspartame reduces appetite independent of its sweet taste by a physiological action which is currently unknown. Aspartame, and other intense sweeteners, are used in sport drinks to allow adjustment of nutrient profile and tonicity, while maintaining a pleasant level of sweetness. The flavour and sweetness of such products are important for motivating consumption, and thereby achieving desired levels of hydration and nutrient intake [09344].

**Glycine**

Glycine is the smallest amino acid; it is non-essential and can be synthesised from serine. Glycine is present in most proteins and is particularly highly concentrated in collagen. Consequently, one of the highest food sources of glycine is gelatin. Glycine is also one of the three amino acid components of glutathione, which is a key component of the body’s defences against oxidative stress; however, it is thought that glycine availability is not the limiting step in glutathione synthesis. Glycine ingestion increases plasma concentrations of insulin in a similar way to other amino acids. Glycine is also an inhibitory neurotransmitter. There is little research on supplementation with glycine. Research has looked at its potential role in decreasing inflammation. Sport specific research has focused on combining glycine with other nutrients. Glycine-propionyl-L-carnitine (GPLC) has been shown to influence exercise performance, decrease oxidative stress and potentially increase vasodilation through increases in plasma nitrate. At present, there is insufficient evidence to suggest the use of glycine as a supplement to enhance sporting performance. However, further research
on the effectiveness of GPLC is warranted [11241].

**Glycine-arginine-alpha-ketoisocaproic acid**

Oral supplementation with glycine-arginine-alpha-ketoisocaproic acid (GAKIC) has previously been shown to improve exhaustive high-intensity exercise performance. There are no controlled studies involving GAKIC supplementation in well-trained subjects. The aim of one study was to examine the effects of GAKIC supplementation on fatigue during high-intensity, repeated cycle sprints in trained cyclists. After at least 2 familiarization trials, 10 well-trained male cyclists completed 2 supramaximal sprint tests each involving 10 s separated by 50-s rest intervals on an electrically braked cycle ergometer. Subjects ingested 11.2 g of GAKIC or placebo (Pl) during a period of 45 min before the 2 experimental trials, administered in a randomized and double-blind fashion. In contrast to previous studies in untrained individuals, the results suggested that GAKIC has no ergogenic effect on repeated bouts of high-intensity exercise in trained individuals [11242].

Glycine-arginine-alpha-ketoisocaproic acid (GAKIC) is a relatively new supplement that athletes and fitness enthusiasts ingest to enhance performance during anaerobic exercise. Therefore, the purpose of this study was to investigate the potential ergogenic effects of GAKIC ingestion during multiple bouts of resistance exercise. Seven resistance-trained men participated in a randomized, counterbalanced, double-blind study. Participants were randomly assigned to placebo or GAKIC (10.2 g) and performed 5 sets of 75 percent of 1-repetition maximum leg press to failure. Total load volume was calculated by multiplying the 75 percent of 1-repetition maximum mass lifted by the sum of repetitions to failure. One week later, participants ingested the other supplement (placebo or GAKIC) and performed the same exercise protocol. Blood lactate, glucose, and heart rate were determined preexercise and immediately postexercise. GAKIC supplementation significantly increased leg-press total load volume. Heart rate and blood lactate were significantly increased postexercise compared with preexercise but were not significantly different between GAKIC and placebo. No significant changes were detected for one-repetition maximum and blood glucose. These novel findings suggest that GAKIC increases total work performed during repeated bouts of lower-body resistance exercise. Thus, the data suggest that GAKIC ingestion before weight training may increase the training volume of athletes and resistance-trained individuals [13704].

Glycine-arginine-alpha-ketoisocaproic acid (GAKIC) has been proposed to increase anaerobic high-intensity exercise performance in male subjects. However, the effects of GAKIC ingestion in female subjects have not been studied. Therefore, the purpose of this study was to investigate the effects of GAKIC supplementation on total load volume (i.e., mass lifted) and metabolic parameters during repeated bouts of submaximal leg extensions in college-age females. Nine resistance-trained females participated in a randomized, counterbalanced, double-blind study. Subjects were randomly assigned to placebo or GAKIC (10.2 g) and performed six sets of 50 percent of one repetition maximum leg extensions (two legs simultaneously) to failure. One week later, subjects ingested the other supplement and performed the same exercise protocol. Furthermore, blood lactic acid, blood glucose, and heart rate were also measured preexercise and 5 s after the completion of the exercise protocol (postexercise). GAKIC supplementation significantly increased leg extension total load volume. Heart rate and blood lactic acid were significantly increased postexercise compared to preexercise, but were not significantly different between GAKIC and placebo. Blood glucose was significantly decreased postexercise compared to preexercise, but was not significantly different between GAKIC and placebo. Collectively, these findings suggest
that GAKIC increased lower body resistance performance in trained college-age females; however, these findings are not necessarily generalizable [13705].

**alpha-Ketoglutarate**

AKG is a five-carbon dicarboxylic acid produced in the citric acid cycle from the oxidative decarboxylation of isocitrate. AKG plays a central role in the oxidation of glutamine and glutamate in the small intestine and the rate of AKG formation is important in determining the overall rate of the citric acid cycle. AKG functions as an energy donor, ammonium scavenger, provides a source of glutamine that stimulates protein synthesis, inhibits protein catabolism in muscle and constitutes an important metabolic fuel for cells of the gastrointestinal tract. The majority of studies investigating the effects of AKG have been conducted in animals, however, in humans, AKG has been shown to increase plasma levels of several hormones such as insulin, growth hormone and insulin-like growth factor I significantly, although the mechanisms responsible are poorly understood. Research investigating the effects of AKG on athletic performance is scant and the limited studies that have been conducted have examined the effects of AKG co-ingestion with L-arginine and/or with creatine. Twelve grams of L-arginine α-ketoglutarate (AAKG) per day in three daily doses of 4 g (2 g L-arginine and 2 g AKG) over 8 weeks was found to be safe, well tolerated and positively influenced 1RM bench press and Wingate peak power performance but did not affect body composition or aerobic capacity. Creatine and AAKG supplementation (0.1 g/kg/day creatine+0.075 g/kg/day) for 10 days was found to improve upper body muscle endurance and peak power output on repeated Wingate tests compared to a placebo. These findings, however, should be interpreted with caution because studies investigating the effects of creatine and L-arginine supplementation without the addition of AKG have found positive effects on exercise performance. Currently, there is insufficient evidence to support the use of AKG supplementation to inhibit protein degradation in the muscle, increase muscle mass, promote positive training adaptations and enhance athletic performance. Further research is necessary to investigate the effects of AKG supplementation alone and AKG co-ingestion with other nutrients [11288].

**alpha-Ketoisocaproate**

KIC is a metabolite of the amino acid leucine. beta-Hydroxymethylbutyrate (HMB) is produced from KIC by the enzyme KIC dehydrogenase, and this route of HMB formation is dependent on liver KIC dioxygenase. KIC has been found to have protein-sparing and antinflammatory properties through inhibition of muscle proteolysis and enhancement of protein synthesis. alpha-Ketoisocaproic acid (alpha-KIC) supplementation has been suggested to enhance exercise performance by attenuating exercise-induced muscle soreness and promoting energy supply to the muscle. More specifically, it has been purported that the liver can convert KIC to ketone bodies thereby increasing the energy supply during exercise and alpha-KIC supplementation has also been suggested to spare glucose utilisation by the muscle. Very few studies have examined the effects of alpha-KIC supplementation alone on athletic performance. Acute alpha-KIC supplementation at doses of 1.5 and 9.0 g ingested immediately prior to exercise failed to improve moderate- and high-intensity exercise performance in resistance trained men. Other studies have investigated the co-ingestion of alpha-KIC with other nutrients. alpha-KIC, glycine and L-arginine co-ingestion has been found to enhance high-intensity exercise performance. alpha-KIC and HMB co-ingestion was found to attenuate exercise-induced muscle damage and preserve fat-free mass. However, it is unclear whether these benefits could be attributed to α-KIC supplementation or the other
compounds. At present, there is insufficient evidence to support the use of alpha-KIC supplementation in enhancing exercise performance [11288].

**Proline**

Proline is not considered essential in adult humans, although early work demonstrated a potential benefit of proline when arginine was limiting. Proline is readily available in dairy, meat and eggs, and most plant proteins but can also be synthesised endogenously by two pathways, one arising from ornithine and arginine, or one from glutamine and glutamate. Although the glutamate pathway is considered to be the major route, it is not known how much body proline is derived from the diet or made de novo. Since proline and hydroxyproline (formed post-translationally) comprise approximately 25 percent of the amino acids in collagen, proline is important to skin, bone, cartilage, tendons, ligaments and connective tissues. Proline is degraded via proline oxidase (dehydrogenase) to glutamate or ornithine, and the final fates include polyamines, arginine and entry into the TCA cycle. Proline is an osmoprotectant, a source of superoxide (in the immune system), and plays a role in sensing both energy availability and maintaining protein homeostasis. Hydroxyprolines are present in proteins other than collagen where they play a role in oxygen sensing while hydroxyproline, released from protein degradation, is an antioxidant. Given the importance of proline in growth and wound repair, including the muscle hypertrophy of training, it has been proposed that proline may be conditionally essential. Indeed, proline has been marketed as a supplement for bodybuilders and weight lifters, and for recovery after strenuous exercise. However, there is no direct evidence to support these claims. It is notable that, while circulating proline concentrations decrease during burn injury, dietary supplementation with proline has no effect on plasma proline levels in such patients. Very few studies have looked directly at proline supplementation, though in patients with gyrate atrophy, supplements of up to 488 mg/kg/d are well tolerated. It is, however, not possible to make any claims about the safety or even effectiveness of proline supplements due to an almost complete lack of data. An alternative approach to increase proline availability would be to provide proline precursors (glutamine, ornithine, arginine) as dietary supplements but again there is little evidence that these are effective or even result in increased proline synthesis [12405].

**Amino acids and creatine**

It was aimed to examine the acute hormonal and performance responses to resistance exercise with and without prior consumption of an amino acid/creatine/energy supplement. Eight men performed a resistance-exercise protocol at baseline (BL), 20 min after consuming a supplement (S) consisting of essential amino acids, creatine, taurine, caffeine, and glucuronolactone or a maltodextrin placebo (P). Venous blood samples were obtained before and immediately after (IP), 15 min (15P), and 30 min (30P) after each protocol. Area under the curve of resistance-exercise volume revealed that BL was significantly less than S (10 %) and P (9 %). For fatigue rate, only S was significantly lower than BL. Total testosterone (TT) and growth hormone (GH) were significantly elevated at IP and 15P in all conditions. The GH response was significantly lower, however, in S and P than in BL. The TT and GH responses did not differ between S and P. These results indicated that a supplement consisting of amino acids, creatine, taurine, caffeine, and glucuronolactone can modestly improve high-intensity endurance; however, the anabolic-hormonal response was not augmented [07345].

**Fat**

1823
One study compared the fat metabolism between "a single bout of prolonged exercise" and "repeated bouts of exercise" of equivalent exercise intensity and total exercise duration. Seven men performed three trials: a single bout of 60-min exercise (Single); two bouts of 30-min exercise, separated by a 20-min rest between exercise bouts (Repeated); and rest. Each exercise was performed with a cycle ergometer at 60 percent of maximal oxygen uptake. In the Single and Repeated trials, serum glycerol, growth hormone, plasma epinephrine, and norepinephrine concentrations increased significantly during the first 30-min exercise bout. In the Repeated trial, serum free fatty acids (FFA), acetoacetate, and 3-hydroxybutyrate concentrations showed rapid increases during a subsequent 20-min rest period. During the second 30-min exercise bout, FFA and epinephrine responses were significantly greater in the Repeated trial than in the Single trial. Moreover, the Repeated trial showed significantly lower values of insulin and glucose than the Single trial. During the 60-min recovery period after the exercise, FFA, glycerol, and 3-hydroxybutyrate concentrations were significantly higher in the Repeated trial than in the Single trial. The relative contribution of fat oxidation to the energy expenditure showed significantly higher values in the Repeated trial than in the Single trial during the recovery period. These results indicate that repeated bouts of exercise cause enhanced fat metabolism compared with a single bout of prolonged exercise of equivalent total exercise duration [07362].

Medium-chain triglycerides (MCT) are triglycerides with a fatty acid chain length varying between 6 and 10 carbon atoms. MCT differ from long-chain triglycerides as they are relatively soluble in water and, hence, rapidly hydrolysed and absorbed. MCT are transported in the blood through the portal system, consequently they bypass adipose tissue that makes them less susceptible to hormone-sensitive lipase and deposition into adipose tissue stores. Due to these properties, MCT have been researched for both benefits to exercise performance and health. One review aimed to assess whether MCT are beneficial in either of these situations. MCT have been proposed as a means to maximizing an athlete's ability to maintain their glycogen stores so they can be more competitive. However, only two studies to date have shown an improvement in exercise performance. From a health perspective, MCT increase fat oxidation and energy expenditure as well as reduce food intake and beneficially alter body composition. Results indicate that MCT feeding is ineffective in improving exercise performance and future work should focus on the health benefits and applications of MCT [10518].

Fatty acids are a major component of most diets and can be synthesised endogenously in the human body. They are found in all cells and tissues and are transported between tissues in the bloodstream. Fatty acids are usually linked to other structures, frequently but not exclusively by ester linkages, to form more complex lipids such as triglycerides, phospholipids and sphingolipids. Non-esterified fatty acids (NEFAs), often called "free fatty acids", circulate in the bloodstream and are an important source of energy for skeletal muscle and heart cells. All fatty acids have a common general structure (a hydrocarbon chain (termed the acyl chain) with a carboxyl group at one end and a methyl group at the other)\(^1\): the reactive carboxyl group readily forms ester links. Individual fatty acids are distinguished by the length of their hydrocarbon chain, and by the absence, presence, number and configuration (cis or trans) of double bonds within that chain. Fatty acids have systematic and trivial names, and there are also several shorthand nomenclatures based upon structural features. Saturated and monounsaturated fatty acids can be synthesised de novo from precursors such as glucose. This occurs mainly in the liver and is promoted by insulin. The simplest polyunsaturated fatty acids (PUFAs), linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3), cannot be synthesised in animals including humans. However, they can be synthesised in plants, often abundantly. Because they have important roles in animals but cannot be synthesised de novo, linoleic and alpha-linolenic acids are essential in the diet.
Essential fatty acid deficiency is manifested by typical nutrient deficiency symptoms. However, this condition is rare in humans, being avoided at relatively low intakes of the essential fatty acids. Animals can metabolise essential fatty acids further, inserting additional double bonds (desaturation) and extending the hydrocarbon chain (elongation). Through these processes, linoleic acid can be converted to arachidonic acid (20:4n-6) and alpha-linolenic acid to EPA (20:5n-3). Further metabolism to longer-chain, more unsaturated derivatives is possible (eg, of EPA to DHA (22:6n-3)), although the extent of this conversion is not clear in humans. There is competition between the n-6 and n-3 fatty acid families for metabolism, and so the ratio or balance between these fatty acids appears important. Fat makes an important contribution to dietary energy intake, typically providing 30-40 percent in most Western diets. Most fat occurs as fatty acids esterified into triglycerides. All diets contain many different fatty acids, the relative abundance reflecting the fatty acid composition of the foods eaten. Triglycerides must be extensively hydrolysed before the body can assimilate their constituent fatty acids. This hydrolysis is catalysed by lipase enzymes, chiefly pancreatic lipase, operating in the small intestine. The process of triglyceride digestion is very efficient in most humans. After entering the absorptive cells (enterocytes), fatty acids with hydrocarbon chains of <12 carbons are absorbed directly into the portal blood. However, transport of longer chain fatty acids is more complex. They are first re-esterified into triglycerides, then packaged with phospholipids and apolipoproteins to form chylomicrons, which are secreted into the lymphatic circulation and then enter the bloodstream, having bypassed the liver. Fatty acids within chylomicrons are targeted for uptake and storage in adipose tissue, promoted by lipoprotein lipase action at the endothelial surface. Fatty acids taken up are re-esterified to triglycerides within the adipose tissue and stored in this form. These processes are promoted by insulin. Hydrolysis of stored triglyceride releases the fatty acids, which enter the bloodstream in the non-esterified form: this is promoted by adrenaline, noradrenaline and other stress hormones. Some triglyceride stored within skeletal muscle serves as a local reservoir of fatty acids. The principal roles of fatty acids are as energy sources and membrane constituents. Many types of fatty acid can fill these roles. Certain fatty acids have additional, specific roles, such as serving as precursors for the synthesis of bioactive lipid mediators (eg, prostaglandins), and influencing membrane and intracellular signalling processes, the activation of transcription factors and gene expression. Through these different actions, fatty acids are able to influence cellular functions and thus physiological responses. Fatty acids are oxidised by the process of beta-oxidation which occurs within mitochondria. Oxidation of fatty acids generates more energy than the oxidation of glucose (about 9 cal/g vs about 4 cal/g). Two key points control the rate of fatty acid oxidation:

- intracellular fatty acid concentration which, in turn, is determined by their concentration in the blood, so that a rise in circulating NEFA concentration increases fatty acid oxidation in the tissues using them
- transport of NEFAs (as their coenzyme A esters) from the cytosol into the mitochondria via the carnitine acyl transferase system.

NEFAs become important energy sources during starvation, endurance exercise and other situations where carbohydrate supply is limiting. Glucose and NEFA oxidation is inversely related, so that energy demands of different tissues in different physiological situations can be met by an appropriate, but changing, fuel supply. Importantly, the oxidation of fatty acids requires concurrent glucose oxidation because the movement of acetyl-coenzyme A from fatty acid beta-oxidation into the Krebs cycle needs a supply of oxaloacetate provided from pyruvate, derived from glucose. Fatty acid oxidation cannot maintain the same power output as glucose oxidation, and so performance is decreased as fatty acids contribute increasingly to meeting the demand for energy. It is well known that endurance training regimens can enhance the number of mitochondria and enzyme activities of the Krebs cycle and the beta-
oxidation pathway, so improving NEFA utilisation as a fuel. However, other strategies that have been investigated do not enhance fatty acid oxidation. Among these are “fat loading” (high fat consumption), which may bring about metabolic alterations but does not improve performance, perhaps even impairing the ability to undertake high-intensity exercise when required during an endurance event. Oral L-carnitine has poor bioavailability, and when consumed by itself, it has little impact on tissue carnitine content; only if future studies can resolve this problem will it be able to have a role in increasing fatty acid oxidation. Caffeine promotes NEFA release from stored triglycerides, but this appears to have a limited role in explaining its effects as an ergogenic aid. Increased dietary intake of fatty acids normally consumed in low amounts (eg, long-chain n-3 PUFAs like EPA and DHA) results in the incorporation of these fatty acids into cell membranes, and this might be one mechanism by which dietary fat affects cell function, physiological responses and health. One study suggested that differing fatty acid profiles of skeletal muscle between trained and untrained individuals, despite similar dietary fatty acid composition was a direct consequence of changes in fatty acid metabolism due to increased physical activity. There is no role for supplements aimed at providing saturated or monounsaturated fatty acids or linoleic acid, since these are all consumed in significant amounts from a mixed healthy diet, and the former two classes of fatty acid can be synthesised de novo. There may be a role for supplements aimed at providing those fatty acids which are consumed in lower amounts from the diet and which have general or specific physiological functions or roles in human health or athletic performance. Examples would be alpha-linolenic acid, found in significant amounts in certain plant oils (eg, flaxseed), which acts to increase the status of its derivative EPA: EPA status increase is linearly related to the amount of alpha-linolenic acid provided. Also the long-chain highly unsaturated n-3 PUFAs EPA and DHA found in fish oils and similar supplements and conjugated linoleic acids may be an option [10519].

One study compared the fat metabolism between “a single bout of 30-min exercise” and “three bouts of 10-min exercise” of the same intensity (60 % maximal oxygen uptake) and total exercise duration (30 min). Nine healthy men participated in three trials: (1) a single 30-min bout of exercise (Single), (2) three 10-min bouts of exercise, separated by a 10-min rest (Repeated) and (3) rest (Rest). Each exercise was performed with a cycle ergometer at 60 percent of maximal oxygen uptake, followed by 180-min rest. Blood lactate concentration increased significantly after exercise in the Single and Repeated trials, but the Single trial showed a significantly higher value during the recovery period. No significant difference was observed in the responses of plasma glycerol concentration. The Repeated trial produced a smaller increase in the ratings of perceived exertion during the exercise. During the exercise, no significant difference was observed in respiratory exchange ratio (RER) between the Single and Repeated trials. However, the RER values during the recovery period were significantly lower in the Repeated trial than in the Single and Rest trials, indicating higher relative contribution of fat oxidation in the Repeated trial. These results suggest that the repetition of 10-min of moderate exercise can contribute to greater exercise-induced fat oxidation compared with a single 30-min bout of continuous exercise [11286].

Fish oil and conjugated linoleic acid (CLA) belong to a popular class of food supplements known as “fat supplements”, which are claimed to reduce muscle glycogen breakdown, reduce body mass, as well as reduce muscle damage and inflammatory responses. Sport athletes consume fish oil and CLA mainly to increase lean body mass and reduce body fat. Recent evidence indicates that this kind of supplementation may have other side-effects and a new role has been identified in steroidogenensis. Preliminary findings demonstrate that fish oil and CLA may induce a physiological increase in testosterone synthesis. The aim of this review is to describe the effects of fish oil and CLA on physical performance (endurance and resistance exercise), and highlight the new results on the effects on testosterone
biosynthesis. In view of these new data, it can be hypothesized that fat supplements may improve the anabolic effect of exercise \[13706\].

**Medium-chain triglycerides**

Medium-chain triglycerides (MCTs) are fats in which the fatty acids joined to the glycerol backbone are 6-14 carbon molecules in length. These fats are digested and metabolised differently from the long-chain fatty acids that make up most of our dietary fat intake. Specifically, MCTs can be digested within the intestinal lumen with less need for bile and pancreatic juices than long-chain triglycerides, with the liberated medium chain fatty acids (MCFAs) being absorbed via the portal circulation. MCFAs are then taken up into the mitochondria without the need for carnitine-assisted transport. MCT supplements derived from palm kernel and coconut oil are used in clinical nutrition situations as an energy source for patients who have various digestive or lipid metabolism disorders. Sports related applications of MCTs includes their use by body builders as an easily absorbed and oxidized fuel source that is less likely to deposit as body fat. There has been little investigation of such chronic use apart from some evidence that it may be associated with deterioration in blood lipid profiles. The best studied use of MCTs by athletes is as a source of rapidly accessible fat that can be consumed during exercise to increase fat availability during endurance and ultra-endurance events. In such events, there is both time to consume a fat source and the potential benefits if this leads to a sparing of muscle glycogen use. The maximum rate of oxidation of MCTs occurs after about 120-180 min of exercise and co-ingestion with carbohydrate can increase this rate, possibly by increasing the rate of MCT absorption.\[17\] The literature on supplementation with MCT and carbohydrate during ultra-endurance exercise is inconsistent, with the results appearing to depend on the amount of MCT that can be ingested and the prevailing hormonal conditions. Studies in which the intake of large amounts of MCT raised plasma free fatty acid concentrations and allowed glycogen sparing reported enhancement of a performance trial undertaken at the end of prolonged exercise.\[18\] However, these metabolic (and performance) benefits may be compromised when exercise is commenced with higher insulin levels, as is the case following a carbohydrate-rich pre-exercise meal. The effectiveness of MCT is mostly limited by the inability of subjects to tolerate the substantial amount of MCT oils required to have a metabolic impact. A total intake of about 30 g appears to be the limit of gastrointestinal tolerance of MCT, which would limit its fuel contribution to 3-7 percent of the total energy expenditure during typical ultra-endurance events. Gastrointestinal reactions to larger intakes range from insignificant\[18\] to performance-limiting.\[19\] Differences in gastrointestinal tolerance between or within studies may reflect differences in the type and intensity of exercise, the mean chain length of MCTs found in the supplements or increased tolerance in some athletes due to chronic exposure to MCTs. Despite some support for use during prolonged exercise, MCTs appear to have limited application to most sporting situations [11393].

**Maximal lipid oxidation**

For patients with metabolic diseases, as with other diseases, exercise training is a fully recognized therapy. Such training helps obese patients stabilize weight after slimming. For patients with type 2 diabetics, it is both a prevention and a glucose-lowering treatment and reduces health care costs. It was proposed a targeted training for individuals at the level of maximal lipid oxidation (LIPOX_{max}) with a protocol of exercise calorimetry (four 6-min workloads) based on Brooks and Mercier's crossover concept. Calorimetric interpretation of gas exchange at the fifth and sixth minutes of each stage shows a bell-shaped curve for lipid oxidation that peaks at LIPOXmax, a point that varies considerably among individuals. As well, glucose oxidation is a linear function of power (carbohydrate cost of the watt). Such a
calculation predicts fairly actual lipid oxidation over 45 min at the same level. Other protocols, with 3-min workloads used in sports medicine, are not reliable for patients with metabolic diseases. For obese adults and teenagers, as well as those with type 2 diabetes, 2 months' training at the LIPOXmax (three sessions at 45 min per week) results in a net loss of fat mass, with preserved fat-free mass, and increased ability to oxidize lipids. At the end of this period, training can be "re-targeted" to be more effective and, possibly, associated with other strategies with stronger exercise intensities. Therefore, metabolic training is a viable option for patients with metabolic diseases, but the full concept is still evolving. However, the major challenge remains to transform inactive individuals into active ones.

In cyclists

The objective of one work was to determine whether the young cyclists follow the optimal fats and minerals intake, based on the recommended dietary guidelines. The appropriate intake of fats, fibre, and minerals (sodium, calcium, magnesium, potassium) in their diets; may reduce the risk to develop hypertension and cardiovascular diseases, in the long-term. The correct rehydration is essential in cycling during summer. Nutrients intake questionnaire of 7 consecutive days, applied to 34 young cyclists. The evaluation results are compared with the enKid study of Spanish young people. The diet has been evaluated at the beginning of the cyclist season. A percentage of cyclists in the present study, consume excessive quantities of cholesterol (94 % of cyclists), saturated fats (74 %), and sodium (47 %); while they do not consume the recommended quantities of unsaturated fats (100 %); fibre (67 %), calcium (29 %), magnesium (10 %) and potassium (44 %). This work contributes to the knowledge of the diets followed by very active young people. Their diets show nutritional unbalances; therefore the need to educate the cyclists, their parents and coaches, to follow diets based on the Mediterranean diet; rich in vegetable, fruits, fish, nuts, and olive oil, in order to increase the intake of MUFA, PUFA and minerals, that will protect them against possible risk of cardiovascular diseases.

Fish oil

Both fish oil supplementation and physical exercise are able to induce benefits to mental health by providing an improvement in cognitive performance and enhancing neuropsychology and protection against neurological lesions. The aim of one study was to investigate the cognitive effects in rats of the: a diary and prolonged fish oil supplementation (85 mg/kg/day) initiated from prenatal period to the midlife (300 day/old); moderate physical exercise in treadmill initiated from adolescent period to midlife, and association of fish oil supplementation and moderate physical exercise protocol during the same period. Animals were submitted to the habituation in the open-field, object recognition and to the plus-maze discriminative avoidance tasks. The results demonstrated that a diary and prolonged fish oil supplementation can facilitate the persistence of the long-term habituation and recognition memories without, however, affecting the discriminative avoidance memory. Conversely, although the program of physical exercise exerted no effects on habituation or objects recognition, it was able to potentiate the persistence of the discriminative avoidance memory. Such promnestic effects (induced by both fish oil supplementation and physical exercise) were not accompanied by alterations in emotionality or locomotor activity. The findings suggest that fish oil supplementation, initiated from prenatal period to midlife, and physical exercise program applied throughout the life induced distinctly a better cognitive performance.
Fish oil (FO) supplementation prevents the development of obesity and insulin resistance, and upregulate the expression of UCP3 in skeletal muscle in rodents. This may represent indirect evidence that FO promotes fat oxidation and/or alter energy efficiency. The aim of one study was to evaluate whether such effects can be observed in humans. The metabolic effects of FO were assessed during exercise in order to obtain a direct measurement of energy efficiency. Eight healthy male volunteers were studied with and without supplementation with 7.2 g/day FO (including 1.1 g/day eicosapentaenoic acid and 0.7 g/day docosahexaenoic acid) during 14 days. Their VO$_2$ max was measured on cycle ergometer. Thereafter, energy metabolism (substrate oxidation, energy expenditure and energy efficiency) was assessed during a 30 min cycling exercise at 50 percent VO$_2$ max performed 2 h 30 after a standardized, high carbohydrate breakfast. VO$_2$ max was 39 ± 2 after FO and 38 ± 2 (mL/kg and min) in control conditions, which was a non-significant difference. Basal plasma glucose, insulin and NEFA concentrations, and energy metabolism were similar with FO and in controls. During exercise, the increases in plasma NEFA concentrations, energy expenditure, glucose and lipid oxidation, and the decreases in glycaemia and insulinemia were not altered by FO intake. Energy efficiency was similar in both groups. In order to ascertain that the absence of effects of FO was not due to consumption of a carbohydrate meal immediately before exercise, 4 of the 8 subjects were re-studied in fasting conditions, FO also failed to alter energy efficiency in this subset of studies. It was concluded that FO supplementation did not significantly alter energy metabolism and energy efficiency during exercise in healthy humans.

To investigate the effects of docosahexaenoic-(DHA)-rich fish oil (FO) supplementation on lymphocyte function before and after a marathon race 22 athletes participated in a study. Eight marathon runners were supplemented with 3 g of FO daily for 60 d (FO group), and 13 athletes were not supplemented (C group). The following measures of lymphocytes were taken before and after the marathon: cell proliferation, cytokine production (IL-2, IL-10, TNF-alpha, and IL-4), and signs of cell death. In the C group, the marathon had no effect on lymphocyte proliferation, DNA fragmentation, or mitochondrial membrane polarization; however, the marathon increased phosphatidylserine externalization (by 2.5-fold), induced a loss of plasma membrane integrity (by 20 %), and decreased IL-2, TNF-alpha, and IL-10 production (by 55 %, 95 %, and 50 %, respectively). FO supplementation did not prevent lymphocyte death induced by the marathon, as indicated by cell viability, DNA fragmentation, and phosphatidylserine externalization. However, FO supplementation increased lymphocyte proliferation before and after the marathon, and before the race, FO supplementation decreased IL-2, TNF-a, and IL-10 production in concanavalin-A-stimulated lymphocytes (by 55 %, 95 %, and 58 %, respectively) compared with cells from the C group. The production of cytokines was not altered before or after the race in the FO group. DHA-rich FO supplementation increased lymphocyte proliferation and prevented a decrease in cytokine production, but it did not prevent lymphocyte death induced by participation in the marathon. Overall, DHA rich-FO supplementation has beneficial effects in preventing some of the changes in lymphocyte function induced by marathon participation.

**Omega-3 fatty acids**

Polyunsaturated fatty acids are popular dietary supplements advertised to contribute to weight loss by increasing fat metabolism in liver, but the effects on overall muscle metabolism are less established. It was evaluated the effects of conjugated linoleic acid (CLA) or combination omega 3 on metabolic characteristics in muscle cells. Human rhabdomyosarcoma cells were treated with either DMSO control, or CLA or combination omega 3 for 24 or 48 hours. RNA was determined using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Mitochondrial content was determined using flow cytometry and immunohistochemistry. Metabolism was quantified by measuring extracellular
acidification and oxygen consumption rates. Omega 3 significantly induced metabolic genes as well as oxidative metabolism (oxygen consumption), glycolytic capacity (extracellular acidification), and metabolic rate compared with control. Both treatments significantly increased mitochondrial content. It was concluded that omega 3 fatty acids appear to enhance glycolytic, oxidative, and total metabolism. Moreover, both omega 3 and CLA treatment significantly increase mitochondrial content compared with control [12431].

Human beings evolved consuming a diet that contained about equal amounts of omega-6 and omega-3 essential fatty acids. Today, in Western diets, the ratio of omega-6 to omega-3 fatty acids ranges from approximately 10:1 to 20:1 instead of the traditional range of 1:1 to 2:1. Studies indicate that a high intake of omega-6 fatty acids shifts the physiologic state to one that is prothrombotic and proaggregatory, characterized by increases in blood viscosity, vasospasm, and vasoconstriction, and decreases in bleeding time. omega-3 Fatty acids, however, have anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic, and vasodilatory properties. Excessive radical formation and trauma during high-intensity exercise leads to an inflammatory state that is made worse by the increased amount of omega-6 fatty acids in Western diets, although this can be counteracted by eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). For the majority of athletes, especially those at the leisure level, general guidelines should include EPA and DHA of about 1 to 2 g/d at a ratio of EPA:DHA of 2:1 [07366].

Increased dietary LCn-3PUFA (long-chain n-3 polyunsaturated fatty acid) intake stimulates muscle protein anabolism in individuals who experience muscle loss due to aging or cancer cachexia. However, it is not known whether LCn-3PUFAs elicit similar anabolic effects in healthy individuals. To answer this question, we evaluated the effect of 8 weeks of LCn-3PUFA supplementation (4 g of Lovaza®/day) in nine 25-45-year-old healthy subjects on the rate of muscle protein synthesis (by using stable isotope-labelled tracer techniques) and the activation (phosphorylation) of elements of the mTOR (mammalian target of rapamycin)/p70S6K (p70 S6 kinase) signalling pathway during basal post-absorptive conditions and during a hyperinsulinaemic-hyperaminoacidaemic clamp. We also measured the concentrations of protein, RNA and DNA in muscle to obtain indices of the protein synthetic capacity, translational efficiency and cell size. Neither the basal muscle protein fractional synthesis rate nor basal signalling element phosphorylation changed in response to LCn-3PUFA supplementation, but the anabolic response to insulin and amino acid infusion was greater after LCn-3PUFA (i.e. the muscle protein fractional synthesis rate during insulin and amino acid infusion increased). In addition, the muscle protein concentration and the protein/DNA ratio (i.e. muscle cell size) were both greater after LCn-3PUFA supplementation. It was concluded that LCn-3PUFAs have anabolic properties in healthy young and middle-aged adults [11438].

The effect of fish-oil supplementation (FO-S) on the immune responses of elite swimmers was investigated. In a randomized placebo-controlled trial, swimmers received either fish-oil capsules (n=10) containing long chain polyunsaturated fatty acids (FA) of n-3 (LCPUFA n-3) or placebo capsules (n=10), both for 6 weeks. Plasma FA, immunological markers, insulin and cortisol were evaluated. The FO-S resulted in an increase in LCPUFA n-3 and a decrease in arachidonic n-6 FA in plasma and a reduction in the production of interferon-gamma by cultured cells. A reduction in the production of tumor necrosis factor-alpha was observed in both groups. An increase in interleukin-2 production and no significant difference in interleukin-4 were also observed. FO-S was able to attenuate the exercise-induced increases in prostaglandin E2. Circulating concentrations of insulin did not change, while cortisol and glucose showed increase after the study period. These results suggest that FO-S influence exercise-associated immune responses in competitive swimmers [07367].
Long-distance migrants have evolved specific adaptations that make their athletic records possible. Unique mechanisms explaining their amazing capacity for endurance exercise have now been uncovered, particularly with respect to energy storage, mobilization, transport and utilization. Birds are champions of migration because flying offers a key compromise: it allows more rapid movement than swimming, but has a lower cost of transport than running. High efficiency for muscle contraction, pointed wings, low wingloading, travelling in V-formations, storing fuel as energy-dense lipids and atrophy of non-essential organs are some of their strategies to decrease the cost of transport. The ability to process lipids rapidly also emerges as a crucial component of the migrant phenotype. High lipid fluxes are made possible by lipoprotein shuttles and fatty acid binding proteins that accelerate lipid transport and by upgrading the metabolic machinery for lipolysis and lipid oxidation. Preparation for long flights can include natural doping on n-3 polyunsaturated fatty acids from unique invertebrate diets. Muscle performance is improved by restructuring membrane phospholipids and by activating key genes of lipid metabolism through peroxisome proliferator-activated receptors (PPARs). The physiological secret to long migrations does not depend on a single 'magic' adaptation but on the integration of multiple adjustments in morphology, biomechanics, behavior, nutrition and metabolism. Research on the physiology of migrants improves the fundamental knowledge of exercise biology, but it also has important implications for wildlife conservation, treating obesity and improving the performance of human athletes [09350].

Wild semipalmated sandpipers (Calidris pusilla) eat n-3 fatty acids to prime their muscles for long migrations. Sedentary bobwhite quails (Colinus virginianus) were used as a model to investigate the mechanisms for this natural doping. The goal of one study was to characterize the stimulating effects of n-3 eicosapentaenoic acid (EPA) and n-3 docosahexaenoic acid (DHA) on oxidative capacity. Mechanisms linked to changes in membrane composition and in gene expression for peroxisome proliferator-activated receptors (PPAR) were investigated. Dietary n-3 fatty acids stimulated the activities of oxidative enzymes by 58-90% (citrate synthase, cytochrome oxidase, carnitine palmitoyl transferase and hydroxyacyl dehydrogenase), and sedentary quails showed the same changes in membrane composition as sandpipers preparing for migration. EPA and DHA have the same doping effect. The substitution of n-6 arachidonic acid by n-3 EPA in membrane phospholipids plays an important role in mediating the metabolic effects of the diet, but results provide no significant support for the involvement of PPARs (as determined by changes in gene expression). The fatty acid composition of mitochondrial membranes and sarcoplasmic reticulum can be monitored by measuring total muscle phospholipids because all phospholipids are equally affected by diet. Only extreme regimes of endurance training can lead to increments in oxidative capacity matching those induced here by diet. As they prepare for long migrations, semipalmated sandpipers improve their physical fitness by eating! Choosing n-3 fatty acid doping over endurance training strikes us as a better strategy to boost aerobic capacity when rapid storage of energy is critical [09351].

The purpose of one study was to test the influence of 2.4 g/d fish oil n-3 polyunsaturated fatty acids (n-3 PUFA) over 6 weeks on exercise performance, inflammation, and immune measures in 23 trained cyclists before and after a 3-d period of intense exercise. Participants were randomized to n-3 PUFA (n=11; 2,000 mg eicosapentaenoic acid, EPA, 400 mg docosahexaenoic acid, DHA) or placebo (n = 12) groups. They ingested supplements under double-blind methods for 6 weeks before and during a 3-day period in which they cycled for 3 hr/d at about 57 percent W,max with 10-km time trials inserted during the final 15 min of each 3-hr bout. Blood and saliva samples were collected before and after the 6 weeks supplementation period, immediately after the 3-hr exercise bout on the third day, and 14 hr postexercise and analyzed for various immune-function and inflammation parameters. Supplementation with n-3 PUFA resulted in a significant increase in plasma EPA and DHA.
but had no effect on 10-km time-trial performance; preexercise outcome measures; exercise-induced increases in plasma cytokines, myeloperoxidase, blood total leukocytes, serum C-reactive protein, and creatine kinase; or the decrease in the salivary IgA:protein ratio. In conclusion, 6 weeks supplementation with a large daily dose of n-3 PUFAs increased plasma EPA and DHA but had no effect on exercise performance or in countering measures of inflammation and immunity before or after a 3-day period of 9 hr of heavy exertion [09352].

The authors evaluated the role of a high-protein, low-calorie, polyunsaturated fatty-acid (PUFA)-supplemented diet on anthropometric parameters, erythrocyte-membrane fatty-acid composition, and plasma antioxidant defenses of nonprofessional volleyball athletes. The athletes were divided in two groups: One (n=5) followed the Mediterranean diet, and the other (n=6) followed a high-protein, low-calorie diet with a 3-g/day fish-oil supplementation. All the athletes had anthropometric measurements taken, both at the beginning and at the end of the study, which lasted for 2 months. Body-mass index and total body fat were significantly diminished in the second group, while they remained unchanged in the first. Plasma total antioxidant activity (TAA) was significantly increased in the plasma of both groups, with no differences between the groups, suggesting that physical activity, not the different diets, is the main contributor to the increase of plasma TAA. The second group showed a significant increase in erythrocyte-membrane PUFA content and in the unsaturation index value (UI) because of the fish-oil supplementation. A high-protein, low-carbohydrate, fish-oil-supplemented diet seems to be useful only when the aim of the diet is to obtain weight loss in a short-term period. The significant increase in the UI of erythrocyte membranes indicates the potential for harm, because a high intake of PUFA might increase susceptibility to lipid peroxidation not counterbalanced by a higher increase in TAA. Adherence to the Mediterranean diet seems to be the better choice [08423].

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have beneficial effects on cardiovascular function. It was tested the hypotheses that dietary supplementation with DHA (2 g/day) + EPA (3 g/day) enhances increases in stroke volume (SV) and cardiac output (CO) and decreases in systemic vascular resistance (SVR) during dynamic exercise in healthy subjects. It was concluded that safflower oil treatment had no effects on MAP, HR, SV, CO or SVR at rest or during exercise. DHA + EPA-induced increases in stroke volume and cardiac output imply that dietary supplementation with these fatty acids can increase oxygen delivery during exercise, which may have beneficial clinical implications for individuals with cardiovascular disease and reduced exercise tolerance [08424].

The purpose of one investigation was to determine the effects of eicosapentaenoic and docosahexaenoic acid (EPA and DHA) supplementation on resting and exercise-induced inflammation and oxidative stress in exercise-trained men. Fourteen men supplemented with 2224 mg EPA + 2208 mg DHA and a placebo for 6 weeks in a random order, double blind cross-over design (with an 8 week washout) prior to performing a 60 minute treadmill climb using a weighted pack. Blood was collected pre and post exercise and analyzed for a variety of oxidative stress and inflammatory biomarkers. Blood lactate, muscle soreness, and creatine kinase activity were also measured. Treatment with EPA/DHA resulted in a significant increase in blood levels of both EPA and DHA, while no differences were noted for placebo. Resting levels of CRP and TNF-alpha were significantly lower with EPA/DHA compared to placebo. Resting oxidative stress markers were not different. There was a mild increase in oxidative stress in response to exercise. No interaction effects were noted. However, a condition effect was noted for CRP and TNF-alpha, with lower values with the EPA/DHA condition. It was concluded that EPA/DHA supplementation increases blood levels of these fatty acids and results in decreased resting levels of inflammatory biomarkers in exercise-trained men, but does not appear necessary for exercise-induced attenuation in either inflammation or oxidative stress. This may be due to the finding that trained men
Fish oils contain the long-chain highly unsaturated ω-3 (n-3) fatty acids EPA and DHA, although EPA and DHA amounts and their ratio vary according to origin (type of fish, season, location where the fish is caught, etc). Many commonly available fish oils contain about 30 percent EPA plus DHA; more concentrated preparations are available. Most fish oils present fatty acids in triglyceride form, although some supplements provide them as phospholipids, free fatty acids or ethyl esters. All forms have good bioavailability. Fish liver oils, such as cod liver oil, contain higher amounts of vitamins A and D. Typical daily intakes of EPA and DHA are likely to be <200 mg/day, perhaps much less than this, which is below the recommendations made (about 500 mg/day). Thus, supplements can make a substantial contribution to meeting the recommended n-3 intake. When fish oil supplements are consumed, EPA and DHA become enriched within blood lipids, cells and tissues, and influence many aspects of metabolism and physiology; the changes induced are considered to lead to improved health or lowered risk of disease. A daily intake of at least several hundred milligrams of EPA and DHA is apparently required to induce health benefits, but clear threshold doses and dose–response relationships are not established.

Exercise- or athlete-specific benefits of fish oils are not clear because of inconsistencies in the scientific literature. They may improve metabolic changes that occur with exercise and reduce exercise-induced inflammation. Enhanced cardiac function during exercise, perhaps resulting in better oxygen delivery to tissues, has been described following fish oil supplementation in healthy non-athletes. Other studies report no effect of fish oil on maximum aerobic power, anaerobic threshold or exercise performance in athletes. One study reported that fish oil reduced exercise-induced delayed onset muscle soreness, but another study found no effect. These studies typically used moderate (1.8 g/day) to high (4 g/day) doses of EPA plus DHA over several weeks or months, but many studies have studied a small number of subjects. One area yielding some positive results for fish oil supplementation is exercise-induced bronchoconstriction (EIB). A high dose (3.2 g EPA plus 2.0 g DHA daily) for 3 weeks markedly improved lung function postexercise in non-atopic elite athletes with EIB and in asthmatic athletes. Cell culture work suggests that EPA rather than DHA may be responsible, and the benefit may involve novel EPA-derived mediators. Although there are plentiful supplies of body fat on even the leanest athletes, the best efforts of sports scientists to find a way to use supplements to achieve and translate enhanced fat oxidation into performance benefits have not been successful. Therefore, general supplementation of dietary fat by athletes is not warranted. However, interest in supplementation with key fatty acids remains: for example, the fish oil n-3 series, proposed to enhance activities including cardiac function, anti-inflammatory responses and EIB management [10519].

The aim of one investigation was to assess the effects of 6 weeks of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) supplementation on resting and exercise-induced lipid peroxidation and antioxidant status in judoists. Subjects were randomly assigned to receive a placebo or a capsule of polyunsaturated fatty acids (PUFAs; 600 mg EPA and 400 mg DHA). Blood samples were collected in preexercise and postexercise conditions (judo-training session), both before and after the supplementation period. The following parameters were analyzed: α-tocopherol, retinol, lag phase , maximum rate of oxidation (Rmax) during the propagating chain reaction, maximum amount of conjugated dienes (CDmax) accumulated after the propagation phase, nitric oxide (NO) and malondialdehyde (MDA) concentrations, salivary glutathione peroxidase activity, and the lipid profile. Dietary data were collected using a 7-day dietary record. A significant interaction effect between supplementation and time (p < .01) on triglycerides was noted, with values significantly lower in the n-3 long-chain-PUFA (LCPUFA) group after supplementation than in the placebo group. Significant interaction effects between supplementation and time on resting MDA
concentrations and Rmax were found, with elevated values in the n-3 LCPUFA group after supplementation and no change in the placebo group's levels. The authors observed a significantly greater NO and oxidative-stress increase with exercise (MDA, Rmax, CDmax, and NO) in the n-3 LCPUFA group than with placebo. No main or interaction effects were found for retinol and α-tocopherol. These results indicate that supplementation with n-3 LCPUFAs significantly increased oxidative stress at rest and after a judo-training session [10520].

Based on the findings of epidemiological data and recent clinical trials, omega-3 fatty acids seem to have a preventive and therapeutic effect on depression. It was examined the effect of omega-3 fatty acids on the forced-swimming test (FST) in two groups of Sprague-Dawley rats after a six-week treatment with two different diets. Behavioral responses were observed and recorded during the 5-min test. The fatty acid composition from the whole brain tissue and the RBC membrane of the rats were analyzed. Comparing to control diet, omega-3 fatty acid diet significantly decreased the immobility time and increased behaviors of swimming and climbing during the FST. The group in omega-3 fatty acid diet had higher levels of docosahexaenoic acid (DHA, 50% increase) and alpha-linolenic acid (ALA, 63% increase) in the brain, and of eicosapentaenoic acid (EPA, 27% increase) in the peripheral RBC membrane. The level of brain DHA is negatively correlated to the immobility time and is positively correlated to the swimming time. The result shows that omega-3 fatty acids have a beneficial effect on preventing the development of depression-like behaviors in rats with the FST [06269].

**Multi-ingredient performance supplements (MIPS)**

Multi-ingredient performance supplements (MIPS) intended for consumption in close proximity to resistance exercise are extremely popular among young males and athletes. The composition of MIPS varies widely, but the principle ingredients generally include creatine monohydrate, caffeine, beta alanine, the branched chain amino acids (BCAAs) leucine, isoleucine, and valine, as well as L-citrulline, and L-arginine. Resistance training (RT) enhances muscle protein synthesis and hypertrophy while increasing strength and power. Some multi-ingredient performance supplements have been shown to augment the physiological improvements associated with RT. The purpose of one study was to investigate the impact of specific pre- and post-workout MIPS on anabolic hormones, body composition, muscle strength, and power in resistance-trained men participating in a periodized RT program. Twenty-four 24 years) resistance-trained men completed 6 wks of periodized RT (3 times a week). Participants were assigned to one of two groups based upon maximal voluntary contraction of the quadriceps (Biodex) to lean mass (LM) ratio. Group 1 (n=13; MIPS) consumed one serving of NO-Shotgun® (whey protein, casein protein, branched-chain amino acids, creatine, beta alanine, and caffeine) before each workout and one serving of NO-Synthesize® (whey protein, casein protein, branched-chain amino acids, creatine, and beta alanine; Vital Pharmaceuticals, Inc., Davie, FL) immediately after each workout and on non-RT days. Group 2 (n=11; Placebo; PLA) consumed a flavor-matched isocaloric maltodextrin placebo. Serum insulin-like growth factor 1, human growth hormone, testosterone, body composition (DXA), circumferences, 1-repetition maximal strength (1RM) of the upper (chest press) and lower body (leg press), and anaerobic power (Wingate test) were assessed before and after the intervention. Statistical analysis included a 2 x 2 (group x time) ANOVA with repeated measures. There was a main time effect (p = 0.035) for testosterone to increase, but no differences between groups were observed. There were no differences in the other blood hormones. Group x time interactions were observed for LM. Only a main effect of time was noted for circumference measures. Both groups increased upper and lower body 1RM strength to a similar degree. MIPS significantly increased peak
anaerobic power while PLA remained unchanged. Consumption of MIPS during the course of a periodized RT program facilitated training-induced improvement in LM in trained males, whereas the consumption of PLA did not. MIPS improved measures of anaerobic power while PLA did not [12452].

To investigate the effects of an acute multinutrient supplement on game-based running performance, peak power output, anaerobic by-products, hormonal profiles, markers of muscle damage, and perceived muscular soreness before, immediately after, and 24 h following competitive rugby union games 12 male rugby union players ingested either a comprehensive multinutrient supplement (SUPP), or a placebo (PL) for 5 d. Participants then performed a competitive rugby union game (with global positioning system tracking), with associated blood draws and vertical jump assessments pre, immediately post and 24 h following competition. SUPP ingestion resulted in moderate to large effects for augmented 1st half very high intensity running (VHIR). Further, moderate increases in 2nd half VHIR distance and VHIR mean speed in SUPP condition were also apparent. Postgame aspartate aminotransferase (AST) and creatine kinase (CK) measures demonstrated increased values in the SUPP condition, while AST and CK values correlated with 2nd half VHIR distance. Elevated C-reactive protein (CRP) was observed postgame in both conditions; however, it was significantly blunted with SUPP. It was concluded that multinutrient supplement may assist in the maintenance of VHIR during rugby union games, possibly via the buffering qualities of multinutrient supplement ingredients. However, correlations between increased work completed at very high intensities and muscular degradation in multinutrient supplement conditions, may mask any anticatabolic properties of the supplement [10234].

The purpose of one study was to examine changes in performance and metabolic parameters in collegiate soccer players during preseason preparation and to determine the impact of a nutraceutical blend proposed to reduce oxidative stress. Male Division I college soccer players (n=22) performed a progressive maximal treadmill test at the beginning and end of preseason to assess changes in VO\(_{2\text{max}}\), velocity at lactate threshold (VLT), time-to-exhaustion, lipid hydroperoxide (LPO), 8-isoprostane, and creatine kinase (CK) response. After baseline testing, athletes were randomly assigned to receive the nutraceutical blend (EXP; n=12) or an isocaloric equivalent (CON; n=10) for 20 days of preseason training. Delta VO\(_{2\text{max}}\), DeltaVLT, and Delta time-to-exhaustion were improved across groups, but no significant effect of supplementation on performance was seen. Changes in resting levels of CK from the beginning to end of preseason were significantly lower in EXP than in CON. Additionally, EXP demonstrated a significant decrease in the magnitude of the 8-isoprostane response at trial 2 compared with trial 1. A similar pattern was seen for LPO. Preseason training in male college soccer players resulted in significant improvements in VO\(_{2\text{max}}\), VLT, and time-to-exhaustion. Supplementing with a proprietary antioxidant and nutraceutical blend may enhance some of these effects as indicated by magnitude of the responses. However, it appears that the most notable effects of supplementation were seen for reduced CK and oxidative stress, at least with short-term supplementation [10235].

The purpose of one study was to determine the effects of the pre-workout supplement Assault™ (MusclePharm, Denver, CO, USA) on upper and lower body muscular endurance, aerobic and anaerobic capacity, and choice reaction time in recreationally-trained males. Subjective feelings of energy, fatigue, alertness, and focus were measured to examine associations between psychological factors and human performance. Twelve recreationally-trained males participated in a 3-week investigation. Subjects reported to the human performance laboratory on three separate occasions. All participants completed a baseline/familiarization day of testing that included a maximal graded exercise test for the determination of aerobic capacity (VO\(_{2\text{max}}\)), one-rep maximum (1-RM) for bench and leg press to determine 75 percent of 1-RM, choice reaction tests, and intermittent critical velocity
familiarization. Choice reaction tests included the following: single-step audio and visual, one-tower stationary protocol, two-tower lateral protocol, three-tower multi-directional protocol, and three-tower multi-directional protocol with martial arts sticks. Subjects were randomly assigned to ingest either the supplement (SUP) or the placebo (PL) during visit 2. Subjects were provided with the cross-over treatment on the last testing visit. Testing occurred 20 min following ingestion of both treatments. Significant main effects for the SUP were observed for leg press, perceived energy, alertness, focus, choice reaction audio single-step, choice reaction multi-direction 15 s, and multi-direction for 30 s. It was concluded that ingesting the SUP before exercise significantly improved agility choice reaction performance and lower body muscular endurance, while increasing perceived energy and reducing subjective fatigue. These findings suggest that the SUP may delay fatigue during strenuous exercise [12463].
NUTRITIONAL SUPPLEMENTS: TRACE ELEMENTS, VITAMINS AND OTHER OXIDANTS

The purpose of one study was to assess the effect of relative exercise intensity on various plasma trace elements in euhydrated endurance athletes. Twenty-seven well-trained endurance athletes performed a cycloergometer test: after a warm-up of 10 min at 2.0 W/kg, workload increased by 0.5 W/kg every 10 min until exhaustion. Oxygen uptake, blood lactate concentration, and plasma ions (Zn, Se, Mn and Co) were measured at rest, at the end of each stage, and 3, 5 and 7 min post-exercise. Urine specific gravity was measured before and after the test, and subjects drank water ad libitum. No significant differences were found in Urine specific gravity between, before, and after the test or in any plasma ion level as a function of intensity. There were weak significant correlations of Zn and Se with blood lactate concentration but no relationships were established between blood lactate concentration and VO$_2$, fat oxidation, carbohydrate oxidation, energy expenditure from fat, from carbohydrates and total energy expenditure and plasma ion levels. Acute exercise at different submaximal intensities in euhydrated well-trained endurance athletes does not provoke a change in plasma trace element levels, suggesting that plasma volume plays an important role in the homeostasis of these elements during exercise [11287].

Iron

Iron status was assessed in 70 female athletes aged 18-25 years participating in collegiate cross-country track, tennis, softball, swimming, soccer, basketball, and gymnastics. No significant differences in mean hemoglobin, hematocrit, serum iron, total iron-binding capacity, transferrin saturation, and ferritin were found among teams. The mean concentrations of each parameter for each of the teams were within the normal ranges. However, several athletes from different sports had suboptimal iron status indexes. Of 17 athletes with a serum ferritin concentration < 15 microg/L, 8 (4 freshmen, 2 sophomores, 2 unknown) also exhibited low serum iron concentrations (< 60 microg/dL) and low transferrin saturation (< 16 %). Thirteen (6 freshmen, 3 sophomores, 2 juniors, 2 seniors) of 51 (25 %) athletes failed to consume two-thirds of the Recommended Dietary Allowance for iron and exhibited suboptimal serum concentrations of ferritin, iron, and/or transferrin saturation. Of nine athletes taking iron supplements, one exhibited suboptimal iron status. In summary, nonanemic iron depletion was present among female collegiate athletes involved in many different sports and in all years of participation (freshmen, sophomore, junior, and senior athletes). Female athletes should continue to be individually and routinely evaluated for nutritional deficiencies throughout their collegiate athletic careers [06291].

Decrements in iron status have been reported in female soldiers during military training. Diminished iron status adversely affects physical and cognitive performance. It was determined whether iron supplementation could prevent decrements in iron status and improve measures of physical performance and cognitive status in female soldiers during basic combat training. In this 8-wk randomized, double-blind, placebo-controlled trial, soldier volunteers (n=219) were provided with capsules containing either 100 mg ferrous sulfate or a placebo. Iron status indicator assays were performed pre- and post-training. Two-mile running time was assessed post-training; mood was assessed by using the Profile of Mood States questionnaire pre- and post-training. The training course affected iron status: red blood cell distribution width and soluble transferrin receptor were significantly elevated, and serum ferritin was significantly lowered after training. Iron supplementation significantly
attenuated the decrement in iron status; group-by-time interactions were observed for serum ferritin and soluble transferrin receptor. Iron supplementation resulted in significantly improved vigor scores on the Profile of Mood States post-training and in faster running time in volunteers reporting to training with iron deficiency anemia. Thus, iron supplementation may prove to be beneficial for mood and physical performance during the training period [09363].

Iron is an ubiquitous metal of vital importance to normal physiologic processes, is one of these substances. Although the risk of iron deficiency or depletion in athletes is limited to specific situations, namely the female sex, increased training stress, increased iron losses, and poorly balanced vegetarian diets, iron-supplementation therapy is commonplace in athletes to counterbalance physiological or pathological anemia and to prevent physiological dysfunction. Iron supplementation may be initiated on the basis of unproven evidence of performance improvement or it may accompany the administration of erythropoiesis-stimulating substances, as iron bioavailability is crucial in enhancing for example the efficacy of rHuEpo. While an athlete who is iron deficient may not be overtly anemic, he or she may have a hemoglobin level that is less than optimal. Correcting iron deficiency anemia is not blood doping and may improve an athlete's performance. However, taking excessive oral iron supplements cannot increase hemoglobin above the reference range and may occasionally result in iron overload, which is associated with serious metabolic risks. While transferrin saturation is considered an indicator of an underlying genetic defect associated with primary hemochromatosis, the linear relationship between body iron load and serum ferritin makes the measurement of ferritin concentration a suitable approach to assess the total body iron content and detect potential misuse, especially in endurance athletes. Serum ferritin measurement, ordinarily performed in athletes entering elite training programs to identify hematologic and iron-related abnormalities, allows early recognition of iron overload, which may persist for a long time in most athletes after retirement from competition and cessation of excessive iron supplementation [10353].

One study examined iron status and nutrient intake in highly active (physical activity per week, PPA) and sedentary women. Participants completed a 7-day weighed-food record (energy, protein, fiber, alcohol, and micronutrients), 7-day pedometer/activity log, and fasting blood draw (hemoglobin, hematocrit, red blood cell indices, C-reactive protein, serum iron, percent transferrin saturation, total iron-binding capacity, ferritin, transferrin receptor, sTfR, and sTfR index). Significantly lower serum ferritin and mean cell hemoglobin concentrations were found in active than in sedentary women. Significantly higher mean sTfR and sTfR index values were found in the active women. No significant differences were found between groups for the other blood markers. Serum ferritin concentrations (storage iron) indicated iron depletion (stage I) in 21 percent of active and 18 percent of sedentary participants. Nonetheless, 50 percent of active and 18 percent of sedentary participants were iron depleted as evidenced by the sTfR index (ratio of functional-to-storage iron). Elevated sTfR concentrations (functional iron) were observed in 25 percent of active and 3 percent of sedentary participants. Although the active women reported significantly greater iron but similar heme iron intakes, they had higher mean sTfR, higher sTfR index, and lower serum ferritin concentrations than the sedentary women. Assessment of iron status may require measures not commonly used in routine assessments [09364].

Iron deficiency is the most prevalent nutritional disorder in the world and is particularly pertinent in athletes. An altered iron status may range in severity from iron depletion (serum ferritin (SF) <35 microg/L, haemoglobin (Hb) >115 g/L, transferrin saturation >16 %) to iron deficiency anaemia (SF <12 microg/L, Hb <11.5 g/L, transferrin saturation <16 %). It is recognised that optimal physical performance is dependent on the efficient delivery and
utilisation of oxygen by the exercising muscle. Iron is a fundamental element to both of these processes due to its role as the functional component of haemoglobin and myoglobin, as well as being a critical constituent of mitochondrial enzymes and cytochromes that promote oxidative phosphorylation. Insufficient iron stores may thus lead to feelings of lethargy and decrements in athletic performance. There are several reasons why athletes may present with low iron levels, including plasma volume expansion, low dietary iron intake, low iron bioavailability and excessive iron excretion/loss. Furthermore, female athletes are at greater risk of iron deficiency, due to menstrual blood loss and suboptimal dietary intake. During exercise, iron losses can occur from several avenues such as red blood cell haemolysis, haematuria and sweating. Such iron loss mechanisms are dependent on the exercise mode, intensity and duration. Additionally, recent research has demonstrated that the hormone hepcidin may also have an impact on an athlete’s iron stores in the acute postexercise period. As a result of these aforementioned mechanisms of iron loss in athletes, iron replacement in this population is of utmost importance. However, the human body has no internal mechanism to generate its own iron stores, and as such, dietary intake and/or supplementation is the only solution to replacing these iron losses. To maintain sufficient iron stores, it is recommended that a dietary iron intake of 8 mg/day for men and 18 mg/day for women is consumed. The food choices used to attain this daily iron requirement may vary, and will include the ingestion of the more efficiently absorbed haem iron (derived from meat sources; accounting for about 20% of the daily dietary iron intake), and the less efficiently absorbed non-haem iron (derived from plant foods such as spinach, leafy greens, lentils and beans; accounting for about 80 percent of the daily dietary iron intake). With regard to iron supplementation, previous research has demonstrated that an oral iron supplement provided at a dose of 100 mg of ferrous sulphate per day may increase SF levels by 30-50 percent over a 6-8-week period. The absorption of this iron supplement is considered to be more efficient when taken in conjunction with ascorbic acid (i.e. orange juice) and less efficient when taken with caffeinated products (i.e. tea and coffee). As a result, the practitioner may wish to consider the prescription of an oral iron supplement that also contains Vitamin C (i.e. ferro-gradumet C). Although it is recommended, and commonly practiced, to prescribe oral iron supplementation at 100 mg of ferrous sulphate on a daily basis, an ill-tolerance of this supplement may result in side effects such as gastrointestinal distress, constipation and/or black-coloured stools. In the event of such a response, the supplementation protocol may be reduced in consultation with the sports physician, to represent a dosage more tolerable to the individual athlete. Alternatively, the use of intramuscular iron injections may be considered. Ideally, the supplementation regime should be monitored by a blood test every 8-12 weeks in order to screen the athlete’s SF levels. Once the SF has returned to a level that suggests a healthy iron status, the supplementation should cease, since the oxidative nature of excessive iron stores may be toxic to the body. Such a consequence also highlights the dangers of unsupervised and unwarranted iron supplementation. Although it is accepted that the consumption of an oral iron supplement might be effective in improving the physical performance of iron-deficient, anaemic athletes, there is still much debate on the efficacy of such a regime in enhancing the physical performance of iron-deficient, non-anaemic athletes. Some studies have demonstrated significant improvements in running energy efficiency, 15 km running time-trial time and maximal oxygen uptake as a result of oral iron supplementation in iron-deficient, non-anaemic athletes. However, other studies have demonstrated that there is no improvement in athletic performance, despite a regime of oral iron supplementation significantly increasing the SF levels. The likely contrast in such outcomes may result from the different performance parameters and athlete characteristics measured within these studies, thus suggesting that more research is warranted in this area. However, despite the equivocal research to suggest the influence of an oral iron supplement given to iron-depleted, non-anaemic athletes, it is still encouraged that iron supplementation continues to be prescribed to such populations on the basis of preventing iron depletion from potentially progressing to iron deficiency anaemia. As established above, the use of an oral
Iron supplement may take at least 6 to 8 weeks to improve the iron status of an athlete. However, should a faster increase in iron status be required (due to impending competition, extreme fatigue, etc), the use of an intramuscular iron injection may be considered by the sports physician. When comparing such a technique to the ingestion of oral iron tablets, it has been shown that 5×2 mL intramuscular injections of Ferrum H (equivalent to 100 mg of elemental iron), provided over a 10-day period, resulted in a significantly faster and greater increase in SF levels when compared to the ingestion of an oral iron tablet (105 mg of elemental iron), which was consumed over a 30-day period. Although a significantly faster way to improve the iron status of an athlete, to date, the evidence for an improvement in athletic performance from such a regime is negligible, and more research is required into this area of iron supplementation. Additionally, intravenous iron infusions are obviously an even faster method of increasing iron status. However, intravenous infusions are considered a banned method by the World Anti-Doping Agency (WADA) and unless one can justify this method over oral or intramuscular iron replacement. In conclusion, iron-deficient, non-anaemic athletes should, in the first instance, increase their dietary intake of iron (preferably in the form of haem iron) on the advice of a sports dietitian. In addition, depending on the SF levels, clinical symptoms of fatigue, poor performance and response to dietary intake, oral iron supplementation or intramuscular iron injections should be considered in consultation with a sports physician. Finally, there appears to be no indication for iron supplementation in the absence of iron deficiency, and there certainly may be adverse effects with ongoing, unsupervised, iron supplementation [11288].

Iron supplementation for the iron-depleted nonanemic athlete is a controversial issue. Athletes may be iron deficient due to poor dietary intake, significant or obligatory blood loss, or deficiency via increased need secondary to intense physical activity. Athletes who are found to be anemic secondary to iron deficiency do benefit and show improved performance with appropriate iron supplementation. There is contradictory evidence for iron supplementation and improving performance in the iron-depleted nonanemic athlete. An athlete's iron status is usually monitored via serum ferritin. Currently, there is no standardized ferritin level at which supplementation is recommended, nor is there a consensus as to the appropriate maintenance of ferritin. Screening endurance athletes or female athletes in general for iron deficiency and also educating these athletes regarding the importance of a balanced diet to maximize performance would seem prudent and beneficial. Based on the literature, supplementation for the iron-depleted nonanemic athlete does not appear to be justified to solely improve performance [07378].

To evaluate the effect of iron supplementation on physical performance in children (0-18 years) through systematic review of randomised controlled trials (RCTs). RCTs with interventions that included oral or parenteral iron supplementation, fortified formula milk, or cereals were evaluated. The physical performance outcomes studied were heart rate, treadmill endurance times, blood lactate, and oxygen consumption. A total of three studies were included, in all of which iron was supplemented in the form of oral medicinal iron. At 5, 6 and 7 miles per hour running speeds, the pooled weighted mean difference (WMD) in the heart rate (per minute) between the iron and the placebo, following exercise was -7, -6, and -8 respectively. After excluding the study with nonanemic subjects, the corresponding figures were -13, -14, and -13), respectively. Oxygen consumption, estimated in two studies, showed no significant difference between the treatment groups. Blood lactate levels were estimated in one study only at two different doses of iron, and were significantly lower in iron supplemented group in comparison to placebo both before (7.7 and 7.6 mg/dL versus 8.4 mg/dL) and after (14.4 and 14.4 mg/dL versus 16.5 mg/dL) exercise. Treadmill endurance time was significantly better in iron supplemented group when compared with placebo in one study. It was concluded that iron supplementation may have a positive effect on the physical performance of children, as evaluated through the post exercise heart rate in anemic
subjects, blood lactate levels and treadmill endurance time. In view of the limited data availability, this finding cannot be considered conclusive [07379].

To determine the effect of iron supplementation on iron status and endurance capacity 20 iron-deficient (serum ferritin, sFer<16 microg/l; serum transferrin receptor, sTfR>8.0 mg/l; or sTfR/log sFer index >4.5), nonanemic (hemoglobin, Hb>120 g/l, women; >130 g/l, men) men and women (18-41 years) were recruited via fliers and newspaper advertisements; 20 of 31 eligible subjects participated. A 30 mg measure of elemental iron as ferrous sulfate or placebo was given daily for 6 weeks. Dietary iron intake and physical activity did not differ between groups before or after supplementation. Iron supplementation significantly increased sFer compared to placebo, but did not affect Hb or hematocrit. Iron supplementation prevented the decline in ventilatory threshold (VT) observed in the placebo group from pre- to post-supplementation; this effect was greater in individuals with lower sFer before intervention. Changes in sFer from pre- to post-treatment were positively correlated with changes in VT, independent of supplementation. The iron group significantly increased gross energetic efficiency during the submaximal test. Changes in sFer were negatively correlated with changes in average respiratory exchange ratio during the submaximal test. It was concluded that iron supplementation significantly improves iron status and endurance capacity in iron-deficient, nonanemic trained male and female subjects [07380].

This case study examines the impact of low serum ferritin (sFe) on physiological assessment measures and performance in a young female 1500-m runner undertaking approximately 95-130 km/wk training. The study spans 4 race seasons and an Olympic Games. During this period, 25 venous blood samples were analyzed for sFe and hemoglobin (Hb); running economy, VO$_{2\text{max}}$, and lactate threshold were measured on 6 occasions separated by 8-10 mo. Training was carefully monitored including 65 monitored treadmill training runs (targeting an intensity associated with the onset of blood lactate accumulation) using blood lactate and heart rate. Performances at competitive track events were recorded. All data were compared longitudinally. Mean sFe was 24.5 ± 7.6 microg/L (range 10-47), appearing to be in gradual decline with the exception of 2 data points (37 and 47 microg/L) after parenteral iron injections before championships, when the lowest values tended to occur, coinciding with peak training volumes. Each season, 1500-m performance improved, from 4:12.8 in year 1 to 4:03.5 in year 4. VO$_{2\text{max}}$ (69.8 ± 2.0 mL/kg/min) and running economy (%VO$_{2\text{max}}$ at a fixed speed of 16 km/h; max 87.8%, min 80.3%) were stable across time and lactate threshold improved (from 14 to 15.5 km/h). Evidence of anemia (Hb <12 g/dL) was absent. These unique data demonstrate that in 1 endurance athlete, performance can continue to improve despite an apparent iron deficiency. Raising training volume may have caused increased iron utilization; however, the effect of this on performance is unknown. Iron injections were effective in raising sFe in the short term but did not appear to affect the long-term pattern [13709].

**Screening**

The aim of one prospective, cohort study was to determine the clinical and performance related utility of hematological and iron-related screening in elite athletes. Three hundred and three male and 273 female elite athletes underwent routine medical screening over a three-year period. In association with a standard medical consultation, a full blood count and iron-related variables were measured. Ten male athletes had a serum ferritin less than 30 ng/mL and satisfied AIS criteria for iron supplementation. In only one case was a disorder identified which was not expected following the clinical history and examination. Fifty-two female athletes had a serum ferritin less than 30 ng/mL and satisfied AIS criteria for iron supplementation. In the females, there were no instances in which a medical condition was
identified which was not expected following the clinical history and examination. In both groups, clinically non-significant abnormalities were generally minor or isolated reductions in hemoglobin and/or hematocrit, and alterations in red cell parameters or single measures of iron status. It was concluded that screening for hematological and iron-related abnormalities in athletes has a low yield. Due to the critical nature of the effects of anaemia and low serum ferritin on some aspects of performance it is reasonable to perform a haemoglobin and a serum ferritin on male and female athletes entering an elite training program. Isolated abnormalities which are close to the limits of their normal ranges and not accompanied by symptoms or signs of illness can almost certainly be ignored [07381].

Boron

Boron, an element with the atomic number 5, is an essential nutrient for plants, but the physiological role in humans is not fully understood. Boron can be considered as an ultra trace element therefore requirements are micrograms per day. Dietary daily human intake is estimated to range from 2.1 to 4.3 mg of boron per kilogram body weight per day. Boron enters the food chain via its incorporation into plant structure and subsequent consumption by humans. There is little evidence of boron deficiency in humans, however, oral boron supplementation is used in the general health and sports markets. Boron has been linked to increased endogenous testosterone levels, but there is little evidence in the literature using an athletic population to support this. Research investigating boron supplementation in postmenopausal women (after a boron-restricted diet) focused on mineral, estrogen and testosterone metabolism. A supplementation of 3 mg of boron per day was associated with an increase in testosterone levels. No proposed mechanism for increased testosterone was reported; however, the authors suggested that maintenance of adequate boron levels in postmenopausal women might prevent calcium loss and bone demineralisation. Few studies have investigated the effects of boron supplementation on increasing testosterone levels in an athletic population. In a randomised placebo-controlled design including participants who regularly weight-trained for at least 1 year (however, no data were reported describing the trained status of the participants). The volunteers were given either 2.5 mg/day of boron (n=10) or placebo (n=9) for a 7-week period. The results suggested an increase in strength (based on one repetition maximum, squat and bench press), increases in lean tissue and testosterone in both groups. However, there was no evidence that the boron supplementation had any additional effect. Therefore, the changes could be regarded as only due to a training effect. A 10-month boron supplementation study in young female athletes, versus sedentary women, produced a modest effect on mineral status. This included a decrease in serum phosphorus and increased urinary calcium excretion which was greater in athletes than in controls. There is currently little evidence to support the use of boron supplementation in the athletic population [09344].

Boron possesses widespread properties in biochemistry and nutrition. Acute supplementation with 11.6 mg of boron resulted in a significant increase in plasma boron concentration. Given such a fast bioavailability, the objective was to determine whether acute (hourly or daily), and weekly supplementation could have any significant biological effects on the steroid hormones and further on some inflammatory biomarkers. Eight healthy male volunteers attended the laboratory on three occasions (days 0, 1 and 7). On the first day (day 0), a blood sample collection at 8.00 A.M was followed by ingestion of placebo with the breakfast. On the next day (supplementation day 1), similar procedure was followed by ingestion of a capsule containing 10mg of boron. On both occasions blood was collected every 2h for the next 6h. Subjects were requested to consume a capsule of 10mg boron every day with their breakfast, and on the day 7, the blood collection was carried out at 8.00
A.M. again. Boron in plasma increased significantly following hours and weekly consumption. Six hours supplementation showed a significant decrease on sex hormone binding globulin high sensitive CRP, and TNF-alpha level. After one week (in samples taken at 8.00 A.M, only), the mean plasma free testosterone increased and the mean plasma estradiol decreased significantly. Dihydrotestosterone, cortisol and vitamin D was elevated. Also, concentrations of all three inflammatory biomarkers decreased after supplementation. Of note, despite decreased proinflammatory cytokines, based on recent clinical data, this must be the first human study report to show an increase level of free testosterone after boron consumption [11289].

Calcium

Exercise-induced sweat calcium losses have been reported as substantial in male athletes. The first aim of the study was to quantify the increase in 24-h total dermal calcium losses and the net changes in calcium retention in active sportswomen after a 1-h strenuous exercise session. A second aim was to determine the effectiveness of calcium supplementation to offset any calcium loss. Twenty-six premenopausal sportswomen completed three 8-d intervention phases in a randomized-order, crossover design. The three phases were placebo+no exercise (control), placebo+exercise, and 400 mg of calcium as calcium carbonate (TUMS Ultra) twice daily + exercise. The supervised exercise was 1 h/day cycling at 65-70 percent of heart rate reserve. A controlled diet of approximately 450 mg/day of calcium and 24-h pooled urine and fecal collections allowed determination of calcium balance on days 5-8 of each phase. Twenty-four-hour dermal collections were made at the end of each phase using a whole-body washdown procedure. Exercise increased dermal calcium losses, which was no longer significant when calcium supplementation was provided. Higher urinary calcium excretion during calcium supplementation is suggestive of higher net calcium absorption. Exercise did not affect urinary calcium excretion indicating lack of compensation for dermal losses. Net calcium retention was positive only during the exercise+calcium supplementation intervention period. It was concluded that calcium supplementation can correct for negative calcium balance attributable to low calcium dietary intake and additional dermal losses from exercise [07387].

Calcium plays a major role in forming and maintaining healthy bone tissue. Food sources of calcium include milk and milk products, such as yogurt and cheese, foods prepared with milk, vegetables, fruits, fish with bones, dried beans and calciumfortified foods and supplements. Low-fat or non-fat versions of milk and milk products have the same amount of calcium as whole milk but a lower fat content. In some countries, a few foods such as cereal and orange juice are fortified with calcium. In addition, vegetables and some fruits can also provide calcium. If adequate calcium is not consumed from foods or beverages, a supplement can help meet calcium needs. Absorption of calcium reaches a plateau at doses of about 500 mg. Doses spaced throughout the day appear to result in a greater total calcium absorption than if one larger dose is taken only once during the day. Since many nutrients in addition to calcium and vitamin D play a role in bone health, it is important that athletes consume a well-balanced diet containing a variety of food, rather than just focusing on one or two bone-related nutrients. A diet that contains vegetables, fruits, low-fat milk and milk products, whole grains and adequate levels of protein and calories to maintain a healthy body weight can provide the nutrients needed for the formation and maintenance of healthy bones. For otherwise healthy athletes, if calcium or vitamin D needs are not met from foods and beverages, then supplements are recommended at levels that meet but do not exceed recommended intakes for these nutrients when added to intakes from foods [10252].
Cobalt

Soluble cobalt (Co) supplements with recommended daily doses up to 1000 microg Co/day are increasingly being marketed to consumers interested in healthy living practices. For example, some athletes may consider using Co supplements as blood doping agents, as Co is known to stimulate erythropoiesis. However, the distribution and excretion kinetics of ingested Co are understood in a limited fashion. It was used a Co-specific biokinetic model to estimate whole blood and urine Co levels resulting from oral exposure or ingestion of Co in amounts exceeding typical dietary intake rates. Following 10 days of Co supplementation at a rate of 400 to 1000 microg/day, predicted adult Co concentrations range from 1.7 to 10 microg/L in whole blood, and from 20 to 120 microg/L in urine. Chronic supplementation (≥ 1 year) at a rate of 1000 microg Co/day is predicted to result in blood levels of 5.7 to 13 microg/L, and in urine levels from 65 to 150 microg/L. The model predictions are within those measured in humans following ingestion of known doses. The methodology presented in this paper can be used to predict urinary or blood Co levels following acute or chronic occupational incidental ingestion, medicinal therapy, supplemental intake, or other non-occupational exposures [12444].

Copper

The importance of the mineral copper for human health can be deduced from its role as a cofactor in numerous metalloenzymes involved in antioxidant defence, oxygen transport and utilisation, immune function, catecholamine and connective tissue synthesis. Copper deficiency in adults has been described as secondary to malabsorption, zinc supplementation and excessive soft drink consumption. Severe copper deficiency is associated with wide ranging clinical manifestations: iron resistant anaemia, pancytopenia, neuropathy, hypercholesterolaemia and osteoporosis. However, copper toxicity has been associated with water contamination over 1.6 mg/l. Dietary requirements for adults have been set at 1.2 mg/day in the UK (tolerable upper intake 10 mg/day) with no specific recommendations for athletes. Copper status studies in male and female athletes across a variety of sports via blood/dietary analysis have yielded mixed results, but confirm that self-reported dietary intakes cannot reliably predict actual micronutrient status. Copper deficiency affects immune function. Indeed, athletes who restrict total energy and nutrient intake for long periods to reduce their body mass may be at greater risk of copper deficiency and its associated immunological effects. Copper supplementation may help to reduce loss of bone mineral density. It is advised that copper status should be assessed in athletes with chronically restricted energy intakes, who report persistent fatigue, frequent infections and stress fractures. Despite potential for poor copper status in some athletes, copper supplementation should not be undertaken without clinical justification, given the fact that it can be toxic [10251].

Chromium

Chromium picolinate, a complex of trivalent chromium (a trace mineral) and picolinic acid, has been heavily marketed for both muscle building and fat loss, but the overwhelming majority of the data do not support these purported benefits [13676].

Chromium enhances insulin signaling and insulin-mediated glucose uptake in cultured cells. It was investigated the effect of chromium on glycogen synthesis and insulin signaling in
humans. Sixteen overweight men (BMI 31) were randomly assigned to supplement with 600 microg/d chromium as picolinate (Cr; n=8) or a placebo (Pl; n=8). After 4 weeks of supplementation, subjects performed a supramaximal bout of cycling exercise to deplete muscle glycogen, which was followed by high-glycemic carbohydrate feedings for the next 24 h. Muscle biopsies were obtained at rest, immediately after exercise, and 2 and 24 h after exercise. Elevations in glucose and insulin during recovery were not different, but the lactate response was significantly higher in Cr. There was a significant depletion in glycogen immediately after exercise, an increase at 2 h, and a further increase above rest at 24 h. The rate of glycogen synthesis during the 2 h after exercise was not different between groups. Glycogen synthase activity was significantly increased immediately after exercise in both groups. Muscle phosphatidylinositol 3-kinase (PI 3-kinase) activity decreased immediately after exercise and increased at 2 h, with a trend for a lower PI 3-kinase response in Cr. Thus, chromium supplementation did not augment glycogen synthesis during recovery from high-intensity exercise and high-carbohydrate feeding, although there was a trend for lower PI 3-kinase activity [06292].

Chromium (Cr$^{3+}$) is a required trace mineral that potentiates the effect of insulin. A small number of patients on total parenteral nutrition have developed severe chromium deficiencies and subsequently presented with symptoms of diabetes. However, this was reversed with chromium supplementation. Chromium levels in food are quite low, with the highest levels found in egg yolk, Brewer's yeast and beef. Although physical activity may increase chromium losses, and intestinal chromium absorption is low (0.5-2.0 %), chromium deficiencies are uncommon, and supplementation is generally not warranted. The adequate intake of chromium is 35 and 25 μg/day for adult (19–50 years) men and women, respectively. Chromium picolinate, a complex of trivalent chromium and picolinic acid, is better absorbed (2-5 %) than dietary chromium. As a dietary supplement, chromium picolinate has been heavily marketed for muscle building, fat loss and, more recently, to reduce insulin resistance/type 2 diabetes risk. Based on these claims, sales of chromium picolinate supplements have soared and representing 6 percent of mineral supplement sales (second behind calcium) in the USA. The overwhelming majority of the data does not support the purported benefits of chromium picolinate supplementation, and thus, supplementation is not recommended. Adverse events related to chromium picolinate supplementation in humans are rare and based on case studies, but negative effects on iron status were noted in one double-blind placebo-controlled trial. Data are not available regarding the safety of high-dose, long-term chromium picolinate ingestion. There is currently little evidence to support the use of chromium picolinate supplementation by athletes [10253].

**Magnesium**

Magnesium (Mg) is an essential biological element that is predominantly located in bones (approx 52 %), muscle cells (28 %), soft tissue (19 %), serum (0.3 %; concentration range 0.75–1.1 mmol/L) and red blood corpuscles (0.5 %). Important food sources of Mg include vegetables, fish, nuts and whole grains. Serum acts as a transit pathway between bone stores and actively metabolising tissues and is not representative of the body's Mg status. Intracellular Mg is under hormonal control in some cell types and regulated by a secondary active transport system, the Na$^+$-Mg$^{2+}$-exchanger. In both extra- and intracellular compartments, the equilibrium between ionised Mg$^{2+}$ and bound Mg is established. Only Mg$^{2+}$ is available to react in physiological and biochemical processes during cellular homeostasis by binding to organic substances such as proteins, nucleic acids and nucleotides. In general, Mg$^{2+}$ is an important regulator of three main complexes: enzyme activation, for example during energy metabolism; stabilising membrane function and
integrity; and cell signalling, for example, intracellular calcium signals. There is interest in the effect of exercise on magnesium status. Acute exercise induces hypomagnesaemia (10 out of 18 studies) while total intracellular Mg content of blood cells seems to be unchanged (7 out of 12 studies). Ionised intracellular Mg was seen to decrease after high-intensity exercise while ionised Mg$^{2+}$ increased. Results from animal studies suggest that Mg$^{2+}$ is shifted from plasma into muscle and adipose tissue. Meanwhile, longitudinal and cross-sectional studies suggest that chronic exercise training may be followed by Mg$^{2+}$ depletion and that athletes are prone to Mg$^{2+}$ deficiency; this is most likely due to Mg losses in sweat and urine. Dietary restrictions, especially in athletes participating in sports requiring weight control, may aggravate the situation. One study has reported depleted Mg$^{2+}$ status in a group of athletes compared with a control group, as indicated by enhanced retention of an intravenous dose of Mg$^{2+}$ during a Mg$^{2+}$ loading test. Mg$^{2+}$ deficiency is characterised by an enhanced neuromuscular excitability, including symptoms such as cardiac arrhythmias, headache, nervousness, and cramps of both smooth and skeletal muscle. The latter, however, have to be differentiated from exercise induced muscle cramps for which Mg supplementation has not been proven to be effective for so far. A recent review indicates no significant effect of Mg supplementation on exercise performance; this is the case for athletes with balanced Mg status, although single studies indicate that Mg supplementation may improve exercise economy (lower VO$_{2max}$ values at given intensity; better lactate clearance). However, Mg supplementation should be considered in athletes with Mg deficiency based on findings from animal experiments and in humans which suggest that magnesium deficiency can compromise exercise capacity. Magnesium supplements include both inorganic and organic compounds of which the latter seemed to have a better bioavailability. A range between 350 and 400 mg/day is recommended as the upper limit. In the case of individuals with renal insufficiency, the daily dose will need to be reduced. Common side effects include gastrointestinal problems such as nausea and diarrhoea. Intravenous application may cause hypotension and cardiac arrhythmias [11393].

The effects of magnesium supplementation on plasma magnesium, zinc, and copper levels were determined in young adult tae-kwon-do athletes and sedentary controls at rest and exhaustion. After a 4-week supplementation period with 10 mg/day/kg Mg, the plasma magnesium, copper, and zinc levels significantly increased in sedentary and training (90-120 min training 5 days a week) subjects when compared to nonsupplemented controls [07384].

The effects of magnesium supplementation on blood parameters were studied during a period of 4 weeks in adult tae-kwon-do athletes at rest and exhaustion. Thirty healthy subjects of ages ranging in age from 18 to 22 years were included in the study. The subjects were separated into three groups, as follows: Group 1 consisted of subjects who did not train receiving 10 mg/kg/d magnesium. Group 2 included subjects equally supplemented with magnesium and exercising 90-120 min/d for 5 day/week. Group 3 were subject to the same exercise regime but did not receive magnesium supplements. The leukocyte count (WBC) was significantly higher in groups 1 and 2 than in the subjects who did not receive any supplements. There were no significant differences in the WBC of the two groups under magnesium supplementation. The erythrocyte, hemoglobin, and trombocyte levels were significantly increased in all groups, but the hematocrit levels did not show any differences between the groups although they were increased after supplementation and exercise. These results suggest that magnesium supplementation positively influences the performance of training athletes by increasing erythrocyte and hemoglobin levels [07385].

The effects of a 1-month exercise program and magnesium supplementation on the adrenocorticotropic hormone and cortisol levels were studied in young tae-kwon-do and sedentary subjects both at rest and exhaustion. The hormone levels were compared before and after supplementation with 10 mg of magnesium (as magnesium sulfate) per kilogram of
body weight. Both exercise and magnesium supplements caused significant increases of the adrenocorticotropic hormone. The cortisol levels were increased in training subjects receiving supplements but not so in subjects that either trained or received magnesium supplements in an independent manner. The cortisol levels measured in resting individuals were higher in the supplemented and non-supplemented athletes than those in sedentary subjects. The results of this study show that exercise and/or magnesium supplementation causes a rise of the adrenocorticotropic hormone, whereas cortisol is increased only as a result of combined exhaustion and magnesium supplements [07386].

One study was performed to determine how the magnesium supplementation for a 4-week period affects the glucose and insulin levels at rest and at exhaustion in sportsmen. It was a 4-week study performed on 30 healthy male subjects varying between 18-22 ages. Glucose and insulin parameters of the groups were measured 4 times; at rest and exhaustion in the beginning of the research and at rest and exhaustion after the end of 4 weeks application period. Glucose levels in exhaustion measurements both before and after the supplementation significantly increased compared to resting levels. Significant difference was determined in the glucose values of 1st and 2nd groups supplemented with magnesium in comparison to their first measurements. Insulin values a decrease in all of the 3 groups occurred with exercise both before and after the supplementation [08395].

Potassium

Potassium (K⁺) is the major intracellular cation in the body, with about 98 percent of the body potassium stores located inside the cells with a concentration maintained at about 145 mM. Its major functions are to promote contractility of ciac, smooth and skeletal muscle and to influence the regulation of nerve conduction through the influx of sodium (Na⁺) and efflux of K⁺ on either side of the nerve terminal, occurring at the rate of $10^7$ ions per second. On the surface of a nerve terminal, there are voltage-dependent channels for K⁺. In humans, at least 15 different K⁺-specific voltage-dependent channels have been identified. The Na⁺/K⁺ ATPase, also known as the Na pump, is the primary active transporter system that maintains the high K⁺ and low Na⁺ intracellular concentrations. The plasma membrane ATPase of all mammalian cells catalyses the reaction: ATP→ADP+Pi with obligatory requirements for Na⁺ and K⁺ and Mg²⁺ required for the dephosphorylation of ATP. Muscle has an especially high activity of this P-type Na⁺ pump which has been estimated to use 60 to 70 percent of the ATP synthesised in nerve and muscle cells. For active individuals/athletes, sweat is the major route of loss of K⁺ with an average of 5 mEq/l loss. Early work indicated that the loss of K⁺ from active muscle could contribute to fatigue during prolonged or intense exercise. Potassium efflux from muscles is greater during high intense/short duration exercise and could result in decreased membrane excitability and cause intracellular acidosis. However, the International Olympic Committee (IOC) Consensus on Nutrition for Athletes states that there is no convincing justification for the specific addition of electrolytes other than sodium to fluids consumed for postexercise recovery. Indeed, a sports drink containing 25 mM K⁺ and 60 mM Na⁺ did not have an additive effect on restoring hydration over a drink containing Na⁺. In addition, exercise in the heat was not associated with losses of Ca⁺, Mg²⁺ and K⁺ among tennis players. Potassium should be consumed as a normal component of the diet rather than as a supplement. Foods containing more than 300 mg K⁺/serving include banana, potato, orange juice, avocado, lima beans, cantaloupe, peaches, tomato, chicken, salmon, tuna, turkey and unsalted nuts. Some carbohydrate-electrolyte sports drinks provide higher amounts of K⁺ (90mg) than others (10-30mg) per 8 fl oz (240ml). Consuming supplemental forms of K⁺ could result in hyperkalaemia which could lead to cardiac arrhythmias and even cardiac arrest. The adequate intake from the diet for adults, age 14 to >70 years in the US, is
4.7 g/d but the average intake is about 2.5 g/d. Individuals with kidney failure or compromised kidney function would need to seek a physician's advice before taking any K+ supplement or consuming potassium-rich foods [12458].

**Selenium**

Both obesity and acute high-intensity exercise increase oxidant stress levels. One study investigated whether selenium (Se) supplementation could be a potential effective therapy to reduce obesity-associated oxidant stress and exercise-induced oxidant stress. Ten normal-weight (NW) (22.80 ± 0.41 kg/m²) and ten overweight (OW) healthy subjects (28.00 ± 0.81 kg/m²) were assessed during a randomized double-blind Se supplementation study (200 microg sodium selenite/day for 3 weeks) with a 3-week placebo control and inversion of treatment periods. Blood levels of lipid hydroperoxide (LH), superoxide dismutase (SOD), erythrocyte glutathione (GSH), and total antioxidant status (TAS), were measured at rest, pre-, and postexercise (30 min 70 % VO₂max, before and after treatment (pretreatment (week 0 and 12) and post-treatment (week 3 or 15)). At rest, compared to placebo, Se supplementation had no significant effect on LH, SOD, GSH, and TAS levels. However, Se supplementation decreased LH levels in the OW group, immediately postexercise compared to placebo treatment. Postexercise, with or without Se supplementation, no changes in TAS, SOD, and GSH levels were observed in both the NW and OW group. The study has highlighted a potential benefit of Se in reducing LH levels postexercise in OW individuals. Given that oxidant stress is a predictor of coronary events, it is imperative to better understand oxidant stress-related responses to lifestyle factors (in particular "high-risk" population groups) and potential antioxidant therapy [12446].

To investigate the ameliorative potential of sodium selenite and zinc sulfate on intensive-swimming-induced testicular disorders, 48 Wistar male rats (age, 4 months; mass, 146 g) were randomly divided into 4 groups: the unexercised-control group (n=12); the exercised group (n=12); the control supplemented group (n=12); and the exercised supplemented group (n=12). For 10 weeks, the exercised rats underwent a protocol that consisted of 4 hour swimming per day for 6 days per week; the control rats did not exercise. For 10 weeks, both the supplemented groups received an oral daily dose of a combination of sodium selenite and zinc sulfate (6 and 3 mg/kg body mass, respectively). After 10 weeks, a significant reduction was seen in rats in the exercised group, compared with rats in both control groups, in paired testicular masses; in epididymal sperm count; in testicular delta5, 3beta-hydroxysteroid dehydrogenase (HSD) and 17beta-HSD; in plasma levels of testosterone, luteinizing hormone, follicle-stimulating hormone, and prolactin; in the numbers of preleptotene spermatocytes, midpachytene spermatocytes, and stage 7 spermatids of the stage VII seminiferous epithelium cycle; and in fertility performance. As well, a significant increase was seen in the exercised group, compared with both control groups, in plasma corticosterone levels and in testicular content of malondialdehyde and catalase activity. At the same time, there was a significant reduction in the exercised group, compared with both control groups, in plasma concentrations of zinc and selenium; in the testicular content of glutathione (GSH), the glutathione and glutathione disulphide (GSSG) ratio, ascorbic acid, and alpha-tocopherol; and in testicular activities of superoxide dismutase, glutathione-peroxidase, and glutathione-S-transferase in the testes. No significant changes were seen in the number of spermatogonia-A from the stage VII seminiferous epithelium cycle or the testicular content of GSSG among the groups. Sodium selenite and zinc sulfate supplementation significantly protected against exercise-induced testicular gamatogenic and spermatogenic disorders, prevented testicular oxidative stress, and increased antioxidant status. It can be concluded that intensive-swimming-induced oxidative stress causes
dysfunctions in the male reproductive system, which can be protected by the coadministration of sodium selenite and zinc sulfate [08396].

Vanadium

Vanadium is a trace element that is probably an essential nutrient, although deficiency states in humans have not been described and its precise biological role is yet to be fully defined. It is commonly found in mushrooms, green beans and cereals, with the usual dietary daily intake around 10-60 microg. As a supplement, in the form of vanadyl sulphate (VOSO₄), vanadium has been used for muscle development in weight training athletes at doses up to 60 mg/day, on the basis that muscle growth and body composition are enhanced. There is little evidence to support this practice. Of the five studies identified which investigated the effect of vanadyl sulphate supplementation on anthropometry and/or body composition, all failed to demonstrate a significant effect. For performance enhancement, the only study to demonstrate an improvement was probably due to the treatment group being at a lower baseline fitness than the control group. However, the cationic species of vanadium (VO²⁺) is known to interact with muscle tissue actin at specific binding sites, altering protein conformation and function. The significance of this is unclear, but does suggest some biological basis behind the claims for vanadium effects on muscle. At supraphysiological levels, in vitro and animal studies indicate that vanadate and other vanadium compounds, do exert measurable biological effects. The effects on glucose transport activity and pancreatic β-cell function, via phosphatases and kinases, are the subject of current research into a potential role as antidiabetic therapeutics. The role of vanadium as a transcription modulator of genes in the area of oxidative stress, and oncogenesis has also been described, suggesting a possible future use as an anticancer agent. Vanadium in sulphate form has low toxicity or side effects. One study into the adverse effects of vanadium supplements failed to find any haematological or biochemical changes in 31 athletes taking 0.5 mg/kg/day of vanadyl sulphate. In summary, it would appear that vanadium compounds are biologically active but not in the areas associated with supplement consumption by weightlifting athletes [13676].

Zinc

One study aims to examine the effect of zinc supplementation on free-radical formation and antioxidant system in individuals who are actively engaged in wrestling as a sport. The study registered a total of 40 male subjects, of whom 20 were wrestlers and 20 were sedentary individuals. The subjects were equally allocated to four groups: group 1, zinc-supplemented sportsmen group; group 2, sportsmen group without supplementation; group 3, zinc-supplemented sedentary group; group 4, sedentary group without supplementation. Blood samples were collected from all subjects twice, once at the beginning of the study and once again at the end of 8-week procedures. The blood samples collected were analyzed to determine the levels of malondialdehyde (MDA), serum glutathione (GSH), serum glutathione peroxidase (GPx) activity, serum superoxide dismutase (SOD) activity (ELISA colorimetric method) and zinc (colorimetric method). No difference was found between MDA levels of the study groups in the beginning of the study. The significantly highest MDA value at the end of the study was obtained in group 4. MDA levels in group 2 were established to be significantly higher than those in groups 1 and 3. GSH level, GPx, and SOD activities and zinc level measured in the beginning of the study were not different between groups. Measurements performed at the end of the study showed that groups 1 and 3 (zinc-supplemented groups) had the significantly highest GSH level, GPx, and SOD activities and zinc level. These
parameters were not different in the groups without supplementation (groups 2 and 4). Results obtained at the end of the study indicate that zinc supplementation prevents production of free radicals by activating the antioxidant system. In conclusion, physiologic doses of zinc supplementation to athletes may beneficially contribute to their health and performance [10254].

One study aimed to examine the effect of zinc supplementation on free-radical formation and antioxidant system in individuals who are actively engaged in wrestling as a sport. The study registered a total of 40 male subjects, of whom 20 were wrestlers and 20 were sedentary individuals. The subjects were equally allocated to four groups: group 1, zinc-supplemented sportsmen group; group 2, sportsmen group without supplementation; group 3, zinc-supplemented sedentary group; group 4, sedentary group without supplementation. Blood samples were collected from all subjects twice, once at the beginning of the study and once again at the end of 8-week procedures. The blood samples collected were analyzed to determine the levels of malondialdehyde (MDA), serum glutathione (GSH), serum glutathione peroxidase (GPx) activity, serum superoxide dismutase (SOD) activity (ELISA colorimetric method) and zinc (colorimetric method). No difference was found between MDA levels of the study groups in the beginning of the study. The highest MDA value at the end of the study was obtained in group 4. MDA levels in group 2 were established to be significantly higher than those in groups 1 and 3. GSH level, GPx, and SOD activities and zinc level measured in the beginning of the study were not different between groups. Measurements performed at the end of the study showed that groups 1 and 3 (zinc-supplemented groups) had the highest GSH level, GPx, and SOD activities and zinc level. These parameters were not different in the groups without supplementation. Results obtained at the end of the study indicate that zinc supplementation prevents production of free radicals by activating the antioxidant system. In conclusion, physiologic doses of zinc supplementation to athletes may beneficially contribute to their health and performance [09365].

The aim of one study was to determine how exercise affects thyroid hormones and testosterone levels in sedentary men receiving oral zinc for 4 weeks. The study included 10 volunteers (mean age, 19 years) who did not exercise. All subjects received supplements of oral zinc sulfate (3 mg/kg/day) for 4 weeks and their normal diets. The thyroid hormone and testosterone levels of all subjects were determined at rest and after bicycle exercise before and after zinc supplementation. TT3, TT4, FT3, and total and free testosterone levels decreased after exercise compared to resting levels before supplementation. Both the resting and fatigue hormone values were higher after 4 weeks of supplementation than the resting and fatigue values before supplementation. The results indicate that exercise decreases thyroid hormones and testosterone in sedentary men; however, zinc supplementation prevents this decrease. Administration of a physiologic dose of zinc can be beneficial to performance [07382].

One study evaluated levels of plasma zinc, copper, and leptin, body composition, and their relationship in nine male judo athletes under two different training conditions. Body composition and biochemical measurements (hematological indexes, plasma zinc, plasma copper, and plasma leptin) were analyzed 24 h after intense training and following a 5-d period without training (no-training). Plasma leptin and plasma zinc increased with no-training. Plasma zinc correlated negatively with percent fat mass and positively with plasma leptin in the no-training condition. Plasma copper did not change during the study and correlated positively with plasma leptin and with percent fat mass after training. Percent fat mass was associated negatively with plasma zinc in the no-training condition. Moreover, percent fat mass was negatively associated with the Zn/Cu plasma ratio under both training conditions. These results are consistent with the possible function of zinc as a lipid-mobilizing factor and of copper as a limiting factor in energy metabolism [07383].
The essential trace mineral zinc (Zn) serves multiple biological functions – catalytic, structural, regulatory and substrate – in support of metabolic, endocrine, signal transduction, cellular control, protein stabilization and immune networks and pathways. Overall, approximately 3000 Zn proteins are encoded in the human genome and Zn is a cofactor to more than 300 enzymes; these include superoxide dismutase, alkaline phosphatase and alcohol dehydrogenase, in the liver, and carbonic anhydrase III, AMP deaminase and the matrix metalloproteinases, in skeletal muscle. As one of the most widely distributed metals in the body, 85 percent of Zn resides in muscle and bones, 11 percent in skin and the liver and the remainder in all the other tissues. This clearly demonstrates the overall importance of Zn and would suggest that it may be important in physical performance. The best food sources of Zn are oysters, wheat germ, liver, beef, melon, squash seeds and cocoa [13710].

Improvement in physical performance or aspects of physical performance by administration of Zn can be demonstrated in one of three ways: showing that providing Zn to persons who are Zn deficient restores/improves performance, demonstrating that providing Zn to healthy individuals with adequate Zn status improves some aspect of performance, or showing that Zn supplementation counters a particular health risk that could compromise performance. With regard to Zn deficiency, the multiple functions of Zn suggest that repletion should restore decrements in performance through a variety of mechanisms. One of the earliest mechanisms proposed was related to Zn’s influence on testosterone (T) and growth hormone (GH). In both human and animal studies, it has been clearly shown that a Zn deficiency can adversely affect plasma levels of T, GH and insulin-like growth factor 1 (IGF-1). Specifically, Zn deficiency in rats is associated with lower plasma luteinising hormone and T concentrations, as well as alterations in steroid metabolism and sex steroid hormone receptors when compared to Zn sufficient rats. Moreover, administration of Zn to animals and humans with either Zn deficiency or retarded growth increased serum T and IGF-1 levels [13710].

Studies in humans with Zn deficiency led investigators to evaluate whether administration of Zn would elicit/invoke biological increases in GH, IGF-1 and testosterone. Most studies have indicated that supplemental Zn promotes growth and an anabolic environment only under conditions of Zn deficiency; no effects on plasma testosterone in Zn-sufficient and/or normal growth humans have been noted. It was demonstrated that providing 30 mg of Zn per day for 4 weeks had no effect on testosterone. It was also reported that Zn supplementation for 4 weeks prevented decreases in testosterone caused by fatiguing exercise. However, the dose/serving size used was very high (3 mg/kg/day of Zn sulphate) or about 85 mg/day for a 70 kg person, and higher than the tolerable upper intake level of Zn for adults (40 mg/day). In addition, doses/serving sizes over 100 mg/day have been found to be associated with health problems – headache, abdominal cramps and nausea – as well as resulting in a copper deficiency, which can cause anaemia, leucopaenia and neutropaenia. Importantly, they did not measure performance or show that maintaining serum T conferred any performance benefit [13710].

In addition to the Zn effects on anabolic pathways, others have suggested that Zn supplementation may improve blood rheology, in particular erythrocyte deformability and factors regulating blood viscosity. However, the evidence for any clear performance benefit is weak, and the effects may be specific to those with Zn deficiency, as determined by low serum Zn levels. Importantly, although inconsistent findings have been reported with regard to serum Zn concentrations in athletes, dietary intake of Zn is often below the recommended dietary allowance (8 and 11 mg/day for adult women and men respectively, sedentary or fit) in many athletes and other special populations [13710].

1851
Finally, zinc supplementation may mitigate performance decrements by mediating health barriers, such as the common cold. Although the data are not strong, several studies have indicated that Zn supplementation may provide more rapid relief from cold symptoms and/or shorten the duration of a cold [13710].

**Zinc monomethionine aspartate**

ZMA is a popular nutritional supplement which regularly features in the nutritional supplementation regimens of many athletes, been reported to suffer from Zn and magnesium deficiency. Studies conducted in clinical populations and animals have shown growth retardation, blunted testosterone and IGF-1 concentrations with Zn deficiency. Plasma magnesium concentrations have been shown to be associated with plasma cortisol concentrations, which are believed to lead to inferior gains in muscle mass and strength following resistance training. Collectively, this suggests that Zn and magnesium deficiency may have deleterious effects on skeletal muscle adaptations and exercise performance. With this in mind, it was investigated the hormonal and training responses of US football players following 7 weeks of ZMA supplementation. It was observed a significant increase in the plasma concentrations of Zn and magnesium after ZMA supplementation, together with a significant increase in plasma testosterone and IGF-1. Those athletes who received ZMA also showed improved muscle strength when compared to the placebo group. However, in contrast, two subsequent studies conducted in athletes using similar levels of ZMA supplementation from the same source found no effects. It was also shown that 8 weeks of ZMA supplementation, combined with resistance training, resulted in no significant differences in the plasma concentrations of Zn or magnesium in anabolic (testosterone, IGF-1 and GH) or catabolic hormones (cortisol), gains in muscle mass and strength or cycling anaerobic capacity when compared to placebo. Manufacturers claim that some versions of ZMA improve the quality of sleep. Despite many athletes using ZMA as a sleep aid, no study until now has investigated the effect of ZMA on sleep quality. At present, there is little evidence to support the use of ZMA as an anabolic nutritional intervention or to improve sleep quality [13710].

**Phosphate**

Research into supplementation with sodium phosphate has not investigated the effects of a repeated supplementation phase. Therefore, this study examined the potential additive effects of repeated sodium phosphate (SP) supplementation on cycling time-trial performance and peak oxygen uptake (VO\(_{2\text{peak}}\)). Trained male cyclists (n=9) completed baseline 1,000-kJ time-trial and VO\(_{2\text{peak}}\) tests separated by 48 hr, then ingested either 50 mg/kg fat-free mass/day of tribasic SP or a combined glucose and NaCl placebo for 6 d before performing these tests again. A 14-d washout period separated the end of one loading phase and the start of the next, with 2 SP and 1 placebo phase completed in a counterbalanced order. Although time-trial performance was shorter in SP1 and SP2, effect sizes and smallest-worthwhile-change values did not differ in comparison with baseline and placebo. However, mean power output was greater than placebo during time-trial performance at the 250 kJ and 500kJ time points after the second SP phase. Furthermore, mean VO\(_{2\text{peak}}\) values were greater after the SP1 (3.5-4.3 %), with further improvements found in SP2 (7.1-7.7 %), compared with baseline and placebo. In summary, repeated SP supplementation, ingested either 15 or 35 d after initial loading, can have an additive effect on VO\(_{2\text{peak}}\) and possibly time-trial performance [13711].
Nitrate

Several recent studies indicate that supplementation of the diet with inorganic nitrate results in a significant reduction in pulmonary O\textsubscript{2} uptake during sub-maximal exercise, an effect that appears to be related to enhanced skeletal muscle efficiency. The physiological mechanisms responsible for this effect are not completely understood but are presumably linked to the bioconversion of ingested nitrate into nitrite and thence to nitric oxide. Nitrite and/or nitric oxide may influence muscle contractile efficiency perhaps via effects on sarcoplasmic reticulum calcium handling or actin-myosin interaction, and may also improve the efficiency of mitochondrial oxidative phosphorylation. A reduced O\textsubscript{2} cost of exercise can be observed within 3 h of the consumption of 5-6 mmol of nitrate, and this effect can be preserved for at least 15 days provided that the same ‘dose’ of nitrate is consumed daily. A reduced O\textsubscript{2} cost of exercise following nitrate supplementation has now been reported for several types of exercise including cycling, walking, running, and knee extension exercise. Dietary nitrate supplementation has been reported to extend the time to exhaustion during high-intensity constant work rate exercise by 16-25 percent and to enhance cycling performance over 4, 10, and 16.1 km by 1-2 percent in recreationally active and moderately trained subjects. Although nitrate appears to be a promising “new” ergogenic aid, additional research is required to determine the scope of its effects in different populations and different types of exercise [13712].

One study examined if an elevated nitrate intake would improve VO\textsubscript{2} kinetics, endurance, and repeated sprint capacity in elite endurance athletes. Ten highly trained cyclists (72 ± 4 mL O\textsubscript{2}/kg/min, mean ± standard deviation) underwent testing for VO\textsubscript{2} kinetics (3 × 6 min at 298 ± 28 W), endurance (120 min preload followed by a 400-kcal time trial), and repeated sprint capacity (6 × 20 s sprints, recovery 100 s) during two 6-day periods in randomized order with a daily ingestion of either 0.5 L beetroot (BR) juice to increase nitrate levels or a 0.5 L placebo (PLA) drink with blackcurrant juice. Plasma NO\textsubscript{x} (nitrate + nitrite) levels were higher in BR (147 ± 102 and 159 ± 103 μM after 4 and 6 days of beverage intake, respectively) compared with PLA (41 ± 10 and 40 ± 7 microM). VO\textsubscript{2} kinetics and exercise economy were the same in BR and PLA. Time-trial performance was similar with an average completion time of 18:20 and 18:37 min:s in BR and PLA, respectively, with average power outputs of 290 ± 43 W in BR and 285 ± 44 W in PLA. Peak and mean power during repeated sprinting were similar in BR and PLA. In contrast to observations in moderately trained subjects intake of BR juice had no effect on VO\textsubscript{2} kinetics and performance in elite cyclists [13713].

Vitamins, in general

Master athletes are more than 35 years of age and continue to train as hard as their young counterparts despite the aging process. All life long, they are capable of accomplishing exceptional sporting performances. For these participants in endurance events, matching energy intake and expenditure is critical to maintain health and performance. The proportions of carbohydrate, fat, and protein must be optimized to provide enough calories to sustain the energy requirements of competition or training, and for recovery. In addition, endurance athletes must include adequate vitamins and minerals in their diets to maintain healthy immune function. Vitamins and minerals may be sufficient in the diets of endurance athletes, who have a high energy intake. This would make it unnecessary to use vitamin and mineral supplements. Furthermore, one major limitation for these athletes is the management of oxidative stress, which, when in excess, can be deleterious for the organism. For individuals exposed to oxidative stress, micronutritional supplementations rich in vitamins and minerals
can be also an alternative strategy. Although these supplementations are increasingly used by master athletes, very few data are available on their effects on oxidative stress, muscle recovery, and physical performance. The potential benefits of supplement use in athletes are thus questionable. Some studies indicate no benefits, while others highlight potential negative side effects of vitamin supplementation. Additional studies are warranted in order to design adapted prescriptions in antioxidant vitamins and minerals [13714].

A double-blind study investigated the effects of vitamin and mineral complex supplementation on the neuromuscular function of the knee-extensor muscles after a prolonged trail running race. Twenty-two well-trained endurance runners took either placebo (Pl group) or vitamins and minerals (Vm group) for 21 d before the race and for 2 d after the race. Maximal voluntary contractions (MVC) and surface EMG activity of the vastus lateralis (VL) muscle were recorded before (pre) and 1 h (post), 24 h (post 24) and 48 h (post 48) after the race. Central activation ratio (CAR), neural (M-wave), and contractile (muscular twitch) properties of the quadriceps muscles were analyzed using electrical stimulation techniques. The knee-extensor MVC was significantly reduced after exercise for both groups, but MVC recovery was greater for Vm than Pl after 48 h. The reduced MVC after exercise was associated with a significant reduction in maximal EMG normalized to the M-wave in VL muscle and in CAR for both groups. Characteristics of the muscular twitch were not significantly altered for either groups, whereas M-wave duration increased significantly after exercise. It was concluded that the reduction of MVC immediately after the race appeared to result from peripheral mechanisms such as a failure in muscle membrane excitation and, to a lesser extent, from reduced central activation. The cause of the depressed MVC 24 h after the race seemed to be located within the muscle itself. A dietary supplementation of a vitamin and mineral complex does not attenuate the loss of contractile function immediately after the running exercise, and it may accelerate the recovery of maximal force capacity [06283].

Ultraendurance exercise training places large energy demands on athletes and causes a high turnover of vitamins through sweat losses, metabolism, and the musculoskeletal repair process. Ultraendurance athletes may not consume sufficient quantities or quality of food in their diet to meet these needs. Consequently, they may use oral vitamin and mineral supplements to maintain their health and performance. We assessed the vitamin and mineral intake of ultraendurance athletes in their regular diet, in addition to oral vitamin and mineral supplements. Thirty-seven ultraendurance triathletes (24 men and 13 women) completed a 7-day nutrition diary including a questionnaire to determine nutrition adequacy and supplement intake. Compared with dietary reference intakes for the general population, both male and female triathletes met or exceeded all except for vitamin D. In addition, female athletes consumed slightly less than the recommended daily intake for folate and potassium; however, the difference was trivial. Over 60 percent of the athletes reported using vitamin supplements, of which vitamin C (98 %), vitamin E (78 %), and multivitamins (52 %) were the most commonly used supplements. Almost half (48 %) the athletes who used supplements did so to prevent or reduce cold symptoms. Only one athlete used supplements on formal medical advice. Vitamin C and E supplementation was common in ultraendurance triathletes, despite no evidence of dietary deficiency in these 2 vitamins [10526].

**Vitamin B**

The B-vitamins are water-soluble vitamins that play crucial roles in energy metabolism. There are eight B-vitamins: thiamine (B₁), riboflavin (B₂), niacin (B₃), pantothenic acid (B₅) pyridoxine (B₆), biotin (B₇), involved in energy-producing pathways; folic acid (B₉), cobalamin
involved in synthesising new cells, red blood cells and in cell repair. The richest sources of B-vitamins are unprocessed foods such as whole grains, green leafy vegetables, nuts, dairy products and animal foods such as meat and eggs but, in many countries, foods such as cereals and bread are fortified with these vitamins. It has been reported that inadequate intake and deficiencies in B-vitamins could impair athletic performance. Studies examining dietary intakes of B-vitamins in athletes have found that males typically report higher intakes than females because of their overall higher energy intake. Female athletes on an energy restricted diet and/or who exhibit disordered eating practices have lower intakes of riboflavin, folate and pyridoxine. Limited data on B-vitamin status in athletes exist: the few studies available have examined the status of thiamin, riboflavin, pyridoxine, folic acid and cobalamin. Some studies reported thiamin status to be adequate and, moreover, that exercise training does not appear to alter thiamin status. Similarly, studies on cobalamin also observed adequate status in athletes. However, data have also demonstrated altered cobalamin metabolism in recreational athletes compared with non-exercising controls. Data on riboflavin are mixed, with some studies reporting poor status in athletes and it has been suggested that riboflavin requirements may be higher than the recommended nutritional intake (RNI) for athletes than for the general population. Similarly, poor pyridoxine status in male and female athletes has been documented in some studies. Exercise training has been found to increase pyridoxine requirements due to greater losses and turnover suggesting that athletes undergoing heavy training might require more pyridoxine than current RNI. Studies examining folate status have produced mixed findings, with some researchers reporting adequate status in athletes and others reporting poor folate status. Few studies have investigated the effects of vitamin B supplementation on exercise performance in athletes. Thiamine supplementation does not appear to enhance exercise performance when adequate status is present, similarly, 10 weeks of folate supplementation in female marathon runners had no effect on running performance. Although no studies directly investigated the effects of riboflavin on performance in athletes, it was reported that dieting and exercise increase the intake of riboflavin needed to maintain adequate status. Several researchers have shown that exercise modifies pyridoxine metabolism with one study reporting losses of 1 mg during a marathon race. However, there are no studies on the effects of pyridoxine supplementation on performance per se. High folate and cobalamin intakes in conjunction with regular exercise may reduce plasma homocysteine concentrations which, in turn, can lower the risk factor for cardiovascular disease. Further well-controlled studies are required to investigate the effects of B-vitamin supplementation on exercise performance in athletes, and on physical activity and health, against a background of both adequate and inadequate status [13715].

Vitamin D

The prevalence of seasonal variation in vitamin D status was examined in 20 FA Premier League soccer players residing at a latitude of 53°N. Serum 25-hydroxyvitamin D (25(OH)D) levels decreased between August (104.4 ± 21.1 nmol/L, range 68-151) and December (51.0 ± 19.0 nmol/L), range 22-86), such that levels for 65 percent of the sample were insufficient (<50 nmol/L) in winter. Strategies to augment vitamin D(3) availability may therefore be advantageous for UK soccer players so as to maintain muscle function [12443].

A surprisingly high prevalence of vitamin D insufficiency and deficiency has recently been reported worldwide. Although very little is known about vitamin D status among athletes, a few studies suggest that poor vitamin D status is also a problem in athletic populations. It is well recognized that vitamin D is necessary for optimal bone health, but emerging evidence is finding that vitamin D deficiency increases the risk of autoimmune diseases and
nonskeletal chronic diseases and can also have a profound effect on human immunity, inflammation, and muscle function (in the elderly). Thus, it is likely that compromised vitamin D status can affect an athlete's overall health and ability to train (i.e. by affecting bone health, innate immunity, and exercise-related immunity and inflammation). Although further research in this area is needed, it is important that sports nutritionists assess vitamin D (as well as calcium) intake and make appropriate recommendations that will help athletes achieve adequate vitamin D status: serum 25(OH)D of at least 75 or 80 nmol/L. These recommendations can include regular safe sun exposure (twice a week between the hours of 10 a.m. and 3 p.m. on the arms and legs for 5-30 min, depending on season, latitude, and skin pigmentation) or dietary supplementation with 1,000-2,000 IU vitamin D3 per day. Although this is significantly higher than what is currently considered the adequate intake, recent research demonstrates these levels to be safe and possibly necessary to maintain adequate 25(OH)D concentrations [08406].

Vitamin D insufficiency is prevalent in various populations worldwide but with scarce data on physically active individuals. Vitamin D is important to athletes, affecting bone mass, immunity, and physical performance. This study evaluated the prevalence of vitamin D insufficiency and deficiency among young athletes and dancers. Data on 98 athletes and dancers (age, 15 ± 3 years: 53 % men), who had undergone screening medical evaluations, were extracted from medical records. Mean serum 25(OH)D concentration was 25 ± 8 ng/mL. Seventy-three percent of participants were vitamin D insufficient. Prevalence of vitamin D insufficiency was higher among dancers (94 %), basketball players (94 %), and Tae Kwon Do fighters (67 %) and among athletes from indoor versus outdoor sports (80 % vs 48 %). 25(OH)D levels adjusted for age and sex correlated with serum ferritin and season. In this study, conducted among young athletes and dancers from various disciplines in a sunny country, a high prevalence of vitamin D insufficiency was identified. A higher rate of vitamin D insufficiency was found among participants who practice indoors, during the winter months, and in the presence of iron depletion. Given the importance of vitamin D to athletes for several reasons, we suggest that athletes and dancers be screened for vitamin D insufficiency and treated as needed [10412].

While vitamin D deficiency is well recognized in Middle Eastern women as a result of cultural norms of remaining covered, Middle Eastern men are an under-reported group. Vitamin D is now known to have multiple effects, including an impact on muscle function, thereby increasing the relevance for sportsmen. The aim of one study was to evaluate serum 25-hydroxyvitamin D (25(OH)D) levels in young male Middle Eastern athletes in a cross-sectional study. Ninety-three Middle Eastern men presenting to hospital for an annual screening undertook a blood test to evaluate their vitamin D status. Ninety-one percent of athletes were found to be deficient in 25(OH)D (serum concentration <20 ng/ml). Athletes with severe deficiencies were significantly younger than those with less marked deficiency. A subset of athletes underwent bone mineral density assessment and 59 percent were shown to have at least one Z-score less than -1; despite this, however, no athletes reported a stress fracture. There was no correlation between 25(OH)D concentration and sunlight exposure, skin coverage and skin colouring. Thus, the study revealed that 25(OH)D deficiency is very common among otherwise healthy Middle Eastern male athletes. Given the potentially significant long- and short-term effects of 25(OH)D deficiency, serum 25(OH)D evaluation should be part of the routine assessment in this region [10528].

Vitamin D plays a major role in bone health by helping to maintain skeletal calcium balance in bone tissue. Vitamin D also helps to maintain adequate amounts of serum calcium for bone formation by actions on the intestines and kidney, allowing normal functioning of parathyroid hormone to help maintain serum calcium at normal levels. Sources of vitamin D include sunlight and dietary intake. Vitamin D is synthesised in the skin with exposure to
sunlight. For some individuals, who get enough exposure during the warmer months, the sun can provide adequate levels of vitamin D throughout the entire year. Athletes are among the groups of people who have been shown to be unable to get adequate levels of vitamin D from exposure to sunlight, especially if they live in northern latitudes, have dark skin pigmentation or participate in an indoor sport. These individuals should obtain intake from foods or supplements. Liver, cod liver oil, fatty fish and egg yolks all contain vitamin D. In some countries, a few foods, such as milk and cereals, are fortified with vitamin D. However, the nutrient composition should be checked, since some products make claims for vitamin D levels that are unlikely to make a substantial contribution to total needs. For athletes living in northern latitudes, especially athletes who exercise indoors, vitamin D status should be assessed [10252].

Vitamin D is an important factor for calcium and phosphorus homeostasis. A negative relationship has been observed between vitamin D status and diseases such as cancer, arthritis, diabetes, and muscle fiber atrophy. However, the relationship between vitamin D and prevention of skeletal muscle damage has not been clearly elucidated. The purpose of this study was to investigate the effects of vitamin D on exercise-induced muscle changes. Rats were divided into 3 groups: (1) sedentary control (C: n=10), (2) high-intensity exercise (HE: n=10), and (3) high-intensity exercise with vitamin D supplementation (HED: n=10; i.p. 1000 IU/kg body weight). Rats were trained for 30 min/day on treadmills (5 days/week for 8 weeks) with the running speed gradually increased up to 30 m/min at a 3° incline. At the end of the training period, the running speed was 38 m/min at a 5° incline. The high-intensity exercise significantly increased plasma creatine kinase (CK) and lactate dehydrogenase (LDH) activity. In addition, IL-6 and TNF-alpha levels as well as phosphorylation of AMPK, p38, ERK1/2, IKK, and IκB were significantly increased. Vitamin D-treated rats showed a significant decrease in plasma CK level, phosphorylation of AMPK, p38, ERK1/2, IKK, and IκB, and gene expression of IL-6 and TNF-alpha. Furthermore, the protein expression of vitamin D receptor (VDR) was highly increased in the muscles of HED-treated rats, respectively. Therefore, it was concluded that vitamin D may play a pivotal role in exercise-induced muscle damage and inflammation through the modulation of MAPK and NF-kappaB involved with VDR [13718].

Vitamin D is well known for its role in calcium regulation and bone health, but emerging literature tells of vitamin D's central role in other vital body processes, such as: signaling gene response, protein synthesis, hormone synthesis, immune response, plus, cell turnover and regeneration. The discovery of the vitamin D receptor within the muscle suggested a significant role for vitamin D in muscle tissue function. This discovery led researchers to question the impact that vitamin D deficiency could have on athletic performance and injury. With over 77 percent of the general population considered vitamin D insufficient, it's likely that many athletes fall into the same category. Research has suggested vitamin D to have a significant effect on muscle weakness, pain, balance, and fractures in the aging population; still, the athletic population is yet to be fully examined. There are few studies to date that have examined the relationship between vitamin D status and performance, therefore, this review will focus on the bodily roles of vitamin D, recommended 25(OH)D levels, vitamin D intake guidelines and risk factors for vitamin D insufficiency in athletes. In addition, the preliminary findings regarding vitamin D's impact on athletic performance will be examined [13719].

As research has progressed, the importance and versatility of vitamin D in the body has become quite evident, therefore the prevalence of vitamin D insufficiency has been heavily examined in recent years. Research suggests vitamin D's active role in immune function, protein synthesis, muscle function, inflammatory response, cellular growth and regulation of skeletal muscle. In addition, a common symptom of clinical vitamin D deficiency is muscle
weakness. Due to the many essential roles of vitamin D within the body, it has been suggested that physical performance may be influenced by serum vitamin D status, especially in those who are clinically deficient [13719].

Vitamin D insufficiencies are estimated to affect over one billion people worldwide. The Third National Health and Nutrition Examination Survey (NHANES III) data showed a significant increase in vitamin D insufficiency in the USA over the last 30 years, with over 77 percent of Americans considered vitamin D insufficient. The alarming rates of insufficiency and the vast metabolic properties of vitamin D have led researchers to examine the influence of vitamin D, not only on disease prevention, but also on physical performance and injury. Vitamin D has been identified in most tissues within the body, including skeletal muscle, which has led to further examination of vitamin D’s influence on athletes and physical performance. Because athletes and sports medicine physicians are primarily concerned with performance, the risk of vitamin D insufficiency among athletes has received growing interest and is under current examination by many researchers. In the last decade, researchers have examined 25(OH)D levels among various groups of athletes, ranging from gymnasts to runners to jockeys. Some findings have suggested that vitamin D levels in athletes are comparable to those of the general population; however, results depended largely on geographical location and type of sport (indoor vs. outdoor). It is apparent that the athlete is at an equal risk for vitamin D insufficiency, therefore the potential impact of vitamin D status on performance is now under examination. There are few studies to date that have examined the relationship between vitamin D status and performance, therefore, this review will focus on the bodily roles of vitamin D, recommended serum 25(OH)D level, vitamin D intake guidelines and risk factors for vitamin D insufficiency in athletes. In addition, the preliminary findings regarding vitamin D’s impact on athletic performance will be examined [13719].

It is well recognised that vitamin D plays an important role in calcium regulation and bone health. Emerging evidence, however, also suggests that vitamin D plays important roles in immune and inflammatory modulation and skeletal muscle function, and therefore has the potential to impact upon the health, training and performance of athletes. Although vitamin D is thought of as a “vitamin”, required amounts can be obtained entirely from cutaneous synthesis via exposure to ultraviolet-B (UVB) radiation in sunlight. Cutaneous synthesis of vitamin D, however, is dependent on factors including time of exposure, season, latitude, cloud cover, smog, skin pigmentation, sunscreen coverage and age. Vitamin D is not synthesised during the winter at latitudes greater than 35-37° north or south because insufficient UVB photons reach the earth’s surface during these months. Vitamin D is also obtained in the diet from limited natural and fortified sources. Dietary vitamin D includes vitamin D3 (cholecalciferol, derived from animal sources) and vitamin D2 (ergocalciferol, derived from UVB exposure of fungi and yeast ergosterols). Both forms are readily absorbed (about 50 % bioavailable), except in individuals with malabsorptive disorders. It is well recognised that suboptimal vitamin D status is widespread among the general population worldwide. Among athletes, the prevalence of deficiency varies by sport, training location, skin colour and individual lifestyle habits. For example, a high prevalence of vitamin D deficiency has been observed in gymnasts training in East Germany and Finland, with 37-68 percent of these athletes having serum 25(OH)D concentrations under 10-15 ng/mL (25-38 nmol/L), and in Middle Eastern sportsmen training in Qatar of whom 91 percent have serum 25(OH)D concentrations under 20 ng/mL (50 nmol/L), resulting in high rates of deficiency. In contrast, a low prevalence of insufficiency/deficiency and better overall status has been documented in college athletes training in a high altitude region of the USA. The proportion of athletes found to maintain optimal vitamin D status is observed to be 15-76 percent among some outdoor training populations. Muscle pain and weakness are well-documented but frequently forgotten symptoms of severe vitamin D deficiency improve with vitamin D repletion. Recent evidence from animal and in vitro studies are finding that vitamin D is
important for calcium handling across the sarcolemma and expression of proteins involved in muscle contraction. Although published data on vitamin D status or vitamin D supplementation on muscle performance are not yet available in athletes, provocative evidence from the Russian and German literature at the turn of the 20th century suggests that UVB exposure makes a positive impact upon athletic performance. These studies, however, did not simultaneously measure markers of vitamin D status (which were not yet understood) and were also not conducted using the rigorous scientific standards employed today. Vitamin D supplementation may benefit the health and performance of many athletes but should not be routinely recommended without assessment. Serum 25(OH)D concentration, the best indicator of vitamin D status, should first be evaluated along with anthropometric measurements (including body fat estimation), dietary intake and lifestyle and environmental factors that potentially impact status. Both body fat and body size are important to consider because they are inversely correlated with vitamin D status. This is due to either sequestration or volumetric dilution of ingested or cutaneously synthesized vitamin D by the larger fat mass which increases supplemental dose in larger or fatter athletes. Following assessment, recommendations for achieving/maintaining optimal vitamin D status can be tailored to the individual athlete’s current serum 25(OH)D concentration, diet, lifestyle habits, belief system, lifestyle habits and, if present, clinical symptoms. Habitual exposure to arms, legs and back several times a week for 5 mins (for fair-skinned individuals) to 30 mins (for darker-skinned individuals) at close to solar noon without sunscreen usually leads to sufficient vitamin D synthesis in summer months. Individuals with limited sun exposure require supplementation with at least 1500-2000 IU/day vitamin D to keep 25(OH)D concentrations in the sufficient but not necessarily the optimal range. Higher supplemental doses may be required in those with little sun exposure, regular sunscreen use, dark-pigmented skin and/or excess adiposity. Athletes who live or train at latitudes above/below 35-37° north or south should supplement with at least 600 IU/day during winter, rainy or cloudy seasons even if they maintain adequate stores during sunnier seasons. Regular consumption of vitamin D-fortified foods is not likely to result in sufficient status in the absence of UVB exposure. Although specific guidelines for supplemental vitamin D are not yet established, a rule-of-thumb is to increase supplemental vitamin D by 1000 IU over 3-4 months for every 10 ng/mL elevation in 25(OH)D desired. Thus, a “normal-weight” athlete with a serum 25(OH)D concentration of 20 ng/mL would require an additional 2000 IU daily to increase stores to 40 ng/mL in 3-4 months. Higher doses than estimated, using this rule-of-thumb, may be needed to improve status in some athletes, particularly when adipose stores are high or the starting serum 25(OH)D concentration extremely low. Genetic differences also influence response to supplementation. Supplemental vitamin D can be taken either daily or as a larger bimonthly or monthly dose (i.e. 50 000 IU/month and 1667 IU/day). To replenish stores more rapidly, athletes with deficient status may benefit from short-term, high-dose ‘loading’ regimens under the supervision of a physician. Examples of high-dose regimens include 50 000 IU/week for 8-16 weeks or 10 000 IU/day for several weeks. While supplementation with either D3 and D2 are effective at doses of 1000 IU/day, D3 is recommended for higher supplemental doses (50 000 IU) due to its greater potency at raising and maintaining serum 25(OH)D concentrations. It is interesting to note that Vitamin D deficiency can produce some symptoms similar to those in chronic fatigue syndrome and fibromyalgia. There is still debate about the distinction between deficiency and insufficiency of Vitamin D. On the other hand, taking high doses of Vitamin D supplementation exemplifies the situation in supplementation where more is not necessarily better. It is suggested that supplementation with Vitamin D should be carefully monitored, due to risk of toxicity if allowed to accumulate unchecked. While D3 is recommended for higher doses, it comes from sheep’s wool lanolin and thus is not suitable for vegan athletes. There is good evidence in the literature that Vitamin D, in moderate doses, may be helpful to some athletes [13720].
Despite a wide number of studies performed on the general population, little is known about the Vitamin D status of athletes. A particular influence of many factors, including skin pigmentation, early- or late-day training, indoor training, geographic location and extensive sunscreen use, has been observed in this specific population. The need of supplementation with Vitamin D in athletes is not defined or, when supplementation is needed, even the optimal amount of Vitamin D to be used is not specified. The periodic measurement of Vitamin D is the only procedure capable to define athletes’ status. Although various methods for the measurement of Vitamin D are routinely used, they often give discordant and poorly reproducible results; thus, it is necessary to standardize the various methods, in order to have comparable results. In conclusion, current available data indicate both that little is known about the Vitamin D status of athletes and that is still unclear if supplementation could be desirable. Finally, it must be pointed out that all the papers about Vitamin D status should indicate in detail the method used for really allowing a correct interpretation of data [13721].

One study implemented a two-part design to assess the vitamin D concentration of a large cohort of non-vitamin D supplemented UK-based athletes and 30 age-matched healthy non-athletes and to examine the effects of 5000 IU/day vitamin D(3) supplementation for 8-weeks on musculoskeletal performance in a placebo controlled trial. Vitamin D concentration was determined as severely deficient if serum 25(OH)D < 12.5 nmol/L, deficient 12.5-30 nmol/L and inadequate 30-50 nmol/L. demonstrate that 62 percent of the athletes (38/61) and 73 percent of the controls (22/30) exhibited serum total 25(OH)D < 50 nmol/L. Additionally, vitamin D supplementation increased serum total 25(OH)D from baseline, whereas the placebo showed no significant change. There was a significant increase in 10 m sprint times and vertical-jump in the vitamin D group whereas the placebo showed no change. The current data supports previous findings that athletes living at Northerly latitudes (UK = 53° N) exhibit inadequate vitamin D concentrations (<50 nmol/L). Additionally the data suggests that inadequate vitamin D concentration is detrimental to musculoskeletal performance in athletes. Future studies using larger athletic groups are now warranted [13722].

There is an increasing body of evidence for a multiplicity of roles for the vitamin D endocrine system throughout the human body. Such evidence has developed from an appreciation for the existence of a nuclear receptor (vitamin D receptor, VDR) for the biologically active form of vitamin D (1,25-dihydroxyvitamin D3) and its expression in a large number of tissues. The active metabolite is a potent secosteroid hormone that interacts with more than 200 genes via the VDR, giving it a wide range of actions. One tissue regulated by vitamin D is skeletal muscle. Indeed, in those with a clinical vitamin D deficiency, skeletal muscle myopathy (presenting as a proximal muscle weakness) is often observed. “Normalisation” of 25 hydroxyvitamin D (25[OH]D) concentrations in patients with myopathy has been shown to reverse the myopathy. Histological and electromyographic investigations in vitamin D deficient sample populations have demonstrated muscle fibre atrophy, a reduction in motor unit potentials and muscle weakness. A type II fibre atrophy has also been observed in the non-hemiplegic vastus lateralis muscle of elderly stroke patients, which was corrected with vitamin D supplementation. Vitamin D deficiency is common in the general public and athletic populations and may impair skeletal muscle function. It must be stressed that the recommended “optimum” concentrations are defined in relation to adequate bone function/metabolism and there are currently no accepted guidelines to the optimum levels to maximize neuromuscular performance. Interpretation of existing data is also complicated by the variations in biochemical methods used to measure 25[OH]D. In this regard, recent recommendations state that both vitamins D2 and D3 should be assessed using high performance liquid chromatography (HPLC) mass spectrometry (Tandem MS) to allow an accurate assessment of total 25[OH]D concentration. Despite the equivocal definitions of vitamin D deficiency, it would appear that a large proportion of individuals worldwide are at risk of low 25[OH]D concentrations that may result in subsequent health risks including
myopathy. It was therefore assessed the effects of vitamin D3 supplementation on serum 25(OH)D concentrations and physical performance. Thirty club-level athletes were block randomized (using baseline 25(OH)D concentrations) into one of three groups receiving either a placebo (PLB), 20 000 or 40 000 IU/week oral vitamin D3 for 12 weeks. Serum 25(OH)D and muscle function (1-RM bench press and leg press and vertical jump height) were measured presupplementation, 6 and 12 weeks postsupplementation. Vitamin D deficiency was defined in accordance with the US Institute of Medicine guideline (<50 nmol/L). Fifty-seven percent of the subject population were vitamin D deficient at baseline (mean ± SD value 51±24 nmol/L). Following 6 and 12 weeks supplementation with 20 000 IU (79 ± 14 and 85 ± 10 nmol/l, respectively) or 40 000 IU vitamin D3 (98 ± 14 and 91 ± 24 nmol/L, respectively), serum vitamin D concentrations increased in all participants, with every individual achieving concentrations greater than 50 nmol/L. In contrast, vitamin D concentration in the PLB group decreased at 6 and 12 weeks (37 ± 18 and 41 ± 22 nmol/L, respectively). Increasing serum 25(OH)D had no significant effect on any physical performance parameter. It was concluded that both 20 000 and 40 000 IU vitamin D3 supplementation over a 6-week period elevates serum 25(OH)D concentrations above 50 nmol/L, but neither dose given for 12 weeks improved our chosen measures of physical performance [13723].

Vitamin D is an essential nutrient obtained from the diet and exposure to sunlight. Roles for vitamin D have been established in the function of the cardiovascular, immune, and musculoskeletal systems. An electronic database search was conducted using EMBASE (1967 to August 2012), MEDLINE (1966 to August 2012), SPORTDiscus™ (1975 to August 2012), and the Scientific Electronic Library Online (SciELO) (1998 to August 2012) with no limits of language of publication. Articles that described vitamin D and performance were considered eligible for this review. Recent studies suggest that vitamin D maintains physical performance in athletes and other active populations, e.g. maximal oxygen consumption may be related to vitamin D status. Poor vitamin D status affects muscle strength, and vitamin D may participate in protein synthesis through the actions of the vitamin D receptor in muscle tissue. Vitamin D may protect against overuse injuries, such as stress fracture, through its well-documented role in calcium metabolism [13724].

**Vitamin D and muscle tissue**

The autocrine pathway appears to be of utmost importance and has recently received a great deal of attention in regards to vitamin D’s influence on skeletal muscle function. Vitamin D receptor (VDR) sites have been identified in virtually every tissue within the body. VDR regulates expression in hundreds of genes that perform essential bodily functions. The discovery of VDR within the muscle suggested a significant role for vitamin D in muscle tissue and has since been identified as a regulator of skeletal muscle. There are two proposed mechanisms by which vitamin D status may influence muscular strength. One possible explanation involves the direct role of 1,25-dihydroxyvitamin D [1,25(OH)2D] on VDRs within the muscle cells. A second explanation suggests that vitamin D modifies the transportation of calcium in the sarcoplasmic reticulum by increasing the efficiency or number of calcium binding sites involved in muscle contraction. This indirect mechanism however, has only been examined in rat models. On the contrary, one study disputes the evidence for the presence of VDRs within the skeletal muscle cells and suggests that the immunocytochemical staining to detect VDR may be responsible for the false positives results in previous studies. Furthermore, it has been suggested that vitamin D supplementation in individuals with low vitamin D status may improve muscle strength. This is believed to be due to an increase in the size and amount of type II (fast twitch) muscle fibers associated with vitamin D supplementation. It should be noted that type II fibers are predominant in power and anaerobic activities, and are recruited first to prevent falls,
associated with muscle strength in the aging population. Various researchers have found vitamin D to have a significant effect on muscle weakness, pain, balance and fractures in aging individuals. It is difficult, however to compare the results given the variety of outcome measures and differences in populations used in the studies. Several observational studies have suggested that vitamin D status influences muscular strength and function in the elderly. Contrary to these findings, it was in 2002 found no association between baseline vitamin D status and changes in performance measures over a four year period. Replacing vitamin D stores in the elderly population may be protective against fall risk and declining physical function. Few studies to date have examined this relationship in the adolescent population. However, it was examined the relationship between 25(OH)D status and bone mass, bone turnover, and muscle strength in Chinese adolescent females (n=301) and found that poor vitamin D status (<20 ng/mL) was associated with reduced forearm strength, (using a handgrip dynamometer) when compared to individuals with adequate vitamin D levels (>20 ng/mL). It was also suggested that 25(OH)D levels were positively associated with muscle power, and jump height in postmenarchal females (n=91), however physical activity levels were not taken into consideration. These findings in regard to muscle tissue and function suggest that vitamin D status may have a significant effect on muscle performance and injury prevention, therefore possibly influencing athletic performance. However, further research is warranted to determine the magnitude of effect of vitamin D on muscle strength and performance [13719].

**Vitamin D recommendations (intake abd desirable levels)**

Although the sun is the most plentiful source of vitamin D, there are also some dietary sources. Some common foods contain significant levels of vitamin D, naturally, including salmon, fatty fish, egg yolks, plus, fortified products also exist, such as, milk, cereal and orange juice. While these dietary sources may appear significant, the process of absorbing dietary vitamin D is only about 50 percent efficient; therefore, much of the nutrient value is lost in digestion. The lack of dietary vitamin D is yet another factor that increases the risk of vitamin D insufficiency. Most experts agree that a higher intake of vitamin D, through dietary sources, ultraviolet B (UVB) exposure, and supplementation, is necessary to obtain optimal serum vitamin D levels. In November of 2010, the Institute of Medicine (IOM) released new recommendations for dietary intake of vitamin D, 400-600 IU/day for children and adults (0-70 years), 800 IU/day for older adults (>70 years). These values are only slightly higher than past recommendations. Many experts argue that while IOM intake recommendations may adequately prevent clinical vitamin D deficiency, they are significantly lower than the level necessary to achieve optimal vitamin D status. The Recommended Dietary Allowance (RDA) for Vitamin D, according to the National Institute of Medicine (IOM) is compared to the Endocrine Society’s recommended intake. Many believe that the RDA is grossly underestimated, including the Endocrine Society, who released vitamin intake guidelines that are significantly higher. The Endocrine Society recommends 400-1000 IU/day for infants, 600-1000 IU/day in children (1-18 years) and 1500-2000 IU/day in adults, in addition to sensible sun exposure. Another area of debate among vitamin D researchers is the terminology and reference values used to define optimal vitamin D status, deficiency, and insufficiency. Optimal serum 25(OH)D concentrations have yet to be defined; however, most researchers have similar reference values. Vitamin D deficiency is often defined as <20 ng/mL (50 nmol/L), and insufficiency defined as 20-32 ng/mL (50-80 nmol/L) and optimal levels are >40 ng/mL (100 nmol/L). The term insufficiency “appears to be the currently favored term for the range of marginal deficiency and is the theoretical serum concentration that is not high enough to protect against certain chronic diseases.” Optimal levels of serum 25(OH)D are no exception to the controversy. When serum levels reach >32 ng/mL, parathyroid hormone (PTH) levels become stable and reduce the risk of secondary hypoparathyroidism, which is commonly associated with low vitamin D status. In addition,
intestinal calcium absorption is enhanced, reducing the risk of secondary bone disease. These basic vitamin D functions are efficiently demonstrated at 25(OH)D levels >32 ng/mL; however, superior benefits are observed at even greater levels. For example, only at 25(OH)D levels >40 ng/mL, does vitamin D begin to be stored in the muscle and fat for future use. Therefore, at levels <40 ng/mL, the body relies on a daily replenishment of vitamin D to directly satisfy its daily requirements, which is not likely to be present in the common diet. At levels <40 ng/mL, there appears to be just enough circulating 25(OH)D available for all of the immediate metabolic needs; however, stored vitamin D is not likely available for the advanced processes involved in the critical autocrine pathways. It is estimated that the body requires 3000–5000 IU of vitamin D per day to meet the needs of “essentially every tissue and cell in the body.” The IOM recommends 600 IU of vitamin D for most adults (18–70 years of age) to prevent clinical vitamin D deficiency, defined as 25(OH)D ≤ 20 ng/mL. In contrast, most expert’s recommendations are much higher than 600 IU per day, because their recommendations are designed to help reach optimal 25(OH)D levels of at least 40 ng/mL. Intake levels recommended by most experts not only allow support for daily metabolic requirements, but also allow for vitamin D storage and increased availability, which appears to reduce the risk of many diseases and possibly enhance performance. The recommended daily vitamin D intake, according to most experts, is at least 1000 IU per day to maintain optimal 25(OH)D status; however, more is required if levels begin suboptimal. With over 77 percent of Americans considered insufficient in vitamin D, it is apparent that the current recommendations are suboptimal. Intake recommendations increase with age, pregnancy, and lactation. In addition, experts recommend much higher initial dosages if 25(OH)D levels begin deficient, ranging from 2000 to 200,000 IU, until optimal 25(OH)D levels are met, then 1000-2000 IU/day for maintenance. A commonly prescribed treatment to quickly correct vitamin D deficiency is a weekly dose of 50,000 IU of vitamin D for eight weeks. The tolerable upper limit for vitamin D has been set by the IOM at 4000 IU for adults, compared to 10,000 IU/day by the Endocrine Society. Leading experts have claimed that a daily intake of 10,000 IU would take months, or even years to manifest symptoms of toxicity. A recent publication found no cases of toxicity with daily intakes of 30,000 IU per day for an extended period of time. Regardless of the current dietary intake value, the amount of vitamin D produced from 15 min of unprotected sun exposure is 10,000 to 20,000 IU, in a light-skinned individual, making most experts believe toxicity to be a rare and unlikely event. During the months that UVB rays are available from the sun, five to 15 min of unprotected sun exposure between the hours of 10 a.m. and 3 p.m. appear to provide adequate amounts of vitamin D. There have never been any reported cases of vitamin D toxicity from overexposure to the sun; however, symptoms of intoxication, such as hypercalcemia, have been observed when 25(OH)D levels are greater than 150 ng/mL. Serum 25(OH)D levels in individuals living close to the equator and working outdoors are often around 50 ng/mL, supporting the theory that vitamin D toxicity from the sun is extremely unlikely, and suggesting that any toxicities would result only from over supplementation. Regardless, many experts agree than 1000 IU/day in the absence of proper sun exposure can maintain 25(OH)D levels of at least 32 ng/mL [13719].

Vitamine D status of athletes

The distance from the equator, season, and time of day dictate whether vitamin D is available from the sun. Production of vitamin D from the sun is also dictated by cloud cover, pollution, sunscreen, skin pigment and age. During the summer months, UVB radiation from the sun can be absorbed in adequate amounts to synthesize vitamin D. However, during winter months, the angle of the sun prevents UVB radiation from reaching latitudes greater than 35-37 degrees, therefore, vitamin D cannot be synthesized from in these areas. Research has suggested that low levels of vitamin D are widespread in populations living south of the 35th parallel. Even if one spends ample time in the sun, sunscreen with a sun protection factor (SPF) of 15 results in a 99 percent decrease in vitamin D absorption.
Individuals who spend ample time outdoors may still need vitamin D supplementation to maintain adequate levels during the winter. Many outdoor athletes avoid peak sunlight hours, opting to practice early in the morning or late at night, which greatly reduces UVB exposure, putting them at considerable risk of vitamin D insufficiency. Various studies have found many athletes to be at high risk for vitamin D insufficiencies. It was in 2009 revealed that 90 percent of Middle Eastern sportsmen were vitamin D deficient between April and October. Although these sportsmen were training at favorable latitudes, Qatar (25.4°N), they averaged less than 30 min of sun exposure per day. Another study conducted at favorable latitude (Israel 31.8°N), suggested that 73 percent of athletes were vitamin D insufficient. The majority (83 %) of female, Australian indoor athletes were also found to be vitamin D insufficient. In contrast, a study conducted at less favorable latitude (41.3°N), revealed vitamin D insufficiency in 63 percent of indoor/outdoor athletes during winter, compared to the fall (12 %) and spring (20 %) in indoor and outdoor athletes. Finally, a study conducted even further from the equator (46.9°N), using exclusively outdoor athletes, found 25-30 percent with vitamin D insufficiency from fall to winter. Storlie et al. suggested that 1000 IU/day of vitamin D was not enough to prevent seasonal decline of vitamin D status in this cohort. Although the results are variable, geographical location (latitude) and gender do not appear to be the major risk factors for vitamin D insufficiency in athletes. Lack of sun exposure appears to be the main risk factor, putting indoor athletes and those who avoid peak daylight hours, regardless of latitudinal location, at the greatest risk for vitamin D insufficiency [13719].

**Vitamin D and athletic performance**

Original research concerning vitamin D and athletic performance dates back to the early twentieth century, but current performance trials are quite limited and inconclusive. Russian and German researchers were the first to report the convincing effects of ultraviolet light irradiation for improving athletic performance and decreasing chronic sports related pain. These early European researchers suggested significant improvements in time trials, cardiovascular fitness, and strength with treatment of UVB irradiation prior to performance. German Olympic officials considered these effects significant enough for UVB radiation (vitamin D) to be considered an ergogenic aid. In support of this concept, many athletes claim to peak in physical fitness during the time of year that vitamin D (UVB) levels are at their highest, summer and fall. Unfortunately, there are limited experimental studies available and even fewer that demonstrate a performance enhancement from vitamin D supplementation. However, research examining the aging population (>65 years of age) suggests benefits from vitamin D supplementation. Multiple performance studies in older adults have related low vitamin D levels to decreased reaction time, poor balance, and an increased risk of falling. Furthermore, vitamin D supplementation (800 IU/ day) in older adults showed improvements in strength, and walking distance, and a decrease in general discomfort. These favorable results in older adults support the need for further research on athletic performance and vitamin D. The current research available to support vitamin D’s influence on performance is quite limited. An (n=39), unpublished thesis examined 25(OH)D and maximal oxygen uptake (VO2max) to determine vitamin D’s effect on aerobic fitness in physically active college males. Higher 25(OH)D levels were associated with an increased VO2max, compared to those with lower vitamin D levels. These findings suggest that a favorable vitamin D status may improve aerobic performance. In 2013 it was examined, young, United Kingdom (UK, 53°N) based athletes (n=30), and examined the effects that vitamin D supplementation (20-40,000 IU/week for 12 weeks) had on muscle performance (1-RM bench press, leg press and vertical jump height). Subjects were assigned to a placebo, 20,000 IU/week or 40,000 IU/week of vitamin D for 12 weeks. Muscle performance and 25(OH)D was measured at six and 12 weeks, revealing that six weeks of supplementation was enough to correct vitamin D deficiency, however, it was not enough to
obtain optimal vitamin D levels >40 ng/mL. Contrary to the findings in the elderly population, no significant improvements in muscle performance were observed after 6 or 12 weeks of vitamin D supplementation, although serum 25(OH)D levels significantly increased over this time, from an average of 20 ng/mL to 32-39 ng/mL. In this study, lower baseline concentrations appeared to respond greater to supplementation, therefore, future studies may find more substantial results by dividing subjects into groups based on their baseline levels. Although final 25(OH)D concentrations obtained by the athletes were no longer considered deficient (>20 ng/mL), researchers hypothesized that higher total serum levels may be necessary to document enhanced muscle performance in young athletes. To explain the lack of response, the author suggested that skeletal muscle may require higher serum concentrations for a response, compared to other tissues. The significant response shown in elderly subjects, however, may be explained by sarcopenia. If the elderly were actively losing muscle mass, they may have a more sensitive response to vitamin D supplementation in the skeletal muscle. The authors suggested that more convincing results may be observed by giving supplemental doses of vitamin D to increase serum 25(OH)D above 40 ng/mL. A larger (n=61 athletes, n=31 healthy control subjects) UK-based vitamin D supplementation trial resulted in higher mean 25(OH)D levels, as a result of 5000 IU/day of vitamin D3 for eight weeks and found promising muscle performance results. This supplementation regime significantly increased mean 25(OH)D levels from (mean ± SD) 11.62 ± 10.02 ng/mL to 41.27 ± 10.02 ng/mL, whereas a placebo group showed no significant changes. The supplementation group also displayed significant improvements in 10-meter sprint times and vertical jump (with no improvements in 1-RM bench and squat tests) compared to the placebo group. One athlete’s 25(OH)D levels increased from 22.40 ng/mL to 55.69 ng/mL and showed improvements in all performance areas, this is only one athlete however. These findings support the aforementioned hypothesis that higher serum 25(OH)D levels (>40 ng/mL) may generate more convincing performance improvements. Findings also suggest that a daily dose of vitamin D (5000 IU/day) may be superior in raising 25(OH)D levels when compared to a weekly dose (40,000 IU/week). Based off of these two preliminary studies and guidelines from leading experts, 25(OH)D levels above 40 ng/mL are likely necessary to significantly improve anaerobic athletic performance. There are no studies available that have examined the effect of vitamin D on aerobic or endurance athletic performance. To maintain 25(OH)D levels of 40 ng/mL, vitamin D supplementation, especially during the winter months, is warranted. The 25(OH)D goal of 40 ng/mL is recommended for athletes because at this level, vitamin D begins to be stored in the muscle and fat for future use. Furthermore, at levels below 32 ng/mL, vitamin D is not likely to be readily available for the advanced processes involved in the autocrine pathways, which is the pathway that is most likely to influence performance. This level is also supported by the two comparable Close et al. studies, where the study achieving 25(OH)D levels greater than 40 ng/mL showed significant effects on performance. Besides the two UK based performance trials recent research on vitamin D and athletes has focused on the prevalence of vitamin D insufficiency among athletes, not the effects on performance. Although performance trials are limited, various other studies have resulted in alternative findings to support vitamin D’s positive impact on performance. Although the results of performance trials are not yet convincing enough to support vitamin D as a direct performance enhancer, obtaining optimal 25(OH)D levels can reduce the risk of debilitating stress fracture among athletes, which may indirectly influence performance through prevention of injury. In addition, because of its active role in muscle, resolution of vitamin D insufficiency has the potential to impact performance [13719]

Summer versus winter

Serum 25-hydroxyvitamin D is produced by the exposure of the skin to sunlight. Therefore athletes who train indoors, such as dancers, are vulnerable to vitamin D deficiency. The purpose of the study was to evaluate the serum 25-hydroxyvitamin D status in UK
professional dancers during periods of reduced and increased sunlight exposure (i.e. winter vs summer), and to assess the impact on bone metabolism and risk of injury. Nineteen elite classical ballet dancers (age 26 years; height 1.66 m; mass 54 kg) were monitored over a 6 month period for 25-hydroxyvitamin D, PTH and blood serum bone turnover markers (CTX and PINP) along with injury data. Repeated measure ANOVA and Wilcoxon and Chi-square analyses were used. Significant changes were noted between the winter and summer test dates for 25-hydroxyvitamin D, PTH, and PINP. The oral contraceptive had a significant effect on serum 25-hydroxyvitamin D, PTH and CTX. Soft tissue injuries were significantly lower in summer compared to winter period. It was concluded that professional ballerinas characterized by a high incidence of low serum 25-hydroxyvitamin D levels which improve marginally in the summer. These dancers also demonstrate a higher injury incidence in the winter. Oral contraception seems to increase serum 25-hydroxyvitamin D levels and has a positive effect on bone metabolism [13725].

Experimental

Vitamin D is an important factor for calcium and phosphorus homeostasis. A negative relationship has been observed between vitamin D status and diseases such as cancer, arthritis, diabetes, and muscle fiber atrophy. However, the relationship between vitamin D and prevention of skeletal muscle damage has not been clearly elucidated. The purpose of one study was to investigate the effects of vitamin D on exercise-induced muscle changes. Rats were divided into 3 groups: (1) sedentary control (C: n=10), (2) high-intensity exercise (HE: n=10), and (3) high-intensity exercise with vitamin D supplementation (HED: n=10; i.p. 1000 IU/kg body weight). Rats were trained for 30 min/day on treadmills (5 days/week for 8 weeks) with the running speed gradually increased up to 30 m/min at a 3° incline. At the end of the training period, the running speed was 38 m/min at a 5° incline. The high-intensity exercise significantly increased plasma creatine kinase (CK) and lactate dehydrogenase (LDH) activity. In addition, IL-6 and TNF-alpha levels as well as phosphorylation of AMPK, p38, ERK1/2, IKK, and IkappaB were significantly increased. Vitamin D-treated rats showed a significant decrease in plasma CK level, phosphorylation of AMPK, p38, ERK1/2, IKK, and IkB, and gene expression of IL-6 and TNF-α. Furthermore, the protein expression of vitamin D receptor (VDR) was highly increased in the muscles of HED-treated rats, respectively. Therefore, it was concluded that vitamin D may play a pivotal role in exercise-induced muscle damage and inflammation through the modulation of MAPK and NF-kappaB involved with VDR [13726].

Vitamin A

One study aimed to examine the effects of both physical activity and vitamin A supplementation on trace element metabolism in individuals engaged in taekwondo. The study registered seven healthy male national taekwondo players. The subjects were supplemented with oral administration of 100 mg vitamin A (retinol) for 6 weeks, and concurrently, they were subjected to taekwondo training 5 days a week. Before starting the vitamin A supplementation, blood samples were taken from the subjects twice, once at rest and once after exhaustion. Similarly, at the end of the 6-week vitamin A supplementation, two blood samples were taken from the subjects, once at rest and once after exhaustion, in order to determine (by atomic emission) and compare serum cobalt, molybdenum, calcium, cadmium, chromium, copper, manganese, sodium, nickel, phosphorus, sulfur, iron, boron, and zinc (mg/L) levels. Values of boron and nickel dropped significantly after 6-week vitamin A supplementation. Reduced levels of boron and nickel we obtained in the present study are believed to result from the antioxidant effect of long-term vitamin A supplementation [10250].
Coenzyme Q10

Coenzyme Q10 (originally known as ubiquinone), a coenzyme in the electron shuttle system of the inner mitochondrial membrane, is part of the total antioxidant defence system. It protects different cell structures from free oxygen radicals produced during oxygen stress such as severe physical exercise. Therefore athletes have used antioxidant supplements to strengthen antioxidant defence during training and competition. However, it was recently proposed that free radicals may have useful functions in the body, particularly in the signalling pathways associated with exercise stimulus. Thus, free radical production may be a prerequisite for training effects in muscle. On the basis of available findings, and recent general caution about antioxidant supplementation combined with exercise, it is not recommended that athletes take coenzyme Q10 supplements [10251].

The theoretically beneficial effects of coenzyme Q10 (Q10) on exercise-related oxidative stress and physical capacity have not been confirmed to our knowledge by interventional supplementation studies. The aim of one study was to investigate further whether Q10 supplementation at a dose recommended by manufacturers influences these factors. Using a randomized, double-blind, controlled design, we investigated the effect on physical capacity of 8 wk of treatment with a daily dose of 90 mg of Q10 (n=12) compared with placebo (n=11) in moderately trained healthy men 19 to 44 years old. Two days of individualized performance tests to physical exhaustion were performed before and after the intervention. Primary outcomes were maximal oxygen uptake, workload, and heart rate at the lactate threshold. Secondary outcomes were creatine kinase, hypoxanthine, and uric acid. No significant differences between the groups were discerned after the intervention for maximal oxygen uptake, workload at lactate threshold, or heart rate at lactate threshold were found, neither any differences between the groups were detected for hypoxanthine or uric acid (serum markers of oxidative stress) or creatine kinase (a marker of skeletal muscle damage). It was concluded that although in theory Q10 could be beneficial for exercise capacity and in decreasing oxidative stress, the present study could not demonstrate that such effects exist after supplementation with a recommended dose [12445].

Folate

Adequate folate intake is important for athletes and active individuals because of its role in red blood cell (RBC) production and tissue repair and maintenance. Folate plays a significant part in cell division, especially in tissues with rapid turnover such as RBCs. Folate deficiency leads to anaemia, caused by failure of the red cell precursors to develop into functional RBCs. The result is abnormally large RBCs that cannot effectively transport oxygen or remove carbon dioxide. For many countries, the recommended dietary allowance (RDA) for folate is 400 µg/day for individuals aged >19 years. Folate is found in many foods but is especially high in leafy green vegetables, nuts, legumes and liver. The bioavailability of folate in food is about 50 percent but is reduced by prolonged cooking. Many foods, such as breakfast cereals, are fortified with synthetic folic acid (50-100 % of the RDA for folate), which is highly bioavailable (85 %). Some countries have also made fortification of folic acid in enriched breads, flours and other grain products compulsory. Thus, the diets contain a mixture of food folate and synthetic folic acid with differing levels of bioavailability. Overall, the diets of active males appear to be adequate in folate as long as energy intake is adequate. Conversely, the apparent intake of dietary folate is consistently below recommendations for active females, which can be due to energy restriction for weight loss.
1868

and/or under-reporting of food intake in food records. However, in female marathon runners, although folate supplementation (5 g/day for 10 weeks in conjunction with iron) improved blood folate levels, there was no effect on performance [10527].

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Vitamin K

Vitamin K is of interest to sports performance through its identified potential either to prevent or enhance the healing of bone injury. Vitamin K is a co-enzyme involved in the gamma-carboxylation of certain proteins in bone, including osteocalcin. Vitamin K is found in plant-based food as phylloquinone (K1) and can be synthesised by bacteria as menaquinones (K2). Current recommended dietary intakes for vitamin K are based on a diet sufficient to promote normal blood clotting and it is possible that this is inadequate for optimal bone health. Low vitamin K status has also been linked with increased risk of hip fracture. Reduction in the number of bone injuries or the time taken to recover from these could be a significant performance enhancer in sport by reducing training time lost to injury. The impact of vitamin K on bone health has been examined mostly in postmenopausal women. In a recent meta-analysis the impact of supplementation was assessed against bone loss and fracture risk with the conclusion that vitamin K2 supplementation may decrease fracture risk and that both K1 and K2 reduced bone loss. Another meta-analysis examined the relationship between vitamin K supplementation and bone mineral density (BMD). It was concluded that supplementation with K1 may improve bone density at the lumbar spine but not at the femoral neck, although there was a large heterogeneity in results between studies. Doses studied varied between 1 and 45 mg/day which are well above the current Australian adequate intake of 60-70 microg/day. In both meta-analyses the majority of studies were undertaken in postmenopausal Asian women, which means this may not be generalised to other population groups such as athletes. To date only two studies have been conducted in athletic populations. The first found that K1 supplementation (10 mg/day for 4 weeks) improved markers of bone formation in athletes. Five of eight athletes were classed as deficient in vitamin K at baseline and supplementation had a larger effect on bone markers in these athletes. It is unclear as to why there was such a high incidence of deficiency in this group and whether this is representative of status in athlete populations generally. In another

1868
undertook a 2-year follow-up study looking at supplementation (10 mg/day, K1) in 115 female runners. Menstrual status was assessed and bone loss was measured by BMD. No benefit of supplementation was found. Limitations of the study were infrequent follow-up (6 monthly contact) and no adherence measures. While research relating to vitamin K is interesting, further studies are required before it is routinely considered in the management of bone health in athletes [13715].

Vitamin C

Prolonged physical exertion and environmental heat stress may elicit postexercise depression of immune cell function, increasing upper respiratory tract infection (URTI) susceptibility. It was investigated the effects of acute and short-term vitamin C compared with placebo supplementation on URTI susceptibility, salivary immunoglobulin A, and cortisol responses in healthy individuals following prolonged exercise-heat stress. Twelve participants were randomized in a double-blind design. For 12 days, participants consumed 3 x 500 mg tablets per day, with testing completed at baseline, then following acute (1 d) and short-term (8 d) supplementation. There was a significant linear trend in postexercise cortisol attenuation in the vitamin C group at baseline. No differences were detected in ratio of salivary sIgA to protein or URTI symptoms between groups. These data suggest that vitamin C supplementation can decrease postexercise cortisol in individuals performing exercise similar to that of a half-marathon or marathon in hot conditions. However, no changes in sIgA and URTI were evident, possibly due to previous moderate training and reduced physical and psychological stress compared with athletes participating in ultramarathons [09355].

Epidemiological data suggest that diets rich in antioxidants protect against diseases associated with free radical damage, including cancer, cardiovascular disease and diabetes. Early observations also suggested that vitamin supplements with antioxidant properties, like vitamins C and E, could also prevent or ameliorate pre-eclampsia, but most large randomized clinical trials have failed to show any benefit. Vitamin C given orally, even at high doses, does not achieve sustained serum levels that might be required for effective antioxidant activity. This may explain the failure of the numerous clinical trials involving its use in pre-eclampsia, cancers, cardiovascular diseases, etc. Vitamin C supplementation to stave off pre-eclampsia, cancer and other diseases is a 'nutraceutical' industry-driven myth which should be abandoned. We do not dispute a role for oxidative stress in the pathophysiology of pre-eclampsia, nor the possibility of amelioration of the disease by an anti-oxidant given at the right time and in the correct dosage. It was advocated to make a case that the massive and expensive clinical trials of vitamins C and E should cease until further rigorous scientific research is undertaken [11405].

Ascorbic acid is highly concentrated in the brain, being considered as a neuromodulator. This study investigated the effect of ascorbic acid in the tail suspension test (TST) and in the forced swimming test (FST) in mice and the contribution of the monoaminergic system to its antidepressant-like effect. Moreover, the effects of fluoxetine, imipramine and bupropion in combination with ascorbic acid in the TST were investigated. Ascorbic acid (0.1-10 mg/kg, i.p., 1-10 mg/kg p.o. or 0.1 nmol/mice i.c.v.) produced an antidepressant-like effect in the TST, but not in the FST, without altering the locomotor activity. The effect of ascorbic acid (0.1 mg/kg, i.p.) in the TST was prevented by i.p. pre-treatment with NAN-190 (0.5 mg/kg), ketanserin (62.5 microg/kg), yohimbine (1 mg/kg), prazosin (2 mg/kg), haloperidol (0.2 mg/kg), sulpiride (50 mg/kg), but not with SCH23390 (0.05 mg/kg, s.c.). Additionally, ascorbic acid (1 mg/kg, p.o.) potentiated the effect of subeffective doses (p.o. route) of fluoxetine (1 mg/kg), imipramine (0.1 mg/kg), or
bupropion (1 mg/kg) in the TST. The combined treatment of ascorbic acid with antidepressants produced no alteration in the locomotion in the open-field test. In conclusion, the results show that administration of ascorbic acid produces an antidepressant-like effect in TST, which is dependent on its interaction with the monoaminergic system. Moreover, ascorbic acid caused a synergistic antidepressant-like effect with conventional antidepressants. Therefore, the present findings warrant further studies to evaluate the therapeutical relevance of ascorbic acid for the treatment of depression and as a co-adjuvant treatment with antidepressants [09356].

The risk of upper respiratory infections (URIs) is increased in people who are under heavy physical stress, including recreational and competitive swimmers. Additional treatment options are needed, especially in the younger age group. The aim of one study was to determine whether 1 g/day vitamin C supplementation affects the rate, length, or severity of URIs in adolescent swimmers. It was carried out a randomized, double-blind, placebo-controlled trial during three winter months, among 39 competitive young swimmers (mean age 14 ± 2 years) in Jerusalem, Israel. Vitamin C had no effect on the incidence of URIs (rate ratio 1.01; 95 % confidence interval 0.70 to 1.46). The duration of respiratory infections was 22 percent shorter in vitamin C group, but the difference was not statistically significant. However, it was found a significant interaction between vitamin C effect and gender, so that vitamin C shortened the duration of infections in male swimmers by 47 percent, but had no effect on female swimmers. The effect of vitamin C on the severity of URIs was also different between male and female swimmers, so that vitamin C was beneficial for males, but not for females. The study indicates that vitamin C does not affect the rate of respiratory infections in competitive swimmers [11293].

To test the hypothesis that antioxidants can attenuate high-intensity interval training-induced improvements in exercise performance two groups of recreationally active males performed a high-intensity interval running protocol, four times per week for 4 weeks. Group 1 (n=8) consumed 1 g of vitamin C daily throughout the training period, whereas Group 2 (n=7) consumed a visually identical placebo. Pre- and posttraining, subjects were assessed for VO$_{2\text{max}}$, 10 km time trial, running economy at 12 km/h and distance run on the YoYo intermittent recovery tests level 1 and 2 (YoYoIRT1/2). Subjects also performed a 60 min run before and after training at a running velocity of 65 percent of pretraining VO$_{2\text{max}}$ so as to assess training-induced changes in substrate oxidation rates. Training improved VO$_{2\text{max}}$, 10 km time trial, running economy, YoYoIRT1 and YoYoIRT2 in both groups, although there was no difference between groups in the magnitude of training-induced improvements in any of the aforementioned parameters. Similarly, training also decreased mean carbohydrate and increased mean fat oxidation rates during submaximal exercise in both groups, although no differences existed between training conditions. It was concluded that daily oral consumption of 1 g of vitamin C during a 4 week high-intensity interval training period does not impair training-induced improvements in the exercise performance of recreationally active males [11294].

Vitamin C, also known as ascorbate and ascorbic acid is synthesized by all animals except humans, monkeys, guinea pigs, bats, and several bird species. The chief biologic function of vitamin C is as a water-soluble reducing agent (i.e. electron donor). Vitamin C has the potential to reduce cytochromes a and c of the electron transport chain, as well as molecular oxygen itself. Arguably the most important reaction requiring ascorbic acid as a cofactor involves the hydroxylation of proline residues in collagen. As such, vitamin C is required for the maintenance of normal connective tissue and wound healing. Vitamin C also is needed for the remodeling of bone, because of the presence of collagen in the organic matrix. A number of other metabolic reactions require vitamin C to act as a cofactor; notable among those are the synthesis of epinephrine from tyrosine, and the synthesis of the bile acids.
Also, vitamin C is suspected to have involvement in the process of adrenal steroidogenesis. Other putative biochemical uses of vitamin C include a role in antioxidant protection, thyroxin synthesis, amino acid metabolism, strengthening resistance to infection, and aiding in the absorption of iron. The work of Nobel Prize laureate Linus Pauling stimulated public interest in megadoses of this vitamin to prevent infection with the viruses responsible for the common cold; research on this topic over the past 20 years has offered conflicting results. The classic vitamin C deficiency disease is scurvy, due to the role of the vitamin in the post-translational modification of collagens. Vitamin C deficiency symptoms include small cell-type anemia, atherosclerotic plaques, and pinpoint hemorrhages; bone fragility and joint pain; poor wound healing and frequent infections; bleeding gums and loosened teeth; muscle degeneration and pain, hysteria and depression; and rough skin and blotchy bruises. An excessive intake of vitamin C (e.g. gram doses) may cause diarrhea, abdominal bloating, overabsorption of iron, hyperoxalemia (in dialysis patients), and hemolysis (in patients with glucose-6 phosphate dehydrogenase deficiency). A tolerable upper intake level for vitamin C was set at 2000 mg/d by the Food and Nutrition Board in 2000. The current RDA is 90 mg for men, and 75 mg for women (with an additional 35 mg added for smokers), and at these doses, vitamin C bioavailability is close to 100 percent. Significant food sources include citrus fruits, cabbage-type vegetables, dark green vegetables, cantaloupe, lettuce, tomatoes, potatoes, papayas, and mangoes. Despite its abundance in foods, it is important to note that vitamin C is easily destroyed by light, heat, and oxygen [06284].

Exercise involving lengthening muscle actions, such as downhill running, results in delayed onset muscle soreness (DOMS), which may be attributable to reactive oxygen species (ROS). Although exercise causes oxidative stress, any link between ROS and DOMS remains speculative. There is emerging evidence to suggest that ROS play an important physiological role, assisting in the recovery process and protecting the cell from future damage; however, this has not been fully established. Despite this uncertainty as to the precise role of ROS, attempts to prevent post-exercise ROS production through antioxidant intervention are still common. The study investigated the effects of ascorbic acid supplementation on ROS production and DOMS following downhill running. Subjects were assigned to two groups. The ascorbic acid group (group AA) received 1 g ascorbic acid 2 h pre-, and for 14 d post-downhill running, whilst the placebo group (Pl group) received a placebo. Blood samples were drawn pre-supplement, pre- and post-exercise, and then 1, 2, 3, 4, 7 and 14 d post-exercise for analysis of ascorbate, malonaldehyde and total glutathione. DOMS was assessed using a visual analogue scale and pressure algometry. Muscle function was assessed using isokinetic dynamometry. Plasma ascorbate was elevated throughout in group AA compared with the Pl group. Downhill running resulted in DOMS in both groups. Muscle function was impaired post-exercise in both groups, although a delayed recovery was noted in group AA. Malonaldehyde increased 4 d post-exercise in the Pl group only. Ascorbic acid supplementation attenuates ROS production following downhill running, without affecting DOMS. Furthermore, ascorbic acid supplementation may inhibit the recovery of muscle function [06285].

An increased systemic concentration of stress hormones (of the hypothalamic-pituitary adrenal axis) and some cytokines may contribute to the depression of immune cell function typically observed after prolonged exercise. The aim of the present study was to determine the effect of 2 weeks of supplementation with vitamin C (VC) on cortisol, adrenocorticotropic hormone, interleukin-6, oxidative stress and neutrophil responses to a single bout of endurance exercise. Nine healthy endurance-trained males exercised for 2.5 h at 60 percent VO\textsubscript{2max} after 2 weeks of placebo (PLA) or VC (1,000 mg/day) supplementation. All participants completed both trials utilising a randomised crossover design with a minimum 14 day washout period between trials. There was a significant trial x time interaction effect for plasma cortisol concentration which tended to be lower in the VC trial but post hoc analysis
found no specific between trial differences. There was a significantly lower post-exercise
neutrophilia in the VC trial, compared with the PLA trial. There was no trial x time interaction
for measures of neutrophil function (bacteria-stimulated elastase release, fMLP or PMA-
stimulated oxidative burst). However, there was a trend for higher fMLP-stimulated neutrophil
oxidative burst in the VC compared with PLA trial (trial x time interaction). These results
suggest that supplementation with VC for a period of up to 2 weeks provides little to no
protection against the depression of neutrophil function which typically occurs after
endurance exercise [06286].

Vitamin C is an essential component of the diet and may reduce the adverse effects of
exercise-induced reactive oxygen species, including muscle damage, immune dysfunction,
and fatigue. While supplement sales remain high, the debate as to whether athletes need
supplemental vitamin C continues. Vitamin C certainly plays an important role in immune
function, collagen synthesis, and cortisol synthesis, and it removes free radical intermediates
that initiate damaging cell reactions. Numerous studies have demonstrated an increase in
reactive oxygen species (ROS) or their byproducts with moderate to high intensity exercise.
As signaling molecules, ROS can modify target proteins by oxidizing thiol groups, forming
disulfide bonds that reversibly alter protein structure and function, therefore supplementing
with vitamin C may be expected to enhance athletic performance by reducing the potential
negative consequences of ROS. On the other hand, ROS released in exercise instigates cell
signaling for training adaptations, which vitamin C blocks. Supplementation with vitamin C
may therefore impair performance. The studies included in one review used the search terms
vitamin C, exercise, and athletes. Additional studies were sourced from reference lists in
related articles and books on the topic cited from 1985 to January 2012, with an initial list of
42 articles, with 12 of these included in the final review. From a total of 12 studies, vitamin C
in doses >1 g/day impaired sport performance substantially in four of four studies, possibly
by reducing mitochondrial biogenesis, while a further four studies demonstrated impairments
that were not statistically significant. Doses of about 0.2 g/day of vitamin C consumed
through five or more servings of fruit and vegetables may be sufficient to reduce oxidative
stress and provide other health benefits without impairing training adaptations [12437].

Of the human studies, the results are more varied; of the four studies supplementing with
vitamin C alone, three studies reported performance impairment and one reported
improvement, although all were nonsignificant. In the untrained humans, while the results are
mixed, the magnitude of the performance appears to be greater than in the trained, although
the results are insignificant. Overall, of the seven studies that supplemented with vitamin C
alone, three demonstrated significant performance impairments. Those studies
demonstrating a performance improvement were all nonsignificant outcomes. Regarding the
dose of vitamin C, of the four studies demonstrating significant performance decrements,
three adopted large supplemental doses of vitamin C (>0.5 g/kg daily) and two used a long-
term supplement protocol (>8 weeks). In particular, vitamin C prevented transcription of
genes involved in mitochondrial biogenesis (PGC1>) and the training-induced increase in
cytochrome C concentration (a marker of mitochondrial content) and messenger RNA
(mRNA) expression of the antioxidant enzymes superoxide dismutase and glutathione
peroxidase. It is shown that the exercise-induced rise in ROS is necessary for physiological
adaptations, including PGC1>, to training. The inhibition of mitochondrial growth is one
mechanism by which vitamin C impairs performance, given the important role that the
mitochondria play in aerobic energy metabolism. In addition to the retardation of
mitochondrial growth, ROS has been shown to regulate various intermediate activation
factors, responsible for optimal muscle cell function. ROS increases kinase activity (including
ERK, JNK, and p38) while reducing phosphatase activity (including PTEN and calcineurin),
resulting in the activation of transcription factors (p53, NK-JB, and ATF2). Physiological
changes that occur following moderate ROS production include increases in mitochondrial
growth factors, cell survival proteins (B-cell lymphoma 2), reduction in muscle atrophy and proteins involved in cell death signaling pathways (Bcl-2), and amplification of immune function. It is likely that supplementing with vitamin C may blunt numerous transcription factors responsible for adaptation to training not just PGC1. Distinct from the effects of vitamin C on mitochondrial growth, one study has demonstrated reductions in vascular function with mixed antioxidants. Reductions in exercise-induced redistribution of blood flow to skeletal muscles have been shown to reduce exercise capacity. Thus, vitamin C may reduce exercise-induced blood flow, reducing exercise capacity and performance [12437].

**Optimal vitamin C intake for athletes**

The US recommended intake of vitamin C for healthy individuals of 60 mg/day is based on the needs of nonactive healthy individuals, therefore not necessarily appropriate for athletes. For athletes with low vitamin C concentrations at baseline, will supplementing alter performance outcomes? While it is natural to presume that athletes with low baseline levels will benefit, this issue has not been well answered. Certainly in the general population, patients with ulcers and low vitamin C concentrations who supplemented with 500 mg daily demonstrated improved healing compared with controls. At present, it is safe to advise athletes to aim at the higher end of the recommended fruit and vegetable servings, in an attempt to consume adequate vitamin C, folate, magnesium, potassium, fiber, vitamins A, and other as-yet unidentified phytochemicals. Chronically megadosing with vitamin C is not recommended, although a moderate intake of 0.2 g daily consumed in the form of fruit and vegetables can be justified for health reasons. The impact vitamin C has on sport performance will depend on the exercise duration, dosage period, and background diet of the athlete. As far as the ideal vitamin C dose is concerned, it could be speculated that higher intakes taken for short period of time, such as onset of illness or during training camps, may provide benefit [12437].

**Vitamin C and E**

The antioxidant vitamins C (ascorbic acid) and E (alpha, beta, gamma, and delta tocopherols and tocotrienols) are involved in protecting cellular organelles from oxidative damage. Exercise can increase free radical production by 2- to 4-fold and produce changes in redox status which may exert oxidative stress on muscles and other tissues leading to alteration of lipids, proteins, and genetic material. Short-term exercise results in temporary increases in concentrations of oxidized products, but habitual exercise may result in an augmented endogenous antioxidant system and a reduction in oxidized products. Supporting endogenous antioxidant defense systems with additional oral doses of antioxidants has received much attention as a strategy to reduce oxidative stress, decrease muscle damage, and improve exercise performance. Indeed, a significant number of athletes, including elite athletes, consume vitamin supplements seeking beneficial effects on performance. However, recently, there is a growing evidence of the negative effects of antioxidant supplementation on exercise performance in both animal and human studies. In light of the results of these prolific studies, it was concluded that vitamin C and/or E interfere with the adaptive responses to endurance exercise training. More studies followed trying to delineate the possible effects of antioxidant supplementation on adaptations in exercise performance and/or redox homeostasis. In this context, it was performed the present literature analysis to evaluate whether antioxidant vitamin C and/or E supplementation affect the favorable adaptations of exercise. The detrimental outcomes associated with unregulated and excessive production of free radicals remains a physiological concern that has implications to health, medicine and performance. Available evidence suggests that physiological
adaptations to exercise training can enhance the body's ability to quench free radicals and circumstantial evidence exists to suggest that key vitamins and nutrients may provide additional support to mitigate the untoward effects associated with increased free radical production. However, controversy has risen regarding the potential outcomes associated with vitamins C and E, two popular antioxidant nutrients. Recent evidence has been put forth suggesting that exogenous administration of these antioxidants may be harmful to performance making interpretations regarding the efficacy of antioxidants challenging. The available studies that employed both animal and human models provided conflicting outcomes regarding the efficacy of vitamin C and E supplementation, at least partly due to methodological differences in assessing oxidative stress and training adaptations. Based on the contradictory evidence regarding the effects of higher intakes of vitamin C and/or E on exercise performance and redox homeostasis, a permanent intake of non-physiological dosages of vitamin C and/or E cannot be recommended to healthy, exercising individuals [12438].

Vitamin C and E supplementation has been shown to attenuate the acute exercise-induced increase in plasma interleukin-6 (IL-6) concentration. Here, we studied the effect of antioxidant vitamins on the regulation of IL-6 expression in muscle and the circulation in response to acute exercise before and after high-intensity endurance exercise training. Twenty-one young healthy men were allocated into either a vitamin (VT; vitamin C and E, n=11) or a placebo (PL, n=10) group. A 1-h acute bicycling exercise trial at 65 percent of maximal power output was performed before and after 12 weeks of progressive endurance exercise training. In response to training, the acute exercise-induced IL-6 response was attenuated in PL, but not in VT. However, no clear difference between groups was observed (group × training). Endurance exercise training also attenuated the acute exercise-induced increase in muscle-IL-6 mRNA in both groups. Oxidative stress, assessed by plasma protein carbonyls concentration, was overall higher in the VT compared with the PL group (group effect). This was accompanied by a general increase in skeletal muscle mRNA expression of antioxidative enzymes, including catalase, copper-zinc superoxide dismutase, and glutathione peroxidase 1 mRNA expression in the VT group. However, skeletal muscle protein content of catalase, copper-zinc superoxide dismutase, or glutathione peroxidase 1 was not affected by training or supplementation. In conclusion, the results indicate that, although vitamin C and E supplementation may attenuate exercise-induced increases in plasma IL-6 there is no clear additive effect when combined with endurance training [12439].

In order to study the effects of vitamin E and vitamin C supplementation on the bioenergetic index, 36 male physical education students were selected nonrandomly and assigned to a different supplementation protocol. The average age was 23 years. The period of supplementation lasted 3 weeks. The subjects from group 1 consumed a daily dose of 400 mg of vitamin E, subjects from group 2 ingested 1000 mg of vitamin C, subjects from group 3 ingested 400 mg of vitamin E along with 1000 mg of vitamin C, and subjects from group 4 (control group) consumed a placebo. The tests applied were the running anaerobic sprint test (RAST) and the Cooper 12-min run test. The results indicate that there were no significant differences between groups during the study in anaerobic power assessed by RAST. It was found a significant difference between group's, however, in aerobic power. It was concluded that daily consumption of vitamin E, vitamin C, and a combination of vitamin E and vitamin C for a period of 3 week significantly improved aerobic power [07369].

Like every redox-active compound vitamin E may exert pro-oxidative and antioxidative effects depending on the reaction partners present. In this work we evaluated the intensity of oxidative stress produced by a physical exercise through swimming as well as of protecting action of antioxidant vitamins E and C. Antioxidant systems include antioxidant enzymes: superoxide-dismutase (SOD), catalase (CAT), glutathion peroxidase (GSH-Px), as well as of
components with an antioxidant action of the reduced glutathion type (GSH) and vitamins E and C. We determine the activities of these enzymes in the erythrocytes and heart homogenate. Our results points out a protective effect against oxidative stress produced by swimming in animals treated with vitamins E and C, which are expressed through the diminution of the malondialdehyde (MDA) quantity both in erythrocytes and in the heart, and through the conservation of GSH content in both products. CAT and GSH-Px activities decrease while that of SOD increases on both tissues, but with different intensities in accordance with the variation of protection degree performed by the vitamin couple on these tissues. The obtained data underline the necessity of intensifying the means of endogenous anti-radical defence with exogenous antioxidant vitamins C and E. This study highlights the need of a proper vitamin supplement in organism under stress [07370].

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Intense and exhaustive exercise (IEE) is associated with oxidative stress in skeletal muscle, and it was recently reported that intestine is sensitive to IEE. In one study, it was investigated the possible relationship between the effects of IEE on morphology and oxidative markers in the ileum and isolated mitochondria. Mice were ascribed either to a control group comprising two subgroups, one sedentary and another exercised for 10 days (E10), or to a corresponding supplemented control group again comprising two subgroups, one sedentary and another exercised for 10 days (E10-V). The IEE program consisted of a single daily treadmill running session at 85 percent of V\(_{\text{max}}\), until animal exhaustion. Vitamins C (10 mg/kg) and E (10 mg/kg) were concurrently intraperitoneally administered 2 h before the exercise sessions. IEE was shown to cause impairment of ileum internal membrane mitochondria verified by ultramicrography analysis, and increase in ileum carbonyl content (117 %) and reduction in antioxidant capacity (36 %), and increase in mitochondria carbonyl content (38 %), increase in the percentage of ruptured mitochondria (25 %), increase in superoxide dismutase activity (186 %), and reduction in citrate synthase activity (40 %) compared with control animals. Observations in the vitamin-supplemented exercised animals (E10-V) were healthy appearance of myocyte mitochondria, decrease in ileum carbonyl content (66 %) and increase in antioxidant capacity (53 %), decrease in mitochondria carbonyl content (43 %), decrease in the percentage of ruptured mitochondria (30 %), slight increase in superoxide dismutase activity (7 %), and significant increase in citrate synthase activity (121 %) compared with E10 animals. Therefore, the present results strongly corroborate the hypothesis that IEE leads to marked disturbances in intestinal mitochondria, mainly in redox status, and affects whole intestinal redox status [09357].

There is a considerable commercial market, especially within the sports community, claiming the need for antioxidant supplementation. One argument for antioxidant supplementation in sports is that physical exercise is associated with increased reactive oxygen and nitrogen species (RONS) production, which may cause cell damage. However, RONS production may also activate redox sensitive signaling pathways and transcription factors, which
subsequently may promote training adaptation. The aim was now to investigate the effects of combined vitamin C and E supplementation to healthy individuals on different measures of exercise performance after endurance training. Using a double-blinded placebo-controlled design, moderately trained young men received either oral supplementation with vitamins C and E (n=11) or placebo (n=10) before and during 12 weeks of supervised, strenuous bicycle exercise training of a frequency of 5 days/week. Muscle biopsies were obtained before and after training. After the training period, maximal oxygen consumption, maximal power output, and workload at lactate threshold all increased significantly in both groups. Also, glycogen concentration, citrate synthase (CS), and beta-hydroxyacyl-CoA dehydrogenase (beta-HAD) activity in muscle were significantly higher in response to training in both groups. However, there were no differences between the two groups with regard to any of the physiological and metabolic variables measured. The results suggest that administration of vitamins C and E to individuals with no prior vitamin deficiencies has no effect on physical adaptations to strenuous endurance training [09358].

Exercise practitioners often take vitamin C supplements because intense muscular contractile activity can result in oxidative stress, as indicated by altered muscle and blood glutathione concentrations and increases in protein, DNA, and lipid peroxidation. There is, however, considerable debate regarding the beneficial health effects of vitamin C supplementation. One double-blind and randomized study was designed to study the effect of vitamin C on training efficiency in rats and in humans. Fourteen men (27-36 years old) were trained for 8 week. Five of the men were supplemented daily with an oral dose of 1 g vitamin C. In the animal study, 24 male Wistar rats were exercised under 2 different protocols for 3 and 6 week. Twelve of the rats were treated with a daily dose of vitamin C (0.24 mg/cm² body surface area). The administration of vitamin C significantly hampered endurance capacity. The adverse effects of vitamin C may result from its capacity to reduce the exercise-induced expression of key transcription factors involved in mitochondrial biogenesis. These factors are peroxisome proliferator-activated receptor co-activator 1, nuclear respiratory factor 1, and mitochondrial transcription factor A. Vitamin C also prevented the exercise-induced expression of cytochrome C (a marker of mitochondrial content) and of the antioxidant enzymes superoxide dismutase and glutathione peroxidase. This means that vitamin C supplementation decreases training efficiency because it prevents some cellular adaptations to exercise [08397].

In order to study the effects of vitamin E and vitamin C supplementation on the bioenergetic index, 36 male physical education students were selected nonrandomly and assigned to a different supplementation protocol. The period of supplementation lasted 3 weeks. The subjects from group 1 consumed a daily dose of 400 mg of vitamin E, subjects from group 2 ingested 1000 mg of vitamin C, subjects from group 3 ingested 400 mg of vitamin E along with 1000 mg of vitamin C, and subjects from group 4 (control group) consumed a placebo. The tests applied were the running anaerobic sprint test (RAST) and the Cooper 12-min run test. The results indicate that there were no significant differences between groups during the study in anaerobic power assessed by RAST. It was found a significant difference between group's, however, in aerobic power and it was concluded that daily consumption of vitamin E, vitamin C, and a combination of vitamin E and vitamin C for a period of 3 week significantly improved aerobic power [08398].

The need for energy in strenuous exercises necessitates an increase in oxygen consumption and production of reactive oxygen species. It seems that supplementation of vitamins C and E reduces exercise-induced oxidative stress. Therefore, one study aimed to investigate the effects of vitamin C and E supplementation on muscle damage and oxidative stress in female athletes. The study was a four-week randomized, double-blind clinical trial, conducted on 64 trained female athletes recruited in the Isfahan sports club. They were randomly assigned to
one of the following four groups: (a) vitamin C (250 mg/day), (b) vitamin E (400 IU), (c) vitamin C + vitamin E, and the control (placebo). Aspartate transaminase (AST), creatine kinase (CK), and lactate dehydrogenase (LDH) for assessing muscle damage, and malondialdehyde, were measured before and after the intervention. In the between-groups comparison, only creatine kinase significantly changed at the end of the period. However, in the intergroup comparison creatine kinase was significantly decreased in group 1. As for Aspartate aminotransferase, no significant difference was spotted in any of the comparisons. Lactate dehydrogenase was significantly decreased in group 2. Finally, the study revealed a significant decrease in oxidative stress markers in groups 1, 3, and 4. It was concluded that it is induced from the results that vitamin C and E supplementation plays a role in reducing muscle damage markers of aerobic exercises [13716].

Effects on running

Exercise-induced oxidative stress is implicated in muscle damage and fatigue which has led athletes to embark on antioxidant supplementation regimes to negate these effects. one study investigated the intake of vitamin C (VC) (1 g), blackcurrant (BC) juice (15 mg VC, 300 mg anthocyanins) and placebo in isocaloric drink form on training progression, incremental running test and 5-km time-trial performance. Twenty-three trained female runners (age, 31 years) completed three blocks of high-intensity training for 3 weeks and 3 days, separated by a washout (4 wks). Changes in training and performance with each treatment were analysed with a mixed linear model, adjusting for performance at the beginning of each training block. Markers of oxidative status included protein carbonyl, malondialdehyde (in plasma and in vitro erythrocytes), ascorbic acid, uric acid and erythrocyte enzyme activity of superoxide dismutase, catalase and glutathione peroxidase were analysed. There was a likely harmful effect on mean running speed during training when taking VC (1.3 %). Effects of the two treatments relative to placebo on mean performance in the incremental test and time trial were unclear, but runners faster by 1 SD of peak speed demonstrated a possible improvement on peak running speed with BC juice (1.9 %). Following VC, certain oxidative markers were elevated: catalase at rest (23 %), protein carbonyls at rest (27 %) and superoxide dismutase post-exercise (8.3 %). In conclusion, athletes should be cautioned about taking VC chronically, however, BC may improve performance in the elite [13717].

Side effects

Vitamins A (retinol), D, E, and K are stored in the liver and in body fat stores hence they do not need to be taken every day. Some are toxic if an excess amount is consumed. In a recent meta-analysis of primary and secondary treatment with antioxidant supplements, beta-carotene, vitamin A and E were also found to increase mortality but there was no convincing evidence for harmful effects of selenium or vitamin C [08399]. Despite the fact that water-soluble vitamins (i.e. C, B) are excreted in the urine rather than stored in the body, there is evidence suggesting that vitamin C, especially in combination with iron [08400] can cause damage to the gastrointestinal tract (GI) and initiate or aggravate symptoms associated with chronic GI disorders. A high intake of iron, especially in combination with manganese doubles the risk for Parkinson’s disease [08401]. Unnecessary cobalt ingestion results in enhanced oxidative stress leading to organ damage and dysfunction in liver and kidney, and also impairs thyroid activity and myocardial function [08402]. In addition to the potential hazard of positive doping tests [08403, 08404], contamination may also pose health risks such as lead contamination in calcium products [08405].

Vitamines A, C, and E
Micronutrients, including vitamin supplements, are widely used in the general population and by athletes. A variety of claims or areas of interest have promoted the use of vitamin supplements, with antioxidant claims becoming a popular interest over the past two decades. While the antioxidant issue has typically targeted the prevention of ageing or various diseases associated with oxidative damage, it has also been recognised that exercise is a stimulator of the generation of oxygen radical species. Hence, there has been interest in whether athletes might have additional needs for antioxidant vitamins to counteract such damage. Vitamins A, C and E are often being discussed together because all three have antioxidant properties, though they differ in many other respects. Recent research has focused on these vitamins’ antioxidant prospects because exercise induces free radical (ROS, reactive oxygen species) release which leads to cellular damage, fatigue and overtraining. Literally hundreds of studies on antioxidant vitamin supplementation (AVS) and exercise have been published. Systematic training causes the body to develop adaptations naturally to the increase in exercise-induced ROS, but it has been hypothesised that AVS may provide additional benefits. Studies validate as well as refute this possibility. Validating this possibility, in one study nine sedentary and nine active young adult males were given 50 mg/day V-A+1000 mg/day V-C+800 mg/day V-E for 2 months and performed a treadmill ergometer VO2max test before and after the treatment period. Before supplementation, sedentary subjects (but not trained subjects) exhibited exercise-induced increased white blood cell and granulocyte levels and increased erythrocyte aggregation and deformability; after supplementation, these phenomena did not occur. Both sedentary and trained subjects showed an elevated erythrocyte lipid peroxidation after the VO2max test pre-supplementation but not post-supplementation. This study suggested that AVS augmented the already increased antioxidant capacity found in trained individuals. In a study of adolescent male basketball players undergoing intense training for 35 days, athletes dosed with 500 mg/day V-C+150 mg/day V-E demonstrated higher plasma content of V-A (retinol), V-C and V-E concomitant with elevated erythrocyte glutathione peroxidase (GSH-Px) and reduced glutathione (GSH; both important antioxidant molecules) compared with non-treated controls. Refuting the prospect of AVS providing additional physiological benefits in trained athletes, a different study supplemented 21 sedentary young adult males with 500 mg/day V-C+400 IU/day V-E for 16 weeks. After the first 4 weeks, subjects also performed endurance training for 12 weeks. With results from previous studies, the researchers noted that, although AVS reduced plasma IL-6 levels in response to an acute exercise bout (cycle Pmax test) before training, AVS did not show the same effect after training. Plasma IL-6 levels were, in fact, higher in the supplemented/trained group than the placebo/trained group, indicating that either the supplement only worked in the sedentary condition or that the supplement attenuated the effects of training. Using a model of eccentric training, another team of researchers supplemented 14 young adult males with 1 g/day V-C+400 IU/day V-E for 11 weeks combined with 7 weeks of resistance training and found no differences in acute exercise-induced plasma GSH or other markers of antioxidant capacity and redox status compared with similarly trained but non-supplemented controls. The effects of AVS on performance have also been studied. Using subjects discussed previously, one team showed that maximal performance parameters and muscle biopsy-determined glycogen, citrate synthase and beta-hydroxyacyl-CoA activity levels were no different in AVS-supplemented versus non-supplemented groups. One study of eight active men given 1 g/day V-C for 4 weeks concomitant with aerobic training showed that AVS had no impact on running performance. Another team reported that 10 well-trained young adult male runners given either 500 mg/day V-C+100 IU/day V-E or placebo for 2 weeks in a cross-over design showed no differences in 8 km time trial performance. Intriguingly, this latter study used an environmental chamber that simulated a hot, humid, ozone-polluted environment for the time trials and also reported that total plasma antioxidant V-C and V-E concentrations were higher, whereas plasma and nasal lavage CC (Clara cell protein; a marker for lung cell
damage) levels were lower in the AVS-treated compared with non-treated trials. Addressing just V-C, it appears that those studies dosing athletes at 1 g/day or greater demonstrate negative effects on performance more often than not. Although the data together may appear equivocal, it would be premature to draw any conclusions regarding the effectiveness or lack of AVS in athletes. Experimental parameters (including subject, exercise and dosing characteristics) are variable across studies, rendering it difficult to make direct comparisons. Regardless of this, athletes who consume well-balanced meals congruent with contemporary guidelines should be receiving adequate supplies of these vitamins. Several have opined that athletes should not be likely to receive any additional benefits from exceeding recommended daily intakes (RDI) and that additional supplementation may only be necessary in cases of extreme, prolonged physical exertion under adverse environmental conditions. Nevertheless, multivitamin supplements are readily available to athletes, vary widely in their vitamin doses, and often contain antioxidant vitamins at RDI or greater levels. For example, in an impromptu survey of a dozen different multivitamin supplements available at a central Iowa grocery store in mid-September, V-A content ranged from 1750-6100 IU (35-120 % RDI), V-C 60-250 mg (100-417 % RDI) and V-E 22.5–200 IU (75-667 %). Over-supplementation (toxicity) is more likely to occur with the fat-soluble vitamins. Excess antioxidant vitamins may be detrimental to athletes when they interfere with normal levels of ROS-mediated intracellular signalling, disrupting redox balance and interfering with normal muscle cell function and performance. The current data only suggest that AVS, in the doses used across studies, either improved or had no effect on physiologically relevant markers of antioxidant capacity and either decreased or had no effect on performance outcomes. There is no question that athletes experience increased oxidative stress as a result of their activity levels, but the issue of whether or not athletes need higher dietary antioxidants than less active counterparts remains under debate. There are hypotheses as well as data to support a beneficial use, a neutral outcome in people who are already achieving intakes at recommended levels from dietary sources, and even a detrimental effect on training adaptations. Athletes consuming a balanced diet commensurate with their caloric needs, including vegetables, fruits and whole grains, are likely receiving adequate levels of antioxidant vitamins. For those athletes “errring on the side of caution”, food-based sources of antioxidant vitamins may be preferable to supplement-based sources because the possibility of toxicity is lower when foods are utilised. Although daily multivitamin supplements are generally regarded as safe, over-supplementation may result in disrupted redox balance or diminished performance. Further studies may help to clarify which of these outcomes is the most likely, given the various scenarios of training, environment and diet in which athletes may find themselves [12440].

<table>
<thead>
<tr>
<th>Vitamin A</th>
<th>Vitamin C</th>
<th>Vitamin E</th>
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<tbody>
<tr>
<td>Retinol</td>
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<td>Tocopherol</td>
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<tr>
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<td>900 microg/day</td>
<td>75-90 mg/day</td>
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<tr>
<td>DRI, women</td>
<td>700 μg/day</td>
<td>65–75 mg/day</td>
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<td>Antioxidant, bone and cartilage maintenance, hormone synthesis, immune function and metabolism</td>
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<td>Dietary sources</td>
<td>Dairy products, fruits and green vegetables</td>
<td>Fruits and vegetables</td>
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<td>Body storage</td>
<td>Liver</td>
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1879
Vitamin E

The purpose of one study is to determine the effect following exercise to exhaustion of vitamin E supplementation on oxidative stress in athletic students. Twenty male students voluntarily participated in the study and were randomly assigned (double blind) to either a vitamin E (daily dose of 450 mg of a-tocopherol for a period of 8 weeks) or a placebo group (took capsules containing 450 mg of lactose for 8 weeks). Before and after 8 weeks blood samples were collected at rest and after exercise to exhaustion. Oxidative stress markers were malondialdehyde (MDA), carbonylated proteins (CP) and creatine kinase (CK). Also, the effect of vitamin E on ergometer cycling time, as an example of endurance performance, was evaluated. ANOVA and independent t-tests indicated that vitamin E supplementation did not significantly change MDA, CP and CK values at rest, after exercise to exhaustion, and cycling time, but plasma volume after exercise to exhaustion significantly decreased. It was concluded that although vitamin E supplementation had no effect on exercise performance or capacity in athletic students, further investigation is required using larger numbers of subjects and measures of vitamin E before unequivocal conclusion can be stated [06287].

During endurance exercise, oxygen consumption by the skeletal muscle can increase 100-200 times. It was previously found that during an ultramarathon race (50 km, forest trail through hilly terrain) compared with a day of rest, vitamin E disappeared faster (as measured using 2H-labelled alpha-tocopherol) and lipid peroxidation increased. Therefore, it was hypothesized that prior supplementation with antioxidants (vitamins E and C) would decrease oxidative stress during distance running and, therefore, decrease lipid peroxidation and inflammation, decrease DNA damage, decrease muscle damage and/or improve recovery. To test these hypotheses, it was carried out a randomized, double-blind study in runners (11 females, 11 males) who were participants in an annual ultramarathon race. It was found that supplementation with both vitamins E and C only prevented increases in lipid peroxidation, but had no apparent effect on DNA damage, inflammation or muscle damage. These results suggest that the mechanism of oxidative damage is operating independently of the inflammatory and muscle damage responses [06288].

One study investigated whether vitamin E can attenuate eccentric exercise-induced soleus muscle injury as indicated by the amelioration of in situ isometric force decline following a low-frequency fatigue protocol (stimulation at 4 Hz for 5 min) and the ability of the muscle to recover 3 min after the termination of the fatigue protocol. Adult male Wistar rats were divided into vitamin E-supplemented or placebo-supplemented groups studied at rest, immediately post-exercise or 48 h post-exercise. Daily dl-alpha-tocopheryl acetate intraperitoneal injections of 100 mg/kg body mass for 5 consecutive days prior to exercise doubled its plasma levels. Fatigue index and recovery index expressed as a percentage of the initial tension. FI at 0 h post- and 48 h post-exercise respectively was 88 percent and 89 percent in the vitamin E groups versus 76 percent and 80 percent in the placebo groups. RI was 99 percent and 100 percent in the vitamin E groups versus 82 percent and 84 percent in the placebo groups. Complementally to the traditionally recorded maximal force, low-frequency fatigue measures may be beneficial for assessing injury-induced decrease in muscle functionality [12441].

The aim of one study was to determine the direction of change in performance variables at fixed blood lactate concentrations following vitamin E (VE) supplementation. In a paired-matched design twelve (male: n=8; female: n=4) trained runners were allocated to a VE (n=6; 268 mg/d) or placebo (n=6; glucose) group for 35 days. Participants completed a discontinuous incremental exercise test, pre and post supplementation, to determine peak
oxygen uptake (\(\text{VO}_{2\text{peak}}\)) running velocity and percentage of peak oxygen uptake (%(\text{VO}_{2\text{peak}})) at the lactate threshold (TLAC) and the onset of blood lactate accumulation (OBLA). Participants maintained a standardised training regime throughout the supplementation period. VE supplementation failed to significantly enhance velocity at TLAC and OBLA compared to a placebo treatment. Analogously, VE did not significantly enhance %(%(\text{VO}_{2\text{peak}})) at TLAC and OBLA compared to a placebo treatment. Whilst VE supplementation did not enhance performance it did not impair performance compared to a placebo. Training significantly enhanced velocity at TLAC and OBLA. No training-induced improvements in %\(\text{VO}_{2\text{peak}}\) at TLAC and OBLA were observed. It was thus concluded that daily VE supplementation for 35 days does not enhance or impair physiological performance at fixed blood lactate concentrations. Long-term VE supplementation for the purposes of performance enhancement is not recommended [12442].

**Dietary antioxidants, in general**

Physical exercise induces oxidative stress and tissue damage. Although a basal level of reactive oxygen species (ROS) is required to drive redox signaling and numerous physiologic processes, excess ROS during exercise may have adverse implications on health and performance. Antioxidant nutrients may be helpful in that regard. Caution should be exercised against excess antioxidant supplements, however. This article presents a digest for sports practitioners. The following three recommendations are made:

- it is important to determine the individual antioxidant need of each athlete performing a specific sport
- multinutrient preparations, as opposed to megadoses of any single form of nutrient, seem to be a more prudent path to choose
- for outcomes of antioxidant supplementation, performance should not be the only criteria

Overall well being of the athlete, faster recovery, and minimization of injury time could all be affected by antioxidant therapy [06289].

Athletes should match their energy intake with expenditure in order to maintain lean body mass. It is also important to consume adequate amounts of antioxidant vitamins and minerals to maintain health. To assess the dietary habits of six nationally ranked Australian swimmers physical training load and dietary intake (24 h food recall) and were recorded on a daily basis during a 4 day intensive physical training period. The results showed no significant difference between energy intake and expenditure or the amount of carbohydrate consumed compared to the Australian recommended daily intake (RDI). Athletes reported a significantly greater intake of vitamin A, vitamin C, vitamin E, and protein than the RDI. It was concluded that these elite swimmers have an adequate dietary intake to allow for optimal physical training and performance [12447].

Exercise has been noted in some, but not all, studies to elicit an oxidative stress. The discrepancy in findings may be related to differences in exercise intensity across protocols, as well as to differences in training status of participants. It was compared blood oxidative stress biomarkers in exercise-trained men after three different bouts of exercise of varying intensity and duration, as well as a nonexercise condition. On different days, men (n=12, 21-35 years) performed aerobic cycle exercise (60 min at 70 % HR reserve) and cycle sprints (five 60-s sprints at 100 % maximum wattage obtained during graded exercise testing and ten 15-s sprints at 200% maximum wattage obtained during graded exercise testing). Blood
was collected before and 0, 30, and 60 min after exercise and analyzed for malondialdehyde, hydrogen peroxide (H₂O₂), advanced oxidation protein products, and nitrate/nitrite (NO(x)). As indicators of antioxidant status, Trolox equivalent antioxidant capacity, superoxide dismutase, catalase, and glutathione peroxidase were measured. No differences were noted in malondialdehyde, hydrogen peroxide advanced oxidation protein product, or NO(x) between conditions or across time. Antioxidant capacity was generally highest at 30 and 60 min after exercise and lowest at 0 min after exercise. It was concluded that in trained men, and considering the limitations of the current design (e.g. inclusion of selected oxidative stress and antioxidant biomarkers measured in blood only), strenuous bouts of exercise do not result in a significant increase in blood oxidative stress during the 1-h postexercise period. These findings may be related to attenuation in reactive oxygen species production as an adaptation to chronic exercise training and/or a protective effect of the antioxidant system in response to acute strenuous exercise [12448].

One study examined the acute effects of a single dose of an antioxidant (AO; Lactaway® containing pycnogenol) on time to fatigue (TTF). Nine trained cyclists performed on two separate occasions a continuous protocol of 5 min at 50 percent of peak power output (PPO), 8 min at 70 percent of PPO, and then cycled to fatigue at 95 percent PPO. Four hours prior to the exercise protocol, the subjects consumed the supplement or a placebo (counterbalanced, double blind protocol). Cyclists, on average, rode for 80 s more in the Lactaway trial than they did in the placebo trial. There was considerable evidence (chances ≥95 %) for substantial positive treatment effects for TTF and the other performance-related variables (excluding [BLa] at 95 % PPO). Other studies are necessary to confirm these results and identify the mechanisms underlying the observed effects [12449].

To compare the effects of a 3-week supplementation between two different mixtures of antioxidants (AOX) and placebo on aerobic exercise performance in acute normobaric hypoxia. Seventeen subjects were randomly assigned in a double blind fashion to receive a broad-based AOX supplement containing beta-carotene, ascorbic acid, d-alpha-tocopherol-succinate, N-acetylcysteine, riboflavin, zinc, and selenium (AO group), or a combination of alpha-ketoglutaric acid (alpha-KG) and 5-hydroxymethylfurfural (5-HMF) (CS group), or placebo (PL group). Before and after supplementation subjects performed two incremental cycle-exercise tests until exhaustion. The first test was conducted under normoxic conditions (LA, FiO₂ of 21 %, 547 m) and the second after the 3-week supplementation period under normobaric hypoxic conditions (AHA, FiO₂ of 13 %, 4300m). In CS peak cycling performance (peak power) declined from LA to AHA 7.3% (90% CI: 2.2-12.4) less compared to PL and 7 percent less compared to AO. Better maintenance of aerobic exercise capacity in CS was associated with an attenuated reduction in maximal heart rate in hypoxia. It was concluded that aerobic exercise performance was less impaired in acute normobaric hypoxia after 3 weeks with supplementation of alpha-KG and 5-HMF compared to a broad-based AOX supplement or placebo [13727].

**Theoretical aspects on the cellular level**

Dietary antioxidant supplements are readily available and routinely consumed by the general public. Reasons for supplementation include compensation for dietary insufficiencies, treatment of primary mitochondrial disorders, improved health and overall wellbeing, and the prevention of diseases associated with aging including cancer. Despite estimates that 40 percent of the adult population in the US consumes some form of antioxidants scientific support for their purported benefits is currently inadequate and some reports even suggest that antioxidants may be detrimental to some health outcome. Exercise is believed to result in a transient increase in reactive oxygen species (ROS) production that results from increased demand for ATP, produced by mitochondrial oxidative phosphorylation, to support
increased muscle contractile activity. This increase in ROS may play a role in activating signaling pathways that mediate long-term adaptations to exercise, such as increased mitochondrial biogenesis, that is associated with improved insulin sensitivity. Conversely, excessive ROS production may place muscle in a condition of oxidative stress that adversely affects performance and adaptation. Consequently, the hypothesis has emerged that the consumption of antioxidants during endurance training could attenuate physiological adaptations in muscle (increased antioxidant defense enzyme activity and mitochondrial biogenesis). The latter concept has been controversial since some reports have shown that antioxidant supplementation counteracted the beneficial effects of exercise, reduced performance, and inhibited mitochondrial adaptations to exercise, but others have not [13728].

Important factors that may contribute to differences between reported outcomes include; the complex interactions between antioxidants, the potential for antioxidants to become pro-oxidant, non-redox cellular effects of the “antioxidants”, and/or temporal differences between the state of training (early adaptive or elite acute extreme performance). Although there are many different compounds that fall under the umbrella term “antioxidant”, vitamin E, vitamin C, coenzyme Q₁₀ (CoQ₁₀) and α-lipoic acid are more commonly studied. CoQ₁₀ is an electron-shuttling compound that is vital to the mitochondrial electron transport chain and has potent antioxidant properties. Vitamin E (alpha-tocopherol) is an important component of biological membranes where it is thought to participate in scavenging lipid peroxide radicals, though it may exert some molecular effects independent of its antioxidant properties. alpha-Lipoic acid is an essential cofactor for mitochondrial α-ketoacid dehydrogenases (e.g. pyruvate dehydrogenase) and is crucial for several mitochondrial metabolic pathways. It is also regarded as potent mitochondrial antioxidant and has been studied in numerous oxidative stress-related pathological conditions such as ischemia/reperfusion injury and diabetes. It also has direct effects on skeletal muscle, increasing resting energy expenditure, insulin sensitivity, and glucose uptake. This is mediated primarily through the activation of AMP-activated protein kinase (AMPK), a major energy (ATP/AMP) sensor in the cell, that regulates mitochondrial biogenesis through peroxisome proliferator-activated receptor gamma coactivator 1alpha (PGC-1alpha). Despite the potential effects of these and other antioxidant compounds on metabolism and energetic adaptation, there is no evidence that mitochondrial function can be directly altered by dietary antioxidant supplementation alone. While many of these antioxidants display potent radical scavenging properties in vitro, experiments conducted in vivo are mixed and some studies indicate that they may function as pro-oxidants, rather than antioxidants [13728].

Clinical studies
The beneficial effects of exercise and a healthy diet are well documented in the general population but poorly understood in elite athletes. Previous research in subelite athletes suggests that regular training and an antioxidant-rich diet enhance antioxidant defenses but not performance. To investigate whether habitual diet and/or exercise (training status or performance) affect antioxidant status in elite athletes antioxidant blood biomarkers were assessed before and after a 30-min ergometer time trial in 28 male and 34 female rowers. The antioxidant blood biomarkers included ascorbic acid, uric acid, total antioxidant capacity (TAC), erythrocyte-superoxide dismutase, glutathione peroxidase (GPx), and catalase. Rowers completed a 7-day food diary and an antioxidant-intake questionnaire. Effects of diet, training, and performance on resting biomarkers were assessed with Pearson correlations, and their effect on exercise-induced changes in blood biomarkers was assessed by a method of standardization. With the exception of GPx, there were small to moderate increases with exercise for all markers. Blood resting TAC had a small correlation with total antioxidant intake, and the exercise-induced change in TAC had a trivial to small association with dietary antioxidant intake from vitamin C, vegetables, and vitamin A. Most other dietary
intakes had trivial associations with antioxidant biomarkers. Years of training had a small inverse correlation with TAC and a small association with the exercise-induced change in TAC. Training status correlates more strongly with antioxidant status than diet does [13729].

To ascertain whether reactive oxygen species (ROS) contribute to training-induced adaptation of skeletal muscle, we administered ROS-scavenging antioxidants (AOX; 140 mg/L of ascorbic acid, 12 mg/L of coenzyme Q10 and 1% N-acetyl-cysteine) via drinking water to 16 C57BL/6 mice. Sixteen other mice received unadulterated tap water (CON). One cohort of both groups (CON(EXE) and AOX(EXE)) was subjected to treadmill exercise for 4 weeks (16-26 m/min, incline of 5°-10°). The other two cohorts (CON(SED) and AOX(SED)) remained sedentary. In skeletal muscles of the AOX(EXE) mice, GSSG and the expression levels of SOD-1 and PRDX-6 were significantly lower than those in the CON(EXE) mice after training, suggesting disturbance of ROS levels. The peak power related to the body weight and citrate synthase activity was not significantly influenced in mice receiving AOX. Supplementation with AOX significantly altered the mRNA levels of the exercise-sensitive genes HK-II, GLUT-4 and SREBF-1c and the regulator gene PGC-1alpha but not G6PDH, glycogenin, FABP-3, MCAD and CD36 in skeletal muscle. Although the administration of AOX during endurance exercise alters the expression of particular genes of the ROS metabolism, it does not influence peak power or generally shift the metabolism, but it modulates the expression of specific genes of the carbohydrate and lipid metabolism and PGC-1alpha within murine skeletal muscle [13730].

The beneficial effects of exercise and a healthy diet are well documented in the general population but poorly understood in elite athletes. Previous research in subelite athletes suggests that regular training and an antioxidant-rich diet enhance antioxidant defenses but not performance. To investigate whether habitual diet and/or exercise (training status or performance) affect antioxidant status in elite athletes antioxidant blood biomarkers were assessed before and after a 30-min ergometer time trial in 28 male and 34 female rowers. The antioxidant blood biomarkers included ascorbic acid, uric acid, total antioxidant capacity (TAC), erythrocyte- superoxide dismutase, glutathione peroxidase (GPx), and catalase. Rowers completed a 7-d food diary and an antioxidant-intake questionnaire. Effects of diet, training, and performance on resting biomarkers were assessed with Pearson correlations, and their effect on exercise-induced changes in blood biomarkers was assessed by a method of standardization. With the exception of GPx, there were small to moderate increases with exercise for all markers. Blood resting TAC had a small correlation with total antioxidant intake, and the exercise-induced change in TAC had a trivial to small association with dietary antioxidant intake from vitamin C, vegetables, and vitamin A. Most other dietary intakes had trivial associations with antioxidant biomarkers. Years of training had a small inverse correlation with TAC and a small association with the exercise-induced change in TAC. It was concluded that training status correlates more strongly with antioxidant status than diet does [13731].

Physical exercise has many benefits, but it might also have a negative impact on the body, depending on the training level, length of workout, gender, age and fitness. The negative effects of physical exercise are commonly attributed to an imbalance between the levels of antioxidants (both low molecular weight antioxidants and antioxidant enzymes) and reactive oxygen and nitrogen species due to excessive production of free radicals during physical exercise. In this critical review, we look for answers for three specific questions regarding the interrelationship between physical exercise and oxidative stress (OS), namely

- the dependence of the steady-state level of OS on fitness
- the effect of intensive exercise on the OS
- the dependence of the effect of the intense exercise on the individual fitness
All these questions have been raised, investigated and answered, but the answers given on the basis of different studies are different. In one review, it was tried to explain the reason(s) for the inconsistencies between the conclusions of different investigations, commonly based on the concentrations of specific biomarkers in body fluids. It was thought that most of the inconsistencies can be attributed to the difference between the criteria of the ill-defined term denoted OS, the methods used to test them and in some cases, between the qualities of the applied assays. On the basis of our interpretation of the differences between different criteria of OS, it was considered possible answers to three well-defined questions. Possible partial answers are given, all of which lend strong support to the conclusion that the network responsible for homeostasis of the redox status is very effective. However, much more data are required to address the association between exercise and OS and its dependence on various relevant factors [13732].

*Redox state in athletes*

The purpose of one study was to assess the influence of sport-specific and nonspecific bouts of exercise on athletes' redox state. Blood samples were collected from 14 handball players immediately before and after graded exercise test on the cycle ergometer and handball training. Levels of superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), nitrites (NO$_2^-$) as markers of nitric oxide, index of lipid peroxidation (TBARs), glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) activity were determined. Exercise intensity was assessed by a system for heart rate (HR) monitoring. Average athletes' HR was not significantly different between protocols, but protocols differed in total time and time and percentage of time that athletes spent in every HR zone. The laboratory exercise test induced a significant increase of H$_2$O$_2$ and TBARs as well as the decrease of the SOD and CAT activity, while after specific handball training, levels of NO$_2^-$ were increased and SOD activity decreased. It seems that unaccustomed short intensive physical activity may induce oxidative stress in trained athletes, while sport-specific activity of longer duration and proper warm-up period may not. Further research should show whether the change of protocol testing and the implementation of various supplementations and manual methods can affect the redox equilibrium [12450].

*In rugby players*

One study investigated the biochemical and anthropometric characteristics in elite athletes of rugby union based in the south of France during the different periods of the competition to identify metabolic and biochemical adaptations to particular lifestyle conditions. Participants included 35 players in 2008 and 43 players in 2009. Biochemical variables [creatinine, uric acid, creatine kinase (CK), alanine aminotransferase, aspartate aminotransferase, C-reactive protein] were evaluated. Specific protein levels (albumin, acid alpha-glycoprotein, prealbumin), vitamins (A, E, C), antioxidant enzymes (glutathione peroxidase (GPX), superoxide dismutase (SOD)) oligoelements (Zn, Se, Cu, erythrocyte magnesium), homocysteine (Hcy), carnitine and the distribution of amino acids were specifically determined for our study during a pre-competition period (September 2008 and 2009). Globally, no deficit was observed for vitamins, oligonutrients and amino acids levels. The high SOD and GPx activities in rugby players suggest a presence of oxidative stress of exercise. The evaluation of renal function should be used with caution because of the interaction between creatinine and lean body mass. In addition, a profound effect of intense exercise on the CK values was reported to establish specific reference values for athletes. The analysis of the biological variation allows optimization of the interpretation of the changes from an increased or decreased baseline value from a season to the other one. The conclusions of the study were that there was a necessity of rugby-specific reference intervals.
for CK and creatinine parameters and the use of enzymatic creatinine for Modification of Diet in Renal Disease (MDRD) and CKD-EPI, or cystatin C to improve glomerular filtration rate estimation. Moreover, it should be taken into account the oxidative stress testifying of a bad recovery, and there is a need to better take care the nutritional status of the players by adapting needs and amino acids supplementations but also to consider a follow-up of oxidative stress and antioxidants according the results [12451].

**Phlebodium decamanum**

Strenuous exercise induces muscle damage due to a highly increased generation of free radicals and inflammatory response and therefore, in this type of exercise, it is important to reduce both oxidative stress and inflammation, at least their negative aspects. The purpose of this study was investigate, for the first time, whether a purified, standard water-soluble fraction obtained from Phlebodium decamanum could reduce the over-expression of inflammation and oxidative stress induced by strenuous exercise. The physical test consisted of a constant run that combined several degrees of high effort (mountain run and ultra-endurance), in permanent climbing. Biochemical parameters, oxidative stress and inflammatory mediators were assessed. The results showed that oral supplementation of P. decamanum during high-intensity exercise effectively reduces the degree of oxidative stress (decreased 8-hydroxy-2'-deoxyguanosine and isoprostanes generation, increased antioxidant enzyme activities in erythrocyte and total antioxidant status in plasma). The data obtained also indicate that this supplementation is efficient in reducing the inflammatory response through the decrease of TNF-alpha and increase of sTNF-RII, but kept the levels of IL-6 and IL-1ra. In conclusion, oral supplementation of P. decamanum extract during high-intensity exercise effectively reduces the degree of oxidative stress and has anti-inflammatory protective effects, preventing the over-expression of TNF-alpha but keeping the levels and effects of IL-6. These findings provide a basis for similar Phlebodium supplementation for both professional and amateur athletes performing strenuous exercise in order to reduce the undesirable effects of the oxidative stress and inflammation signalling elicited during high-intensity exercise [12453].

**Resveratrol**

Resveratrol is a natural polyphenolic flavonoid antioxidant which may provide numerous health benefits such as the prevention of cancer, cardiovascular disease and ischaemic injuries, as well as enhancing stress resistance. It is a freely available food supplement and is found in the seeds and skins of grapes, red wine, mulberries, peanuts and rhubarb. Interest in resveratrol in sports medicine arose after animal studies assessed endurance performance of mice and found a dose-dependent increase in exercise tolerance, improved motor skills and increased number and activity of mitochondria in muscle cells. Resveratrol-treated mice had a significantly higher maximum VO\textsubscript{2} rate, suggestive of an increased oxidative capacity. Resveratrol intake increases the ratio of oxidative to non-oxidative type muscle fibres and increases muscle strength in resveratrol-treated mice. The resveratrol effects also seem to be dependent on the length of intake, as one of the actions proposed is a gene switch. There are no established doses for resveratrol but it has been shown in humans that resveratrol administration with doses of 250 mg and 500 mg, resulted in a dose-dependent increase in cerebral blood flow during task performance and enhanced oxygen extraction. Doses of 1600 mg per day in a 70 kg participant are regarded as safe, even long term. Resveratrol as a food supplement in sports medicine has not received much attention despite some basic scientific evidence that this substance could have multiple indications.
related to high-performance sports. Therefore, further studies are required to confirm whether there are similar effects in humans [12470].

Resveratrol (RES) is a well-known phytocompound and food component which has antioxidative and multifunctional bioactivities. However, there is limited evidence for the effects of RES on physical fatigue and exercise performance. The purpose of one study was to evaluate the potential beneficial effects of trans-RES on fatigue and ergogenic functions following physiological challenge. Male ICR mice from four groups (n=8 per group) were orally administered RES for 21 days at 0, 25, 50, and 125 mg/kg/day, which were respectively designated the vehicle, RES-25, RES-50, and RES-125 groups. The anti-fatigue activity and exercise performance were evaluated using forelimb grip strength, exhaustive swimming time, and levels of serum lactate, ammonia, glucose, and creatine kinase (CK) after a 15-min swimming exercise. The exhaustive swimming time of the RES-25 group was significantly longer than that of vehicle group. A trend analysis revealed that RES treatments increased the grip strength. RES supplementation also produced dose-dependent decreases in serum lactate and ammonia levels and CK activity and also an increase in glucose levels in dose-dependent manners after the 15-min swimming test. The mechanism was related to the increased energy utilization (as blood glucose), and decreased serum levels of lactate, ammonia, and CK. Therefore, RES could be a potential agent with an anti-fatigue pharmacological effect [13734].

Resveratrol and quercetin

Resveratrol and quercetin function as antioxidants and anti-inflammatories in vitro, but these mechanisms have been minimally examined in combination in exercising humans. The purpose of this investigation was to examine supplementation as a countermeasure against oxidative stress and inflammation in response to exercise. Fourteen athletes were randomly assigned, in a double-blind crossover design, to a resveratrol and quercetin combination (RQ) (120 mg resveratrol and 225 mg quercetin for 6 days and 240 mg resveratrol and 450 mg quercetin on day 7 just prior to exercise) or to placebo (P). There was a 1-week washout between trials. Blood was taken at baseline, pre-exercise, immediately after exercise, and 1 h after exercise. Plasma was analyzed for oxidative stress (F2-isoprostanes and protein carbonyls), antioxidant capacity (ferric-reducing ability of plasma (FRAP), Trolox equivalent antioxidant capacity (TEAC), oxygen radical absorptive capacity (ORAC)), and inflammation (cytokine interleukin (IL)-8 and C-reactive protein (CRP)). Statistical design utilized a 2 × 3 ANOVA and Student's t test. Pre-exercise values were not different from baseline for any measure. The postexercise increase in F2-isoprostanes was significantly less with RQ (68 %) than with P (137 %). Protein carbonyls, FRAP, ORAC, and TEAC significantly increased after exercise but were not affected by treatment. IL-8 and CRP increased significantly immediately after exercise but were not affected by treatment. These data indicate that RQ significantly reduces exercise-induced lipid peroxidation without associated changes in inflammation or plasma antioxidant status [13735].

N-Acetylcysteine

N-acetylcysteine (NAC) is a reduced thiol donor that supports cellular resynthesis of glutathione, a major antioxidant in skeletal muscle and other human tissues. Glutathione
buffers reactive oxygen species (ROS) produced by muscle. ROS levels increase substantially during strenuous exercise, overwhelming glutathione buffering and depressing contractile function. This contributes to the development of muscle fatigue. NAC opposes this process. By supporting glutathione resynthesis, NAC slows the rise of ROS activity in exercising muscle and delays fatigue. This was first demonstrated in humans by our group in studies of tibialis anterior, an ankle dorsiflexor muscle, during repetitive electrical stimulation. Subsequent studies confirmed that NAC inhibits fatigue during volitional exercise tasks, for example, loaded breathing and handgrip exercise. The studies of greatest physiological relevance were those who showed that NAC delays fatigue of trained athletes during strenuous cycling exercise. Despite performance benefits in the laboratory, several issues limit NAC use as an ergogenic aid. First, only pharmacological doses of 140–150 mg/kg have been shown to limit fatigue. It is not known if lower doses are effective. Secondly, doses that delay fatigue are safe but can have uncomfortable side effects including nausea and diarrhoea. Finally, NAC may have negative effects on athletic training. Data are emerging to suggest that chronic antioxidant supplementation blunts the positive effects of transient oxidative stress, a signal that appears to be essential for muscle adaptation to exercise. Thus, while effects of high-dose NAC are intriguing, it is too soon to conclude that NAC supplementation is beneficial for athletes. Future directions for research include studies to optimize NAC dosage, balancing efficacy versus side effects, and to evaluate novel thiol donors for their effects on fatigue [11523].

Allopurinol

The aim of one research was to examine the impact of the xanthine oxidase (XO) inhibitor allopurinol on the skeletal muscle activation of cell signaling kinases and adaptations to mitochondrial proteins and antioxidant enzymes following acute endurance exercise and endurance training. Male Sprague-Dawley rats performed either acute exercise (60 min of treadmill running, 27 m/min, 5 % incline) or 6 wk of endurance training (5 days/week) while receiving allopurinol or vehicle. Allopurinol treatment reduced XO activity to 5 percent of the basal levels, with skeletal muscle uric acid levels being almost undetectable. Following acute exercise, skeletal muscle oxidized glutathione (GSSG) significantly increased in allopurinol- and vehicle-treated groups despite XO activity and uric acid levels being unaltered by acute exercise. This suggests that the source of ROS was not from XO. Surprisingly, muscle GSSG levels were significantly increased following allopurinol treatment. Following acute exercise, allopurinol treatment prevented the increase in p38 MAPK and ERK phosphorylation and attenuated the increase in mitochondrial transcription factor A (mtTFA) mRNA but had no effect on the increase in peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha), nuclear respiratory factor-2, GLUT4, or superoxide dismutase mRNA. Allopurinol also had no impact on the endurance training-induced increases in PGC-1alpha, mtTFA, and mitochondrial proteins including cytochrome c, citrate synthase, and beta-hydroxyacyl-CoA dehydrogenase. In conclusion, although allopurinol inhibits cell signaling pathways in response to acute exercise, the inhibitory effects of allopurinol appear unrelated to exercise-induced ROS production by XO. Allopurinol also has little effect on increases in mitochondrial proteins following endurance training [13736].

Other antioxidants

High levels of reactive oxygen species (ROS) produced in skeletal muscle during exercise have been associated with muscle damage and impaired muscle function. Supporting endogenous defence systems with additional oral doses of antioxidants has received much
attention as a noninvasive strategy to prevent or reduce oxidative stress, decrease muscle damage and improve exercise performance. Over 150 articles have been published on this topic, with almost all of these being small-scale, low-quality studies. The consistent finding is that antioxidant supplementation attenuates exercise-induced oxidative stress. However, any physiological implications of this have yet to be consistently demonstrated, with most studies reporting no effects on exercise-induced muscle damage and performance. Moreover, a growing body of evidence indicates detrimental effects of antioxidant supplementation on the health and performance benefits of exercise training. Indeed, although ROS are associated with harmful biological events, they are also essential to the development and optimal function of every cell. The aim of this review is to present and discuss 23 studies that have shown that antioxidant supplementation interferes with exercise training-induced adaptations. The main findings of these studies are that, in certain situations, loading the cell with high doses of antioxidants leads to a blunting of the positive effects of exercise training and interferes with important ROS-mediated physiological processes, such as vasodilation and insulin signalling. More research is needed to produce evidence-based guidelines regarding the use of antioxidant supplementation during exercise training. It was recommend that an adequate intake of vitamins and minerals through a varied and balanced diet remains the best approach to maintain the optimal antioxidant status in exercising individuals [11589].

Various nutritional, behavioral, and pharmacological interventions have been previously shown to extend life span in diverse model organisms, including Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster, mice, and rats, as well as possibly monkeys and humans. This review aims to summarize published evidence that several longevity-promoting interventions may converge by causing an activation of mitochondrial oxygen consumption to promote increased formation of reactive oxygen species (ROS). These serve as molecular signals to exert downstream effects to ultimately induce endogenous defense mechanisms culminating in increased stress resistance and longevity, an adaptive response more specifically named mitochondrial hormesis or mitohormesis. Consistently, we here summarize findings that antioxidant supplements that prevent these ROS signals interfere with the health-promoting and life-span-extending capabilities of calorie restriction and physical exercise. Taken together it is suggested that ROS act as essential signaling molecules to promote metabolic health and longevity [1403].

Acute antioxidant supplementation may modulate oxidative stress and some immune perturbations that typically occur following prolonged exercise. The aims of one study were to examine the effects of acutely consuming dark chocolate (high polyphenol content) on plasma antioxidant capacity, markers of oxidative stress and immunoendocrine responses to prolonged exercise. Fourteen healthy men cycled for 2.5 h at about 60 percent maximal oxygen uptake 2 h after consuming 100 g dark chocolate (DC), an isomacronutrient control bar (CC) or neither (BL) in a randomised-counterbalanced design. Dark chocolate enhanced pre-exercise antioxidant status and reduced by trend 1 h post-exercise plasma free (F_{2}-isoprostane) compared with CC (also, F_{2}-isoprostane increased post-exercise in CC and BL but not DC trials). Plasma insulin concentration was significantly higher pre-exercise and 1 h post-exercise in the DC compared with the CC trial. There was a better maintenance of plasma glucose concentration on the DC trial, which decreased post-exercise in all trials but was significantly higher 1 h post-exercise in the DC trial. There were no between trial differences in the temporal responses of hypothalamic-pituitary-adrenal axis stress hormones, plasma interleukin-6, the magnitude of leukocytosis and neutrophilia and changes in neutrophil function. It was concluded that acute DC consumption may affect insulin, glucose, antioxidant status and oxidative stress responses, but has minimal effects on immunoendocrine responses, to prolonged exercise [11290].

While production of reactive oxygen and nitrogen species (RONS) is associated with some of
the beneficial adaptations to regular physical exercise, it is not established whether RONS play a role in the improved insulin-stimulated glucose uptake in skeletal muscle obtained by endurance training. To assess the effect of antioxidant supplementation during endurance training on insulin-stimulated glucose uptake, 21 young healthy (age 29 ± 1 years, BMI 25 ± 3 kg/m²) men were randomly assigned to either an antioxidant (AO) 500 mg vitamin C and 400 IU vitamin E (alpha-tocopherol) daily or a placebo (PL) group that both underwent a supervised intense endurance-training program 5 times/wk for 12 wk. A 3-h euglycemic-hyperinsulinemic clamp, a maximal oxygen consumption (VO₂max) and maximal power output (Pmax) test, and body composition measurements (fat mass, fat-free mass) were performed before and after the training. Muscle biopsies were obtained for determination of the concentration and activity of proteins regulating glucose metabolism. Although plasma levels of vitamin C and alpha-tocopherol increased markedly in the AO group, insulin-stimulated glucose uptake increased similarly in both the AO and the PL group in response to training. VO₂max and Pmax also increased similarly in both groups as well as protein content of GLUT4, hexokinase II, and total Akt. The results indicate that administration of antioxidants during strenuous endurance training has no effect on the training-induced increase in insulin sensitivity in healthy individuals [11291].

The objectives of one study were to evaluate pro-oxidant-antioxidant balance (PAB) associations with the commonly measured parameters of oxidative stress and antioxidative defence in elite female volleyball athletes and to investigate changes in the parameters of oxidative stress during a period of intense training and dietary antioxidant supplementation. Twenty-seven female volleyball players participated in this study. Blood samples were collected the day before the pre-competitive mesocycle training period began. After the first blood sample donation and during the next six weeks fourteen players (supplemented group) received a cocktail of antioxidants while thirteen of them (control group) received no dietary supplementation. The following parameters were measured: reactive oxygen metabolites (ROMs), superoxide anion (O₂⁻), malondialdehyde (MDA), advanced oxidation protein products (AOPP), lipid hydroperoxides (LOOH), biological antioxidative potential (BAP), paraoxonase activity toward paraoxon (POase) and diazoxon (DZOase), superoxide dismutase (SOD), total sulphhydryl group concentration (SH groups) and pro-oxidant-antioxidant balance. Significant associations were observed between biomarkers of oxidative damage with PAB in multiple linear regression model in the supplemented and the control groups (82 % vs 83 %) before training and in the control group (82 %) after training. Significant associations between antioxidative defence parameters and PAB values were found in the supplemented group after six-weeks of training (57 %). It was concluded that in the absence of antioxidant supplementation, PAB values were dependent on the association with biomarkers of oxidative damage before and after training. After a six-week training period and the applied antioxidant supplementation, PAB values were under the influence of non-enzymatic anti-oxidative defence [11292].

Strenuous physical activity is known to generate reactive oxygen species to a point that can exceed the antioxidant defense system and lead to oxidative stress. Dietary intake of antioxidants, plasma enzymatic (superoxide dismutase, glutathione reductase, and glutathione peroxidase, GPx, activities, nonenzymatic (total antioxidant status, TAS, uric acid, alpha-tocopherol, retinol, alpha-carotene, beta-carotene, lycopene, and lutein + zeaxanthin) antioxidants, and markers of lipid peroxidation (thiobarbituric-acid-reactive substances, TBARS) and muscle damage (creatine kinase, CK) were measured in 17 elite male kayakers and canoeists under resting conditions and in an equal number of age- and sex-matched sedentary individuals. Athletes showed significantly increased plasma values of alpha-tocopherol, alpha-carotene, beta-carotene, and superoxide dismutase activity, and a significantly lower TAS level. Antioxidant intake (alpha-tocopherol, vitamin C, and beta-carotene) and plasmatic GPx, glutathione reductase, lycopene, lutein + zeaxanthin, retinol,
and uric acid levels were similar in both groups. Nevertheless, TBARS and CK levels were found to be significantly higher in the kayakers and canoeists. This work suggests that despite the enhanced levels of antioxidants, athletes undergoing regular strenuous exercise exhibited more oxidative stress than sedentary controls [09359].

Effects of exercise training on important determinants of children's long-term health, such as redox and iron status, have not been adequately investigated. The aim of the present study was to examine changes in markers of the redox, iron and nutritional status of boy and girl swimmers during a prolonged period of training. 11 boys and 13 girls, aged 10-11 years, were members of a swimming club. They were assessed at the beginning of the training season, at 13 weeks and at 23 weeks through blood sampling and recording of the diet. Reduced glutathione increased at 13 and 23 weeks, whereas oxidised glutathione decreased at 13 weeks, resulting in an increase of the reduced/oxidised glutathione ratio at 13 and 23 weeks. Total antioxidant capacity, catalase, thiobarbituric acid-reactive substances, hemoglobin, transferrin saturation and ferritin did not change significantly. Carbohydrate intake was below 50 percent of energy and fat intake was above 40 percent of energy. Intakes of saturated fatty acids and cholesterol were excessive. Iron intake was adequate but intakes of folate, vitamin E, calcium and magnesium did not meet the recommended daily allowances. No significant differences were found between sexes in any of the parameters measured. In conclusion, child swimmers improved the redox status of glutathione during training, although the intake of antioxidant nutrients did not change. The iron status was not impaired by training. Suboptimal intake of several nutrients suggests the need for nutritional monitoring and education of children athletes [09360].

It has been difficult to determine, from the published literature, whether men or women have higher levels of exercise-induced oxidative stress. The aim of one study was to compare variations between the sexes in lipid hydroperoxide (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and lactate dehydrogenase (LDH) after 3 different running exercises performed at the same speed. Eligible participants were healthy university students of both sexes. The participants performed running exercise tests at distances of 800, 1500, and 3000 m at a speed of 10 km/h. Blood samples were taken from the participants just before and immediately after the running activities to determine LPO, SOD, CAT, GR, and LDH, and these measures were compared both before and after exercise and between the sexes. A total of 17 young and healthy, but not physically trained, students (n=8 men; n=9 women) participated in this study. Height, weight, and maximum oxygen consumption values were significantly higher in men than in women. Significant gender effects were found in LPO levels at 3000 m and in SOD activity at 800 m, and 3000 m. CAT activity also significantly differed between the sexes at 800 m and 1500 m. However, no significant gender-time interaction effect was observed for any measurement at the 800-, 1500-, and 3000-m distances. Changes in LPO, SOD, and CAT activities at different running distances were not different between men and women over time because of a nonsignificant gender-time interaction. With regard to changes in oxidative stress, men and women had similar responses to exercise at the same absolute workload, despite significant differences in physical characteristics [09361].

Until recently it has been considered free oxygen radical production in the body to be a solely “negative” occurrence that might be countered by supplementation with antioxidant nutrients. The supposed benefits included improved health and mortality, and for athletes, the extra advantages of enhanced performance through a reduction in exercise-induced damage. In fact a Cochrane Review found little or no difference in the normal population between antioxidant administration and those taking a placebo. Mortality was, in fact, slightly higher in the antioxidant group. However, it is well established that physical exercise results in increased free-radical production in active skeletal muscles. Importantly, numerous reports
indicate that exercise-induced free-radical production is responsible for oxidative damage to cells and contributes to muscular fatigue during prolonged exercise. The fact that working skeletal muscles produce radicals has motivated many athletes to begin using antioxidant supplements in hopes of preventing exercise-induced free-radical damage and/or muscular fatigue. However, whether or not antioxidant supplements are helpful or harmful to the athlete remains a highly debated topic. Free radicals are molecules or any chemical species that contain one or more unpaired electrons in their outer orbital. In cells, radicals can be formed by either losing a single electron or gaining an electron. An unpaired electron results in molecular instability, and therefore radicals are highly reactive molecules that can promote oxidative damage to proteins, lipids and DNA. This radical-mediated damage is referred to as “oxidative damage,” and oxidation of cellular constituents can lead to cellular dysfunction and, in extreme cases, cell death. Also, note that reactive oxygen species (ROS) is a general term that not only refers to oxygen-centred radicals but also includes non-radical but reactive derivatives of oxygen (e.g. hydrogen peroxide). The term “oxidative stress” was introduced in 1985 and is commonly defined as a disturbance in the prooxidant-antioxidant balance in favour of oxidants with the imbalance leading to oxidative damage. Indeed, one of the hallmarks of oxidative stress in cells is the appearance of biomarkers of oxidative damage (e.g. oxidised proteins and/or lipids). Note, however, that exercise-induced oxidative stress is a transient occurrence. Indeed, the exercise-induced spike in radical production is short lived, and growing evidence suggests that this spike in oxidants after exercise is a required signalling pathway for training adaptations to occur. This brief rise in oxidants should not be confused with prolonged oxidative stress found in some pathological conditions. Given that all cells produce radicals, it is not surprising that cells contain an endogenous antioxidant system composed of both enzymatic and non-enzymatic antioxidants. Moreover, dietary antioxidants (e.g. vitamin C and vitamin E) cooperate with the endogenous antioxidant defence systems to form a united antioxidant network in muscle fibres. This cooperative interaction between endogenous antioxidants and dietary antioxidants has fuelled the argument that antioxidant supplementation will boost the muscle fibre’s ability to scavenge ROS and protect against exercise-induced oxidative damage and fatigue. Muscle fatigue is commonly defined as a reduction in the ability of a muscle to generate force. Exercise-induced muscle fatigue is a multifactorial process, and the specific causes of fatigue can vary widely. Nonetheless, growing evidence indicates that radical production in skeletal muscles contributes to fatigue during prolonged submaximal exercise (i.e. events lasting >30 min). ROS production increases in contracting skeletal muscles, and low levels of ROS play an essential role in the regulation of muscle force production. Indeed, low levels of ROS in contracting skeletal muscles are required to achieve optimum force production. However, high levels of ROS can induce oxidative damage to muscle proteins and lipids, and diminish muscle force production. Indeed, well-controlled animal studies indicate that scavenging radicals via antioxidants can protect skeletal muscle against oxidative damage and also delay fatigue during prolonged submaximal exercise. In contrast, antioxidant scavengers are not effective in delaying muscle fatigue in animals performing high intensity exercise. A growing number of studies suggest that acute administration of the antioxidant N-acetylcysteine (NAC) delays human muscle fatigue during prolonged submaximal exercise. Specifically, NAC administration has been shown to delay muscular fatigue in humans during submaximal exercise tasks. Similar NAC does not retard human muscle fatigue during exercise close to VO2max. In contrast to the findings that acute NAC administration can delay muscle fatigue during prolonged submaximal exercise, there is little evidence that other more commonly used antioxidant supplements (e.g. beta carotene, vitamin E, and vitamin C) can improve human exercise performance. Based upon current evidence, it can be concluded that NAC may be able to increase performance during prolonged exercise in humans, but there is limited evidence to indicate that vitamin C, vitamin E or beta carotene can improve performance. Regardless of whether antioxidants can delay muscular fatigue, advocates supporting antioxidant supplementation for athletes argue that rigorous exercise training
results in increased ROS production in skeletal muscles. Therefore, they suggest that antioxidant supplements are required to protect skeletal muscle fibres against oxidative damage. Although this suggestion appears reasonable, there are numerous arguments against antioxidant supplementation for athletes. First, there is no evidence that exercise-induced radical production in skeletal muscle is detrimental to human health. Secondly, regular exercise training promotes increased enzymatic and non-enzymatic antioxidants in muscle fibres resulting in improved endogenous protection against exercise-mediated oxidative damage. Therefore, this increase in endogenous antioxidants may be sufficient to protect against exercise-induced oxidative damage. Moreover, if an athlete maintains an isocaloric diet that is nutritionally well balanced, it is feasible that the athlete may not require supplementary antioxidants above those contained in the diet. There is one circumstance that may justify the use of antioxidant supplements in athletes. Specifically, some athletes may not consume well-balanced diets, and therefore these individuals could be deficient in antioxidant intake (e.g. below the recommended daily allowance (RDA) for selected antioxidant vitamins). This is a reasonable point that is supported by studies investigating the nutritional habits of athletes. Perhaps the two strongest arguments against antioxidant supplementation for athletes and other active individuals are the following. First, emerging evidence indicates that antioxidant supplementation prevents important exercise-induced adaptations in skeletal muscle. Indeed, compelling evidence indicates that exercise-induced ROS production serves as a required signal to promote the expression of numerous skeletal muscle proteins including antioxidant enzymes, mitochondrial proteins and heat-shock proteins. Secondly, two recent reports indicate that antioxidant supplementation with high levels of vitamins E and C (about 16 times higher than RDA for adults) can blunt the training adaptation to exercise. Another key argument against antioxidant supplementation in athletes is that current research does not support the notion that antioxidant supplementation is beneficial to human health. Specifically, a meta-analysis of 68 randomised antioxidant supplement trials (total of 232,606 human participants) concluded that dietary supplementation above the RDA with beta carotene, vitamin A and vitamin E does not improve health outcomes and may increase mortality. At present, there is limited scientific evidence to recommend antioxidant supplements to athletes or other physically active individuals. In fact, the current evidence suggests that athletes should use caution when considering supplementation with high doses of antioxidants [09362].

There is a considerable commercial market, especially within the sports community, claiming the need for antioxidant supplementation. One argument for antioxidant supplementation in sports is that physical exercise is associated with increased reactive oxygen and nitrogen species (RONS) production, which may cause cell damage. However, RONS production may also activate redox-sensitive signaling pathways and transcription factors, which subsequently, may promote training adaptation. The aim of one study was to investigate the effects of combined vitamin C and E supplementation to healthy individuals on different measures of exercise performance after endurance training. Using a double-blinded placebo-controlled design, moderately trained young men received either oral supplementation with vitamins C and E (n=11) or placebo (n=10) before and during 12 wk of supervised, strenuous bicycle exercise training of a frequency of 5 d per week. Muscle biopsies were obtained before and after training. After the training period, maximal oxygen consumption, maximal power output, and workload at lactate threshold increased markedly in both groups. Also, glycogen concentration, citrate synthase, and beta-hydroxyacyl-CoA dehydrogenase activity in the muscle were significantly higher in response to training in both groups. However, there were no differences between the two groups concerning any of the physiological and metabolic variables measured. The results suggest that administration of vitamins C and E to individuals with no previous vitamin deficiencies has no effect on physical adaptations to strenuous endurance training [10413].
Dietary antioxidants include vitamin C, vitamin E, carotenoids (mainly beta-carotene), polyphenols (e.g. flavonoids), selenium, glutathione and coenzyme Q10. Changes in plasma endogenous and dietary antioxidants and oxidative stress markers were studied following two 90 min elite female soccer games separated by 72 h of either active or passive recovery. The active recovery group (n 8) trained for 1 h at 22 and 46 h after the first game (low-intensity cycling and resistance training), while the passive group rested (n 8). Blood samples were taken before the games; immediately after the games; 21, 45 and 69 h after the first game; and immediately after the second game. The oxidative stress markers and antioxidants were not affected by active recovery. The oxidative stress marker GSSG increased by the same extent after both the games, while the lipid peroxidation marker diacron-reactive oxygen metabolite remained unchanged. The endogenous antioxidants total glutathione and uric acid and ferric reducing/antioxidant power increased immediately after both the games with the same amplitude, while increases in cysteine, cysteine-glycine and total thiols reached significant levels only after the second game. The changes in dietary antioxidants after the first game were either rapid and persistent (tocopherols and ascorbic acid (AA) increased; polyphenols decreased) or delayed (carotenoids). This resulted in high pre-second game levels of tocopherols, AA and carotenoids. Polyphenols returned to baseline at 69 h, and were not affected by the second game. In conclusion, the soccer-associated dietary antioxidant defence, but not the endogenous antioxidant defence, is persistent. Similar acute oxidative stress and endogenous antioxidant responses and dissimilar dietary antioxidant reactions occur during two repeated female soccer games. Finally, the complex antioxidant response to soccer is not affected by active recovery training [10530].

Antioxidant requirements have neither been defined for endurance nor been defined for ultra-endurance athletes. To verify whether an acute bout of ultra-endurance exercise modifies the need for nutritive antioxidants, it was aimed to investigate the changes of endogenous and exogenous antioxidants in response to an Ironman triathlon and to particularise the relevance of antioxidant responses to the indices of oxidatively damaged blood lipids, blood cell compounds and lymphocyte DNA, and to examine whether potential time-points of increased susceptibility to oxidative damage are associated with alterations in the antioxidant status. Blood that was collected from forty-two well-trained male athletes 2 d pre-race, immediately post-race, and 1, 5 and 19 d later was sampled. The key findings of the study were that immediately post-race, vitamin C, alpha-tocopherol, and levels of the Trolox equivalent antioxidant capacity, the ferric reducing ability of plasma and the oxygen radical absorbance capacity (ORAC) assays increased significantly. Exercise-induced changes in the plasma antioxidant capacity were associated with changes in uric acid, bilirubin and vitamin C. Also, significant inverse correlations between ORAC levels and indices of oxidatively damaged DNA immediately and 1 d post-race suggest a protective role of the acute antioxidant responses in DNA stability. Significant decreases in carotenoids and gamma-tocopherol 1 d post-race indicate that the antioxidant intake during the first 24 h of recovery following an acute ultra-endurance exercise requires specific attention. Furthermore, the present study illustrates the importance of a diversified and well-balanced diet to maintain a physiological antioxidant status in ultra-endurance athletes in reference to recommendations [10531].

Also physical exercise in general is accepted to be protective, acute and strenuous exercise has been shown to induce oxidative stress. Enhanced formation of free radicals leads to oxidation of macromolecules and to DNA damage. On the other hand ultra-endurance events which require strenuous exercise are very popular and the number of participants is continuously increasing worldwide. Since only few data exists on Ironman triathletes, who are prototypes of ultra-endurance athletes, one study was aimed at assessing the risk of oxidative stress and DNA damage after finishing a triathlon and to predict a possible health
risk. Blood samples of 42 male athletes were taken 2 days before, within 20 min after the race, 1, 5 and 19 days post-race. Oxidative stress marker increased only moderately after the race and returned to baseline after 5 days. Marker of DNA damage measured by the SCGE assay with and without restriction enzymes as well as by the sister chromatid exchange assay did either show no change or deceased within the first day after the race. Due to intake during the race and the release by the cells plasma concentrations of vitamin C and α-tocopherol increased after the event and returned to baseline 1 day after. This study indicates that despite a temporary increase in some oxidative stress markers, there is no persistent oxidative stress and no DNA damage in response to an Ironman triathlon in trained athletes, mainly due to an appropriate antioxidant intake and general protective alterations in the antioxidant defence system [10532].

The "Marathon des Sables" is a competition known to induce oxidative stress. Antioxidant vitamins prevent exercise-induced oxidative damages. The purpose of one study was to evaluate daily intake and plasma level of the main antioxidant vitamins (alpha-tocopherol, vitamin C, beta-carotene and retinol) in 19 male athletes who participated in this competition. Data collected before the beginning of the competition included daily dietary intake using a 7-day food record and plasma biochemical measurements (alpha-tocopherol, vitamin C, beta-carotene and retinol). First, total energy intake was obviously lower than the energetic intake usually observed in well-trained endurance athletes. Second, antioxidant vitamins intake was also insufficient. Indeed, the intake was lower than the French Dietary Reference Intakes (DRI) for this population in 18 subjects for vitamin E and 6 subjects for vitamin C, beta-carotene and Retinol Equivalent. As a significant relationship was found between total energy intake and the intake of vitamin E and vitamin C, the low total energy intake contributed partially to the insufficient antioxidant vitamins intake. The dietary questionnaire analysis also revealed a low intake of vegetable oils, fruits and vegetables. However, plasma concentrations of these antioxidant vitamins were similar to the literature data observed in athletes. The study evidenced obvious insufficient energy intake in ultra endurance athletes associated with a low antioxidant vitamin intake [07372].

The effects of antioxidant diet supplements on blood lactate concentration and on the aerobic and anaerobic thresholds and their adaptations to training were analysed. Fifteen amateur male athletes were randomly assigned to either a placebo group or an antioxidant-supplemented group (90 days supplementation with 500 mg/day of vitamin E and 30 mg/day of beta-carotene, and the last 15 days also with 1 g/day of vitamin C). Before and after the antioxidant supplements, the sportsmen performed a maximal exercise test on a cycle ergometer and maximal and submaximal physiological parameters were assessed together with blood lactate concentration. Maximal oxygen uptake (VO\textsubscript{2max}), maximal blood lactate concentration, and the maximal workload attained rose significantly in both groups after the 3 months of training. At the end of the study, maximal blood lactate concentration was lower in the group that took supplements than in the placebo group. The percentage of VO\textsubscript{2max} attained at the anaerobic threshold rose significantly in both groups after 3 months of training, although the final value in the supplemented group was higher than that in the placebo group. Antioxidant diet supplements induced lower increases in blood lactate concentration after a maximal exercise test and could improve the efficiency in which aerobic energy is obtained [07373].

It was examined the influence of 3 consecutive days of high-intensity cycling on blood and urinary markers of oxidative stress. Eight highly-trained male cyclists completed an interval session (9 exercise bouts lasting 30 s each, at 150 % peak power output) on day 1, followed by 2 laboratory-simulated 30 km time trials on days 2 and 3. The cyclists also completed a submaximal exercise trial matched to the interval session for oxygen consumption. Blood was collected pre- and post-exercise for the determination of malondialdehyde (MDA), total
antioxidant status (TAS), vitamin E, and the antioxidant enzyme activity of superoxide dismutase and glutathione peroxidase, while urine was collected for the determination of allantoin. There were significant increases in plasma MDA concentrations, plasma TAS, and urinary allantoin excretion following the high-intensity interval session on day 1, whereas plasma vitamin E concentration significantly decreased. Post-exercise changes in plasma MDA, TAS concentrations, and urinary allantoin excretion were all significantly attenuated over the 3 consecutive days of exercise, whereas resting plasma TAS concentration was elevated. There were no significant changes in plasma MDA, TAS, or allantoin excretion following submaximal exercise and there were no significant changes in antioxidant enzyme activity over consecutive days of exercise or following submaximal exercise. Consecutive days of high-intensity exercise enhanced resting plasma TAS concentration and reduced the post-exercise increase in plasma MDA concentrations [07374].

Cognitive performance

Antioxidant properties of some vitamins and trace elements may help to prevent cognitive decline. The aim of one study was to estimate the long-term effects of antioxidant nutrient supplementation on the cognitive performance of participants in the Supplementation in Vitamins and Mineral Antioxidants (SU.VI.MAX) study 6 y after the end of the trial. This study included 4447 French participants aged 45-60 years who were enrolled in the SU.VI.MAX study (1994-2002), which was a double-blind, placebo-controlled, randomized trial. From 1994 to 2002, participants received daily vitamin C (120 mg), beta-carotene (6 mg), vitamin E (30 mg), selenium (100 μg), and zinc (20 mg) in combination or as a placebo. In 2007-2009, the cognitive performance of participants was assessed with 4 neuropsychological tests (6 tasks). Principal components analysis (PCA) was performed to identify cognitive-function summary scores. Associations between antioxidant supplementation and cognitive functions, in the full sample and by subgroups, were estimated through ANOVA and expressed as mean differences and 95% CIs. Subgroup analyses were performed according to baseline characteristics. Subjects receiving active antioxidant supplementation had better episodic memory scores (mean difference: 0.61; 95% confidence interval 0.02 to 1.20). PCA indicated 2 factors that were interpreted as showing verbal memory and executive functioning. Verbal memory was improved by antioxidant supplementation only in subjects who were nonsmokers or who had low serum vitamin C concentrations at baseline. This study supports the role of an adequate antioxidant nutrient status in the preservation of verbal memory under certain conditions [11404].

Gender differences

The purpose of one study was to determine the influence of gender and antioxidant supplementation on exercise-induced oxidative stress. Twenty-five men and 23 women ran for 30 min at 80 percent VO_2max, once before and once after 2 weeks of supplementation, and again after a 1-week wash-out period. Subjects were randomly assigned to either placebo (P), antioxidant (A: 400 IU vitamin E+1 g vitamin C), or a fruit and vegetable powder (FV) treatment. Blood was obtained at rest and immediately after exercise. Before supplementation, women had higher resting reduced glutathione, total glutathione, and plasma vitamin E compared with men. With both A and FV supplementations, plasma vitamin E gender differences disappeared. Protein carbonyls, oxidized glutathione, and malondialdehyde all increased similarly for both genders in response to exercise. Both A and FV attenuated the reduced glutathione decrease and the oxidized glutathione and protein carbonyls increase compared with P, with no gender differences. 8-hydroxydeoxyguanosine was lower with treatment A compared with FV and P only for men. Plasma vitamin C increased 39 percent (A) and 21 percent (FV) compared with P. These data indicate that
women have higher resting antioxidant levels than men. Markers of oxidative stress increased similarly in both genders in response to exercise of similar intensity and duration. Two weeks of antioxidant supplementation can attenuate exercise-induced oxidative stress equally in both genders [07375].

*Flavonoids (including quercetin)*

Phytochemicals are chemicals produced by plants, and include tannins, lignins and flavonoids. The largest and best studied polyphenols are the flavonoids, with more than 6000 identified and classified into at least six subgroups: flavonols, flavones, flavanones, flavanols (and their oligomers, proanthocyanidins), anthocyanidins and isoflavonoids. Flavonoids are widely distributed in plants and function as plant pigments, signalling molecules and defenders against infection and injury. Dietary intake of flavonoids ranges from 50 to 800 mg/day depending on the consumption of fruits and vegetables and the intake of tea. In the USA, total flavonoid intake averages 210 mg/day, and in Spain 313 mg/day, with important sources including tea, citrus fruit and juice, beers and ales, wines, melon and berries, apples, onions and bananas. A high intake of fruits and vegetables has been linked in numerous studies to a reduced risk of cardiovascular disease and various types of cancer. The disease-reducing influence of fruits and vegetables may be partly due to high levels of flavonoids. Although cell culture and animal scientific evidence is promising in support of the role of flavonoid intake in disease prevention, human studies are mixed and inconclusive when taken as a whole. Part of the problem is that flavonoid data are available on a limited number of foods, hampering efforts to estimate total flavonoid intake in human subjects. Many flavonoids possess strong anti-inflammatory, anti-viral, antioxidant, anti-obesity and anti-carcinogenic properties when studied in vitro using large doses of the purified form. Inflammation and oxidative stress are key mechanisms in the pathogenesis of certain disease states, supporting the proposed strategy of increased flavonoid intake for the prevention of cancer, diabetes mellitus and cardiovascular disease. However, results from randomised, double-blinded studies in humans with large doses of purified flavonoids such as quercetin have been disappointing. Flavonoids vary widely in bioavailability, and most are poorly absorbed, undergo active efflux and are extensively conjugated and metabolically transformed, all of which can affect their bioactive capacities. Despite low bioavailability of the parent flavonoid, some of the in vivo metabolites may accumulate in tissues and produce bioactive influences, but conclusive human data are lacking. For example, animal data indicate that quercetin metabolites accumulate in the vascular tissue and there act as complementary antioxidants, with plasma albumin facilitating the translocation of quercetin metabolites to the vascular target. There is a growing realisation that bioactive influences of individual flavonoids are potentiated when mixed with other flavonoids (e.g. the flavonol quercetin with the flavanol epigallocatechin 3-gallate; EGCG) or are included in a cocktail or extract of other polyphenols and nutrients. Two or more flavonoids ingested together may increase bioavailability and decrease elimination by competitive inhibition of glucuronide and sulphate conjugation in both the intestine and liver, and by inhibiting efflux transporters such as P-glycoprotein, breast cancer resistance protein and multidrug resistance protein 2. The health-protective effects of plant foods are not produced by a single component but rather by complex mixtures of interacting molecules. The polyphenols and natural components provide a multifaceted defensive strategy for both plants and humans. The ‘pharma’ approach of using large doses of a single bioactive molecule is thus seldom successful in the application of nutrition to human health and performance. In addition, a metabolomics or nutrigenomics approach is needed to improve the capacity of investigators to capture the complex and subtle influences of flavonoid supplements or flavonoid-rich extracts, foods and beverages on whole-body metabolism and physiology. Various nutritional agents have been tested for their capacity to attenuate oxidative stress, inflammation and immune changes following intensive exercise, and thus lower the magnitude of physiological stress and the risk of upper
respiratory tract infection (URTI). Some question the value of using nutritional supplements as countermeasures to exercise-induced oxidative stress and inflammation because these may interfere with important signalling mechanisms for training adaptations. Another viewpoint is that nutritional supplements attenuate but do not totally block exercise-induced oxidative stress and inflammation, analogous to the beneficial use of ice packs to reduce swelling following mild injuries. Pure flavonoids such as quercetin, EGCG and isoflavones, or flavonoid-rich plant extracts, are being tested by an increasing number of investigative teams as performance aids and countermeasures to exercise-induced inflammation, DOMS (delayed onset of muscle soreness), oxidative stress, immune dysfunction and URTI. Most studies have focused on the ability of flavonoid-rich tea and fruit and vegetable extracts to counter oxidative stress and, the majority indicates an effective response. The second most common outcome measure is related to inflammation and DOMS, and again, most studies support protective effects when flavonoid mixtures or plant extracts are ingested before demanding bouts of exercise. Results are mixed for performance outcomes, and few studies have included immune and URTI measures. For any particular flavonoid or plant extract studied within an exercise context, few papers are available, and research designs vary widely with regard to the supplementation dose and regimen, the mode of exercise stress and outcome measures. The flavonoid supplementation period in studies varies from 15 min to 60 days before an exercise challenge, with most studies clustered between 7 and 21 days. Nonetheless, the data in general support that flavonoid-rich plant extracts and unique flavonoid-nutrient mixtures (e.g. quercetin with green tea extract and fish oil, or isoflavones with lycopene) help to counter exercise-induced oxidative stress and inflammation/DOMS. More exercise-related research has been conducted with quercetin than any other flavonoid. In one of the earliest studies with exercise-stressed cyclists, supplementation with pure quercetin (1000 mg/day) over a 5-week period reduced illness rates but did not counter postexercise inflammation, oxidative stress, or immune dysfunction. In a follow-up study using a similar design, quercetin supplementation combined with green tea extract, isoquercetin and fish oil did cause a sizeable reduction in exercise-induced inflammation and oxidative stress, with chronic augmentation of innate immune function. Quercetin's role as a performance aid has been tested by several research teams with mixed results. Animal studies support a role for quercetin as an exercise mimic for mitochondrial biogenesis and enhanced endurance performance. One study with untrained human subjects indicated a modest enhancement in skeletal muscle mitochondrial density and endurance performance, but far below what was reported in mice. Although data are limited and supplementation regimens vary widely, most studies have concluded that flavonoid–nutrient mixtures or flavonoid-rich tea, fruit and vegetable extracts ingested acutely or chronically before an exercise challenge attenuate postexercise oxidative stress, inflammation and DOMS. Results are mixed for performance outcomes, and few studies have included immune measures. Current evidence seems to support a higher antioxidant requirement in athletes than non-athletes to help reduce oxidative damage induced by exercise. Flavonoid-rich extracts when consumed just before or chronically for days or weeks before heavy exertion partly counter postexercise inflammation and oxidative stress. Research is needed to define better optimal dosing regimens. It is also important to determine whether unique flavonoid mixtures that include several of the most bioactive flavonoids across different subgroups amplify these influences, while also bolstering immunity and operating as exercise mimetics for mitochondrial biogenesis. Whether athletes need higher dietary antioxidants, including flavonoids, than sedentary people because of a higher turnover of oxygen radicals imposed by high intensity training and competition is still controversial and speculative. In the meantime, the most optimally effective way to increase antioxidant intake is to consume a varied diet focused on foods that are naturally good sources of antioxidants such as fruit, vegetables and whole grains. This strategy also helps minimise the risk of exceeding intake. When there is a perceived need to supplement dietary intake, it seems sensible to choose a product that provides a combination of antioxidants, including flavonoids, at moderate levels,
rather than large doses of a single nutrient [10533].

Astaxanthin

Astaxanthin, a xanthophyll carotenoid, is a nutrient with unique cell membrane actions and diverse clinical benefits. This molecule neutralizes free radicals or other oxidants by either accepting or donating electrons, and without being destroyed or becoming a pro-oxidant in the process. Its linear, polar-nonpolar-polar molecular layout equips it to precisely insert into the membrane and span its entire width. In this position, astaxanthin can intercept reactive molecular species within the membrane's hydrophobic interior and along its hydrophilic boundaries. Clinically, astaxanthin has shown diverse benefits, with excellent safety and tolerability. In double-blind, randomized controlled trials (RCTs), astaxanthin lowered oxidative stress in overweight and obese subjects and in smokers. It blocked oxidative DNA damage, lowered C-reactive protein (CRP) and other inflammation biomarkers, and boosted immunity in the tuberculin skin test. Astaxanthin lowered triglycerides and raised HDL-cholesterol in another trial and improved blood flow in an experimental microcirculation model. It improved cognition in a small clinical trial and boosted proliferation and differentiation of cultured nerve stem cells. In several Japanese RCTs, astaxanthin improved visual acuity and eye accommodation. It improved reproductive performance in men and reflux symptoms in H. pylori patients. In preliminary trials it showed promise for sports performance (soccer). In cultured cells, astaxanthin protected the mitochondria against endogenous oxygen radicals, conserved their redox (antioxidant) capacity, and enhanced their energy production efficiency. The concentrations used in these cells would be attainable in humans by modest dietary intakes. Astaxanthin's clinical success extends beyond protection against oxidative stress and inflammation, to demonstrable promise for slowing age-related functional decline [11538].

Astaxanthin is a carotenoid. It belongs to a larger class of phytochemicals known as terpenes. It was examined the effect of astaxanthin (AST) on substrate metabolism and cycling time trial (TT) performance by randomly assigning 21 competitive cyclists to 28 d of encapsulated AST (4 mg/d) or placebo (PLA) supplementation. Testing included a VO_{2max} test and on a separate day a 2 h constant intensity pre-exhaustion ride, after a 10 h fast, at 5 percent below VO_{2max} stimulated onset of 4 mmol/L lactic acid followed 5 min later by a 20 km TT. Fourteen participants successfully completed the trial. Overall, it was observed significant improvements in 20 km TT performance in the AST group (n=7), but not the PLA (n=7). The AST group was significantly different vs PLA. The AST group significantly increased power output (20 W), while the PLA group did not (1.6 W). The mechanism of action for these improvements remains unclear, as it was observed no treatment effects for carbohydrate and fat oxidation, or blood indices indicative of fuel mobilization. While AST significantly improved TT performance the mechanism of action explaining this effect remains obscure [11539].

In handball

The chronic exposure to regular exercise training seems to improve antioxidant defense systems. However, the intense physical training imposed on elite athletes may lead to overtraining associated with oxidative stress. The purpose of one study was to investigate the effect of different training loads and competition on oxidative stress, biochemical parameters and antioxidant enzymatic defense in handball athletes during 6-months of monitoring. Ten male elite handball athletes were recruited to the study. Blood samples were collected four times every six weeks throughout the season. During most intense periods of training and competitions there were significant changes in plasma indices of oxidative stress.
increased TBARS and decreased thiols). Conversely, chronic adaptations to exercise training demonstrated a significant protective effect against oxidative stress in erythrocyte (decrease in TBARs and carbonyl group levels). Erythrocyte antioxidant enzyme activities were significantly increased, suggesting a training-induced antioxidant adaptation. Biomarkers of skeletal muscle damage were significantly increased during high-intensity training period (creatine kinase, lactate dehydrogenase and aspartate aminotransferase). No significant changes were observed in plasma IL-6, TNF-alpha and uric acid, whereas a significant reduction was found in the IL-1beta concentration and gamma-glutamyl transferase activity. Oxidative stress and antioxidant biomarkers can change throughout the season in competitive athletes, reflecting the physical stress and muscle damage that occurs as the result of competitive handball training. In addition, these biochemical measurements can be applied in the physiological follow-up of athletes [13737].

alpha-Tocopherol, ascorbic acid, and beta-carotene

It was hypothesized that alpha-tocopherol, ascorbic acid, and beta-carotene, either applied individually or in combination, would modulate redox homeostasis and affect the regulation of genes involved in DNA repair under stress conditions. To test this hypothesis, it was analyzed the influence of these vitamins, either supplied individually or in combination, on the plasma lipid peroxide level and the hepatic level of 8-hydroxy-2'-deoxyguanosine in rats. It was also evaluated the expression of p53 and Mdm2 protein in the intestinal epithelium, as these proteins are involved in the cellular regulation of DNA damage repair. Male Wistar rats (n=112) were supplemented with α-tocopherol (2 mg), ascorbic acid (12 mg), and beta-carotene (1 mg), both individually and in combination, for 14 days; 32 control rats were treated with placebo. Half of the animals in each group (n=8) were subjected to 15-minute treadmill running at 20 m/min to cause exercise-induced oxidative stress. A statistically significant reduction in lipid peroxide levels was observed in the plasma of rats subjected to exercise and given 2 or 3 of the antioxidants. Exercise, as well as coadministration of the antioxidants, had no significant effect on the amount of DNA damage. Downward trends in the level of p53 protein expression were observed both in exercised and nonexercised animals, especially when the studied vitamins were administered in combination. Our findings suggest that alpha-tocopherol, ascorbic acid, and beta-carotene, when given concurrently, have primarily antioxidant effects on lipids under stress but do not significantly affect the regulation of p53 gene expression [13738].

Catechins

Catechins, abundant in green tea, exhibit many biological actions for potential clinical applications. The purpose of a study was to explore the potential benefits of catechin ingestion on recovery of physical performance after downhill running. Institute of Cancer Research mice were used to examine the effects of prior catechin ingestion (0.5 % w/w in diet for 3 weeks) on 1) wheel-running activity, 2) running endurance, 3) muscle force, and 4) muscle oxidative stress and inflammation after downhill running (16 m/min for 5 min, 18 m/min for 5 min, 20 m/min for 10 min, and 22 m/min for 130 min). Voluntary wheel-running activity and the contractile force of the isolated soleus muscle decreased after downhill running. Notably, catechin ingestion significantly alleviated the running-induced decrease in voluntary wheel-running activity by 35 percent; the catechin-treated mice maintained endurance running capacity. Furthermore, catechins alleviated the decrease in tetanic force evident in the soleus muscle after downhill running. Catechins suppressed the running-induced increases in plasma creatine phosphokinase levels by 52 percent; this was also true of the carbonylated protein content of the soleus muscle by 17 percent, malondialdehyde levels by 32 percent in the gastrocnemius muscle, and myeloperoxidase activity of the
gastrocnemius by 22 percent. The levels of tumor necrosis factor-alpha, interleukin-1beta, and monocyte chemoattractant protein-1 in the gastrocnemius muscle were significantly lower by 33, 29, and 35 percent, respectively, in treated mice; the expression levels of mRNAs encoding these fell in parallel. The results suggest that long-term intake of catechins, perhaps through their antioxidant properties, attenuates downhill running-induced muscle damage by suppressing muscle oxidative stress and inflammation, hastening recovery of physical performance in mice [13739].

*In chronic obstructive pulmonary disease*

Oxidative stress may contribute to exercise intolerance in patients with chronic obstructive pulmonary disease (COPD). One study sought to determine the effect of an acute oral antioxidant cocktail (AOC, vitamins C and E, and alpha-lipoic acid) on skeletal muscle function during dynamic quadriceps exercise in COPD. Ten patients with COPD performed knee extensor exercise to exhaustion and isotime trials after either the AOC or placebo (PL).

Pre- to postexercise changes in quadriceps maximal voluntary contractions and potentiated twitch forces (Q(tw,pot)) quantified quadriceps fatigue. Under PL conditions, the plasma electron paramagnetic resonance (EPR) spectroscopy signal was inversely correlated with the forced expiratory volume in 1 s to forced vital capacity ratio (FEV1/FVC), an index of lung dysfunction and maximal voluntary contraction force AOC consumption increased plasma ascorbate levels and attenuated the area under the curve of the EPR spectroscopy free radical signal but it did not alter the endurance time or quadriceps fatigue. The ability of the AOC to decrease the EPR spectroscopy signal, however, was prominent in those with high basal free radicals with minimal effects in those with low levels. These data document a relation between directly measured free radicals and lung dysfunction and the ability of the AOC to decrease oxidative stress in COPD. Acute amelioration of free radicals, however, does not appear to affect dynamic quadriceps exercise performance [13740].

*Experimental*

One study aimed to evaluate the effect of selenium supplementation on lipid peroxidation and lactate levels in rats subjected to acute swimming exercise. Thirty-two adult male rats of Sprague-Dawley type were divided into four groups. Group 1, control; group 2, selenium-supplemented; group 3, swimming control; group 4, selenium-supplemented swimming group. The animals in groups 2 and 4 were supplemented with (i.p.) 6 mg/kg/day sodium selenite for 4 weeks. The blood samples taken from the animals by decapitation method were analyzed in terms of erythrocyte-reduced glutathione (GSH), serum glutathione peroxidase (GPx) and superoxide dismutase (SOD), and plasma malondialdehyde (MDA) and lactate using the colorimetric method, and serum selenium values using an atomic emission device. In the study, the highest MDA and lactate values were found in group 3, while the highest GSH, GPx and SOD values were obtained in group 4. Group 2 had the highest and group 3 had the lowest selenium levels. Results of the study indicate that the increase in free radical production and lactate levels due to acute swimming exercise in rats might be offset by selenium supplementation. Selenium supplementation may be important in that it supports the antioxidant system in physical activity [10414].

*In mice*

Antioxidant supplements are widely consumed by the general public; however, their effects of on exercise performance are controversial. The aim of this study was to examine the effects of an antioxidant cocktail (alpha-lipoic acid, vitamin E and coenzyme Q10) on exercise performance, muscle function and training adaptations in mice. C57Bl/J6 mice were placed on antioxidant supplement or placebo-control.
diets (n=36/group) and divided into trained (8 weeks treadmill running) (n=12/group) and untrained groups (n=24/group). Antioxidant supplementation had no effect on the running performance of trained mice nor did it affect training adaptations; however, untrained female mice that received antioxidants performed significantly better than placebo-control mice. Furthermore, antioxidant-supplemented females (untrained) showed elevated respiratory capacity in freshly excised muscle fibers (quadriceps femoris), reduced oxidative damage to muscle proteins, and increased expression of mitochondrial proteins compared to placebo-controls. These changes were attributed to increased expression of proliferator-activated receptor gamma coactivator 1alpha (PGC-1alpha) via activation of AMP-activated protein kinase (AMPK) by antioxidant supplementation. Overall, these results indicate that this antioxidant supplement exerts gender specific effects; augmenting performance and mitochondrial function in untrained females, but does not attenuate training adaptations [13728].

**Hydroxycitrate**

Glycogen stored in skeletal muscle is the main fuel for endurance exercise. The present study examined the effects of oral hydroxycitrate (HCA) supplementation on post-meal glycogen synthesis in exercised human skeletal muscle. Eight healthy male volunteers (aged 22 years) completed a 60-min cycling exercise at 70-75 % VO2_max and received HCA or placebo in a crossover design repeated after a 7 days washout period. They consumed 500 mg HCA or placebo with a high-carbohydrate meal (2 g carbohydrate/kg body weight, 80 % carbohydrate, 8 % fat, 12 % protein) for a 3-h post-exercise recovery. Muscle biopsy samples were obtained from vastus lateralis immediately and 3 h after the exercise. We found that HCA supplementation significantly lowered post-meal insulin response with similar glucose level compared to placebo. The rate of glycogen synthesis with the HCA meal was approximately onefold higher than that with the placebo meal. In contrast, GLUT4 protein level after HCA supplementation was significantly decreased below the placebo level, whereas expression of fatty acid translocase (FAT)/CD36 mRNA was significantly increased above the placebo level. Furthermore, HCA supplementation significantly increased energy reliance on fat oxidation, estimated by the gaseous exchange method. However, no differences were found in circulating NEFA and glycerol levels with the HCA meal compared with the placebo meal. The present study reports evidence that HCA supplementation enhanced glycogen synthesis rate in exercised human skeletal muscle and improved post-meal insulin sensitivity [12457].

**Applephenon**

It was examined the effects of Applephenon and ascorbic acid administration on physical fatigue. In a double-blinded, placebo-controlled, three-way crossover design, 18 healthy volunteers were randomized to oral Applephenon (1200 mg/d), ascorbic acid (1000 mg/d), or placebo for 8 d. The fatigue-inducing physical task consisted of workload trials on a bicycle ergometer at fixed workloads for 2 h on two occasions. During the test, subjects performed non-workload trials with maximum velocity for 10 s at 30 min (30-min trial) after the start of the test and 30 min before the end of the test (210-min trial). The change in maximum velocity between the 30- and 210-min trials was higher in the group given Applephenon than in the group given placebo; ascorbic acid had no effect. These results suggest that Applephenon attenuates physical fatigue, whereas ascorbic acid does not [07376].
Creatine

Athletes, body builders, and military personnel use dietary creatine as an ergogenic aid to boost physical performance in sports involving short bursts of high-intensity muscle activity. Lesser known is the essential role creatine, a natural regulator of energy homeostasis, plays in brain function and development. Creatine supplementation has shown promise as a safe, effective, and tolerable adjunct to medication for the treatment of brain-related disorders linked with dysfunctional energy metabolism, such as Huntington's Disease and Parkinson's Disease. Impairments in creatine metabolism have also been implicated in the pathogenesis of psychiatric disorders, leaving clinicians, researchers and patients alike wondering if dietary creatine has therapeutic value for treating mental illness. One review summarized the neurobiology of the creatine-phosphocreatine circuit and its relation to psychological stress, schizophrenia, mood and anxiety disorders. While present knowledge of the role of creatine in cognitive and emotional processing is in its infancy, further research on this endogenous metabolite has the potential to advance our understanding of the biological bases of psychopathology and improve current therapeutic strategies [12414].

A broad spectrum of beneficial effects has been ascribed to creatine (Cr), phosphocreatine (PCr) and their cyclic analogues cyclo-(cCr) and phospho-cyclocreatine (PcCr). Cr is widely used as nutritional supplement in sports and increasingly also as adjuvant treatment for pathologies such as myopathies and a plethora of neurodegenerative diseases. Additionally, Cr and its cyclic analogues have been proposed for anti-cancer treatment. The mechanisms involved in these pleiotropic effects are still controversial and far from being understood. The reversible conversion of Cr and ATP into PCr and ADP by creatine kinase, generating highly diffusible PCr energy reserves, is certainly an important element. However, some protective effects of Cr and analogues cannot be satisfactorily explained solely by effects on the cellular energy state. Here it was used mainly liposome model systems to provide evidence for interaction of PCr and PcCr with different zwitterionic phospholipids by applying four independent, complementary biochemical and biophysical assays: chemical binding assay, surface plasmon resonance spectroscopy (SPR), (solid-state $^{31}$P-NMR, and (differential scanning calorimetry (DSC). SPR revealed low affinity PCr/phospholipid interaction that additionally induced changes in liposome shape as indicated by NMR and SPR. Additionally, DSC revealed evidence for membrane packing effects by PCr, as seen by altered lipid phase transition. Finally, PCr efficiently protected against membrane permeabilization in two different model systems: liposome-permeabilization by the membrane-active peptide melittin, and erythrocyte hemolysis by the oxidative drug doxorubicin, hypoosmotic stress or the mild detergent saponin. These findings suggest a new molecular basis for non-energy related functions of PCr and its cyclic analogue. PCr/phospholipid interaction and alteration of membrane structure may not only protect cellular membranes against various insults, but could have more general implications for many physiological membrane-related functions that are relevant for health and disease [12415].

Very high blood creatine kinase (CK) concentrations have been observed among recent finishers of the 161-km Western States Endurance Run (WSER), and it has been suggested that there is a link between rhabdomyolysis and hyponatremia. Therefore, the purpose of one study was to compare CK concentrations of finishers in the 2010 WSER with past values, and to determine whether there was an association between blood CK and sodium concentrations. Consenting 2010 WSER finishers provided blood samples at the finish for
determination of blood CK and sodium concentrations. Finish time, age, and gender were obtained from official race results, and running experience was determined from our database as number of prior 161-km ultramarathon finishes. From 216 (66 %) of the 328 finishers, median and mean CK concentrations were found to be 20 850 IU/L and 32 956 IU/L, respectively (range 1500-264 300 IU/L), and 13 (6%) had values greater than 100 000 IU/L. These values were statistically higher than those reported from the 1995 WSER. The CK concentration was not significantly associated with finish time, age, gender, or running experience. Blood sodium concentrations were obtained from a subgroup of 159 runners, and the relationship between blood CK and sodium concentrations did not reach statistical significance. It was concluded that creatine kinase concentrations of 2010 WSER finishers are higher than values previously reported. More research should focus on explaining this observation and on whether there is a possible link between higher CK concentrations and hyponatremia [12416].

The purpose of one study was to examine the effects of 7 days of supplementation with 20 g/day of creatine monohydrate (CM) on mean power (MP) and peak power (PP) from the Wingate anaerobic test (WAnT), body weight (BW), 1-repetition maximum (1RM) bilateral leg extension (LE) strength, and 1RM bench press (BP) strength. The study used a randomized, double-blind, placebo-controlled design. Twenty-two men (age 22 ± 2 years) were randomly assigned to either a supplement (SUPP; n=10) or placebo (PLAC; n=12) group. The SUPP group ingested 20 g/d of CM powder for 7 days, whereas the PLAC ingested 20 g/d of maltodextrin powder. Measurements for the PLAC and SUPP groups included BW, PP, and MP from two 30-second WAnTs (separated by 7 minutes), and 1RM strength for LE and BP. Testing was conducted before (PRE) and after (POST) 7 days of ingesting either the supplement or placebo. The results of this study indicated that there was a significant increase from PRE to POST testing in MP for the SUPP group (5 %) but not for the PLAC group (-0.3 %). There were no between-group differences, however, for 1RM LE and 1RM BP strength. Furthermore, there were no changes in PP or BW for either group. The findings of this study indicated that loading with 20 g/d of CM for 7 days increased MP (5.4 % increase) from the WAnT, but it had no effect on strength (1RM LE and 1RM BP), PP, or BW [12417].

It was aimed to investigate the role of betaine supplementation on muscle phosphoryl-creatine (PCr) content and strength performance in untrained subjects. Additionally, it was compared the ergogenic and physiological responses to betaine versus creatine supplementation. Finally, we also tested the possible additive effects of creatine and betaine supplementation. This was a double-blind, randomized, placebo-controlled study. Subjects were assigned to receive betaine (BET; 2 g/day), creatine (CR; 20 g/day), betaine plus creatine (BET+CR; 2+20 g/day, respectively) or placebo (PL). At baseline and after 10 days of supplementation, it was assessed muscle strength and power, muscle PCr content, and body composition. The CR and BET+CR groups presented greater increase in muscle PCr content than PL (p=0.004 and p=0.006, respectively). PCr content was comparable between BET versus PL (p=0.78) and CR versus BET+CR (p=0.99). CR and BET+CR presented greater muscle power output than PL in the squat exercise following supplementation. Similarly, bench press average power was significantly greater for the CR-supplemented groups. CR and BET+CR groups also showed significant pre- to post-test increase in 1-RM squat and bench press. No significant differences for 1-RM strength and power were observed between BET versus PL and CR versus BET+CR. Body composition did not differ between the groups. In conclusion, it was reported that betaine supplementation does not augment muscle PCr content. Furthermore, it was shown that betaine supplementation combined or not with creatine supplementation does not affect strength and power performance in untrained subjects [12418].
Creatine is one of the most popular and widely researched natural supplements. The majority of studies have focused on the effects of creatine monohydrate on performance and health; however, many other forms of creatine exist and are commercially available in the sports nutrition/supplement market. Regardless of the form, supplementation with creatine has regularly shown to increase strength, fat free mass, and muscle morphology with concurrent heavy resistance training more than resistance training alone. Creatine may be of benefit in other modes of exercise such as high-intensity sprints or endurance training. However, it appears that the effects of creatine diminish as the length of time spent exercising increases. Even though not all individuals respond similarly to creatine supplementation, it is generally accepted that its supplementation increases creatine storage and promotes a faster regeneration of adenosine triphosphate between high intensity exercises. These improved outcomes will increase performance and promote greater training adaptations. More recent research suggests that creatine supplementation in amounts of 0.1 g/kg of body weight combined with resistance training improves training adaptations at a cellular and sub-cellular level. Finally, although presently ingesting creatine as an oral supplement is considered safe and ethical, the perception of safety cannot be guaranteed, especially when administered for long period of time to different populations (athletes, sedentary, patient, active, young or elderly) [12419].

There is an extensive and still growing body of the literature supporting the efficacy of creatine (Cr) supplementation. In sports, creatine has been recognized as the most effective nutritional supplement in enhancing exercise tolerance, muscle strength and lean body mass. From a clinical perspective, the application of Cr supplementation is indeed exciting. Evidences of benefits from this supplement have been reported in a broad range of diseases, including myopathies, neurodegenerative disorders, cancer, rheumatic diseases, and type 2 diabetes. In addition, after hundreds of published studies and millions of exposures creatine supplementation maintains an excellent safety profile. Thus, it is contended that the widespread application of this supplement may benefit athletes, elderly people and various patient populations [12420].

Creatine is a nitrogen-containing substance found naturally in small amounts in animal foods. In recent years, creatine has been synthesized, mainly as creatine monohydrate, and has been marketed to athletes at all levels. Creatine supplements come in various forms (powder, pills, candy, chews, gels, serum, micronized) for both strength and endurance athletes, including products marketed specifically for males, females, and adolescents. Creatine is a very popular sports supplement. Studies of various athletic groups report usage ranging from 17-75 percent. The most cited reasons for taking creatine are enhanced performance and appearance. Indeed, creatine continues to be one of the most popular sports supplements of all time. Oral creatine supplementation, usually as creatine monohydrate, has been reported to increase muscle supplies of free creatine and creatine phosphate (phosphocreatine; PCr), a high-energy phosphagen. A typical creatine loading protocol involves the ingestion of 20 grams of creatine monohydrate over the course of a day, usually in four separate doses of 5 grams each, for 4-6 consecutive days. Once loaded, a daily dose of 2–5 grams helps maintain elevated muscle creatine levels. Consuming carbohydrate with creatine appears to increase muscle creatine stores significantly more than creatine supplementation alone. Some individuals, particularly vegetarians with initially low levels of intramuscular creatine, may respond more effectively to creatine supplementation, while individuals with higher intramuscular creatine and phosphocreatine levels and those with fewer type II muscle fibers may be less responsive to supplementation. It has been suggested that the improvements in performance associated with creatine supplementation are due to parallel improvements in ATP resynthesis during exercise as a consequence of increased PCr availability, particularly within the type II muscle fibers. Creatine is one of the most researched sports supplements, as literally hundreds of studies
have evaluated its effects on various types of sport performance. Most of the research has focused on the ability of creatine supplementation to increase muscle mass and related muscular strength and power applicable to performance in very-high-intensity sports, such as sprinting in track events. Creatine supplementation appears to increase total body and lean body mass; however, short-term gains in total body mass may be primarily water, but long-term gains associated with resistance training appear to be lean muscle mass. Most studies document increases in muscle mass when creatine is supplemented during resistance training. The increase in muscle mass may be associated with a creatine supplementation-induced ability to do more repetitions during training, which may induce favorable genetic adaptations in the muscle. It has been suggested that the increase in lean body mass often reported after creatine supplementation could be mediated by signaling pathways, such as those involving insulin-like growth factor. In a related way, it has been studied the effect of 12 weeks of creatine supplementation and resistance training on muscular strength and myosin heavy chain (MHC) mRNA and protein expression. Compared to both a control and placebo group, the creatine group significantly increased fat-free mass and strength. Additionally, it was found that in general the MHC mRNA and protein expression were significantly higher in the creatine group compared to the other groups, and suggested that the increased strength and muscle size associated with creatine supplementation may be attributed to increase MHC synthesis. Research using muscle biopsy techniques has also shown increases in muscle cell diameter associated with 12 weeks of creatine supplementation and resistance training, which may be associated with the potential for creatine supplementation to amplify the resistance training-induced increase in satellite cell number and myonuclei concentration in skeletal muscle [06239].

Critical reviews of the scientific literature, including a meta-analysis and a monograph generally indicate that creatine supplementation may increase muscular strength and endurance as documented by enhanced performance in 1-repetition maximum strength tests, increased number of repetitions in various isotonic and isokinetic resistance exercise tasks, and increased work output during maximal short term (6-30 seconds) cycle ergometer tasks. In general, activities that involve sprinting, jumping or cycling performance show improved performance following creatine supplementation, but the beneficial effects appear to be less consistent. For example, using a standard creatine loading protocol with well-trained male sprinters as subjects, it was reported significant improvements in 100-meter sprint velocity and time to complete 6 intermittent 60-meter sprints. It was also reported significant increases in peak power and total work production in 10 sets of multiple 6-second bike sprints with varying periods of recovery in an 80-minute time frame following creatine supplementation. Conversely, it was reported no significant improvement in a 70-meter shuttle run sprint power test by well-trained tennis players and no beneficial effects on repeated 10-second skating sprints in ice-hockey players. Some research findings have a direct application to sports competition, such as an increased 1-RM performance in weight lifting and faster 100-meter sprint run times. The laboratory findings for other types of exercise performance are also rather strong, and do support a possible application to actual field competitions. For example, findings of increased muscle power output during intermittent sprint exercises may be applicable to football (soccer) and other sports associated with high-intensity intermittent sprinting. In one such study, it was studied the effects of creatine supplementation on an exercise test protocol designed to simulate match play in soccer (football). The test involved 5 blocks of 11-minute exercise involving sprint running, agility runs, and a precision ball-kicking drill interspersed with recovery walks, jogs and runs. Creatine supplementation improved performance in some repeated sprint and agility tasks even though the subjects increased body mass, but the creatine had no effect on ball kicking accuracy. Thus, creatine supplementation might improve speed in repetitive sprints, important for many sports, but may not necessarily enhance sports skills. In support
of this latter point, several studies reported no significant effects of creatine loading on tennis skill performance as measured by power and precision of their serves [06239].

Creatine is one of the most popular athletic supplements with sales in the US surpassing 400 million dollars in 2004. Due to the popularity and efficacy of creatine supplementation over 200 studies have examined the effects of creatine on athletic performance. Despite the abundance of research suggesting the effectiveness and safety of creatine a fallacy appears to exist in the general public driven by media claims and anecdotal reports that creatine supplementation can result in muscle cramps and dehydration. Although, a number of published studies have refuted these claims, a recent position statement by the American College of Sports Medicine (ACSM) in 2001 advised individuals who are managing their weight and exercising intensely or in hot environments to avoid creatine supplementation. Recent reports now suggest that creatine may enhance performance in hot and or humid conditions by maintaining hematocrit, aiding thermoregulation and reducing exercising heart rate and sweat rate. Creatine may also positively influence plasma volume during the onset of dehydration. Considering these new published findings, little evidence exists that creatine supplementation in the heat presents additional risk and should be taken into consideration as position statements and other related documents are published [08408].

In 1992 a watershed paper was published by Roger Harris and co-workers on the capacity of the muscle to increase its phosphocreatine concentration following supplementation with a creatine product. This led to an explosion of interest in this unique ergogenic aid - a product of apparently genuine value to biochemists, sports scientists, athletes, coaches, clinicians and the supplement industry. Clearly, the story of creatine is a remarkable one. In the space of two decades, the annual production and use of creatine supplements has grown exponentially, and the publication of creatine supplementation trials in the peer-reviewed literature can be measured in the hundreds. Creatine supplements owe their popularity both to the internet age and the evidence base of beneficial uses. As is the case for any supplement, however, it can be expected that the knowledge to continue to evolve, while our caution about individual products will need to be maintained in view of the frailties in the regulation of supplement manufacture and marketing [10406].

Creatine, a derivative from three amino acids, is distributed at approximately 95 percent in skeletal muscle mass; the remainder is located in the brain, the testes and the kidneys. Its synthesis starts mainly in the kidneys from glycine and arginine, forming alpha-methylguanidoacetic acid, which is conducted through the blood to the liver where it reacts with S-adenosylmethionine to synthesise creatine. Approximately 1-2 g of creatine is produced over 24 h and released mainly to the skeletal muscle system. Some creatine is also added to the pool by adequate dietary intake, predominantly from meat and fish, with a typical diet supplying approximately 1-2 g of creatine daily. It may be assumed that there is a total creatine pool of approximately 120 g in a man of 70 kg body weight. In skeletal muscle, creatine is slowly degraded to creatinine (approximately 2 g/day), a reaction without any enzyme intervention, and is released to the blood and the kidney to be expelled through the urine. Creatine is involved in the regulation of cellular energy demand. Under resting conditions, ATP is mainly formed in mitochondria through oxidative phosphorylation with ADP. Transported in sarcoplasm, some ATP molecules react with creatine, via the enzyme phosphorylcreatine kinase, to form phosphorylcreatine and ADP until equilibrium is reached. When ATP is needed for cellular energy, such as for muscle contraction, the phosphorylcreatine kinase reverse reaction replenishes the ATP content. Creatine thus acts indirectly to maintain a phosphorylcreatine reservoir for energy needs, more specifically to supply the muscle system with ATP. In 1992, it was demonstrated that oral creatine supplementation could increase muscle creatine levels approximately 20 percent. Subsequently, many studies have demonstrated that oral creatine supplementation can
maximise muscle creatine levels by either: a loading dose of 20 g/day for approximately 5
days followed by a maintenance dose of 2–3 g/day; or by the maintenance dose of 2–3 g/day
for approximately 30 days. These regimes lead to improved performance of repeated high-
intensity exercise, increased strength and lean body mass and enhanced fatigue resistance
for exercise tasks lasting 30 s or less, particularly when combined with progressive
resistance training. The mechanisms through which creatine supplementation improves
exercise performance and body composition include metabolic enhancements (increased
pre-exercise phosphorylcreatine, increased pre-exercise muscle glycogen), molecular
adaptations (increased gene expression of growth factors) and reduced muscle damage.
However, creatine supplementation does not increase skeletal muscle protein synthesis.
Many sports competitors are using creatine monohydrate as an ergogenic aid to boost their
performance. Being a natural substance, synthesised by mammals, including humans,
creatine has never been included on any doping list [10406].

It is worth commenting that creatine supplements have potentially greater and more
mainstream value than as performance enhancers for athletes. Creatine supplementation
can improve muscle mass and fatigue resistance in sarcopenic older adults in whom a better
function means an enhanced ability to perform activities of daily living. The benefits of
creatine ingestion have been extended to patient populations as well, and there are many
reports of improved muscle function in patients with various muscle (e.g. muscular
dystrophy) and degenerative central nervous system disorders (e.g. Parkinson's and
Huntington's diseases). Promising new data have emerged demonstrating that creatine
supplementation can improve cognitive processing in older adults. Despite published
allegations of detrimental effects of oral creatine supplementation on liver metabolism,
studies on humans have not shown any significant increase in plasma urea, nor liver enzyme
activity, during 5 years of creatine supplementation. No reports have observed a modification
of the glomerular filtration rate, nor the presence of microalbuminuria. All values remained
within the normal range adapted for the age range. Experimentally, an excess conversion of
creatine to sarcosine may result in cytotoxic agents such as methylamine. However, in
humans taking up to 20 g creatine per day for 2 weeks urine methylamine excretion remains
largely under the upper limit for healthy individuals. Even if there are no health risks induced
by oral creatine supplementation, it is safer to remain cautious when this substance is
administered chronically. We advise that creatine supplementation should not be used by
individuals with pre-existing renal disease or those with a potential risk of renal dysfunction
(diabetes, hypertension, reduced glomerular filtration rate). Regular check-ups should be
undertaken to monitor potential dysfunction, which could appear with some individuals less
prone to compensate any homeostatic imbalance. Great care should also be taken as far as
the purity of exogenous creatine supplements is concerned. Analytical tests must prove their
unique nutraceutical composition, as safety is not assured in some preparations [10406].

The purpose of the one study was to examine player-movement patterns to determine total
distance covered during competitive Rugby League match play using global positioning
systems (GPSs) and to examine pre, during, and postmatch creatine kinase (CK) and
endocrine responses to competitive Rugby League match play. Seventeen elite rugby league
players were monitored for a single game. Player movement patterns were recorded using
portable GPS units. Saliva and blood samples were collected 24 hours prematch, 30 minutes
prematch, 30 minutes postmatch, and then at 24-hour intervals for a period of 5 days
postmatch to determine plasma CK and salivary testosterone, cortisol, and
testosterone:cortisol ratio (T:C). The change in the dependent variables at each sample
collection time was compared to 24-hour prematch measures. Backs and forwards traveled
distances $5,747 \pm 1,095$ and $4,774 \pm 1,186$ m, respectively, throughout the match. Cortisol
and CK increased significantly from 30 minutes prematch to 30 minutes postmatch. Creatine
kinase increased significantly postmatch, with peak CK concentration measured 24 hours
Cortisol displayed a clear pattern of response with significant elevations up to 24 hours postmatch, compared with 24 hours prematch. The GPS was able to successfully provide data on player-movement patterns during competitive rugby league match play. The CK and endocrine profile identified acute muscle damage and a catabolic state associated with Rugby League match play. A return to normal T:C within 48 hours postmatch indicates that a minimum period of 48 hours is required for endocrine homeostasis postcompetition. Creatine kinase remained elevated despite 120 hours of recovery postmatch identifying that a prolonged period of at least 5 days modified activity is required to achieve full recovery after muscle damage during competitive Rugby League match play [10407].

The effects of creatine supplementation on muscle metabolism and exercise performance during a simulated endurance road race were investigated. Twelve adult male endurance-trained cyclists completed a simulated road race on a cycle ergometer (Lode), consisting of a two-hour cycling bout at 60 percent of peak aerobic capacity (VO\textsubscript{2peak}) with three 10-second sprints performed at 110 percent VO\textsubscript{2peak} every 15 minutes. Cyclists completed the 2-hr cycling bout before and after dietary creatine monohydrate or placebo supplementation (3 g/day for 28 days). Muscle biopsies were taken at rest and five minutes before the end of the two-hour ride. There was a 25 percent increase in resting muscle total creatine and 38 percent increase in muscle creatine phosphate in the creatine group. Plasma glucose, blood lactate, and respiratory exchange ratio during the 2-hour ride, as well as VO\textsubscript{2peak}, were not affected by creatine supplementation. Submaximal oxygen consumption near the end of the two-hour ride was significantly decreased by approximately 10 percent by creatine supplementation. Changes in plasma volume from pre- to post-supplementation were significantly greater in the creatine group than the placebo group at 90 minutes of exercise. The time of the final sprint to exhaustion at the end of the 2-hour cycling bout was not affected by creatine supplementation. Power output for the final sprint was increased by about 33 percent in both groups (creatine vs placebo not significant). It can be concluded that although creatine supplementation may increase resting muscle total creatine, muscle creatine phosphate, and plasma volume, and may lead to a reduction in oxygen consumption during submaximal exercise, creatine supplementation does not improve sprint performance at the end of endurance cycling exercise [10408].

During high-intensity exercise, intracellular creatine phosphate (PCr) is rapidly broken down to maintain adenosine triphosphate turnover. This has lead to the widespread use of creatine monohydrate as a nutritional ergogenic aid. However, the increase in intracellular PCr and the concomitant increase in intracellular water have not been investigated with regard to their effect on active range of movement (ROM). Forty male subjects (age, 24+/−3.2 years) underwent restricted randomization into 2 equal groups, either an intervention group (CS) or a control group (C). The CS group ingested 25 g/day of creatine monohydrate for 5 days, followed by 5 g/day for a further 3 days. Before (24 h before starting supplementation (PRE) and after (on the 8th day of supplementation (POST)) this loading phase, both groups underwent goniometry measurement of the shoulder, elbow, hip, and ankle. Data indicated significant reductions in active ROM in 3 movements: shoulder extension, shoulder abduction, and ankle dorsiflexion. There was also a significant increase in body mass for the CS group. The results suggest that short-term supplementation with creatine monohydrate reduces the active ROM of shoulder extension and abduction and of ankle dorsiflexion. Although the mechanism for this is not fully understood, it may be related to the asymmetrical distribution of muscle mass around those joints [10409].

The purpose of one study was to examine the effects of 5 days of creatine loading on the electromyographic fatigue threshold (EMG FT) in college-age men. Sixteen men participated in this double-blind study and were randomly placed into either placebo (n=8) or creatine (5 g
dicreatine citrate plus 10 g of flavored fructose powder per packet; n=8) loading groups. Each participant ingested 1 packet 4 times/d, totaling 20 g/d for 5 days (loading). Before and after loading, each participant performed a discontinuous cycle-ergometer test to determine his EMG FT, using bipolar surface electrodes placed on the vastus lateralis of the right thigh. Four 60-s work bouts (ranging from 200 to 400 W) were completed. Adequate rest was given between bouts to allow for the participants' heart rate to drop within 10 beats of their resting HR. The EMG amplitude was averaged over 5-s intervals for each 60-s work bout. Resulting slopes from each successive work resulted in a nonsignificant interaction for supplement and time. In addition, a significant increase in weight was observed in the creatine group. These data suggest that there was a minimal influence of creatin loading on EMG FT for the participants in this study [08409].

It was present a case of acute cholestatic liver injury associated with the combination of whey protein and creatine supplements. The difficulty of diagnosing drug-induced liver injury is emphasized. The patient was a healthy, 27-year-old man who presented with painless jaundice. He had no occupational exposures to solvents, was not taking prescription medications, and did not use recreational drugs or alcohol. He was an enthusiastic weight-lifter and had been taking creatine for 8 to 9 months and whey protein supplements for 4 weeks prior to the development of symptoms [08410].

The purpose of this study was to determine the effect of 30 days of single-dose creatine supplementation with phosphate salts on body weight and anaerobic working capacity in men. Using a double-blind design, 32 men randomly received 1 serving of either 5 g creatin + 4 g phosphate (n=17) or 20 g of dextrose as placebo (n=15) for 30 days. Results showed no significant differences between groups for working capacity at any time point; however, body weight was significantly increased at 10 days in the creatin group (1.0 kg) versus placebo (0.0 kg), and remained elevated for the duration of the study [08411].

The daily oral ingestion of supplementary creatine monohydrate can substantially elevate the creatine content of human skeletal muscle. This chapter aims to summarize the current knowledge regarding the impact muscle creatine loading can have on exercise performance and rehabilitation. The major part of the elevation of muscle creatine content is already obtained after one week of supplementation, and the response can be further enhanced by a concomitant exercise or insulin stimulus. The elevated muscle creatine content moderately improves contractile performance in sports with repeated high-intensity exercise bouts. More chronic ergogenic effects of creatine are to be expected when combined with several weeks of training. A more pronounced muscle hypertrophy and a faster recovery from atrophy have been demonstrated in humans involved in resistance training. The mechanism behind this anabolic effect of creatine may relate to satellite cell proliferation, myogenic transcription factors and insulin-like growth factor-1 signalling. An additional effect of creatine supplementation, mostly when combined with training, is enhanced muscle glycogen accumulation and glucose transporter (GLUT4) expression. Thus, creatine may also be beneficial in sport competition and training characterized by daily glycogen depletion, as well as provide therapeutic value in the insulin-resistant state [08412].

Creatine, a derivative from three amino acids, is distributed at approximately 95 percent in skeletal muscle mass; the remainder is located in the brain, the testes and the kidneys. Its synthesis starts mainly in the kidneys from glycine and arginine, forming alpha-methylguanidoacetic acid, which is conducted through the blood to the liver where it reacts with S-adenosylmethionine to synthesise creatine. Approximately 1-2 g of creatine is produced over 24 h and released mainly to the skeletal muscle system. Some creatine is also added to the pool by adequate dietary intake, predominantly from meat and fish, with a typical diet supplying approximately 1-2 g of creatine daily. It may be assumed that there is a
total creatine pool of approximately 120 g in a man of 70 kg body weight. In skeletal muscle, creatine is slowly degraded to creatinine (approximately 2 g/day), a reaction without any enzyme intervention, and is released to the blood and the kidney to be expelled through the urine. Creatine is involved in the regulation of cellular energy demand. Under resting conditions, ATP is mainly formed in mitochondria through oxidative phosphorylation with ADP. Transported in sarcoplasm, some ATP molecules react with creatine, via the enzyme phosphorylcreatine kinase, to form phosphorylcreatine and ADP until equilibrium is reached. When ATP is needed for cellular energy, such as for muscle contraction, the phosphorylcreatine kinase reverse reaction replenishes the ATP content. Creatine thus acts indirectly to maintain a phosphorylcreatine reservoir for energy needs, more specifically to supply the muscle system with ATP. In 1992, it was first demonstrated that oral creatine supplementation could increase muscle creatine levels approximately 20 percent. Subsequently, many studies have demonstrated that oral creatine supplementation can maximise muscle creatine levels by either a “loading” dose of 20 g/day for approximately 5 days followed by a “maintenance dose” of 2-3 g/day, or by the “maintenance dose” of 2-3 g/day for approximately 30 days. These regimes lead to improved performance of repeated high-intensity exercise, increased strength and lean body mass and enhanced fatigue resistance for exercise tasks lasting 30 s or less, particularly when combined with progressive resistance training. The mechanisms through which creatine supplementation improves exercise performance and body composition include metabolic enhancements (increased pre-exercise phosphorylcreatine, increased pre-exercise muscle glycogen), molecular adaptations (increased gene expression of growth factors) and reduced muscle damage. However, creatine supplementation does not increase skeletal muscle protein synthesis. Many sports competitors are using creatine monohydrate as an ergogenic aid to boost their performance. Being a natural substance, synthesised by mammals, including humans, creatine has never been included on any doping list. Creatine supplementation can improve muscle mass and fatigue resistance in sarcopenic older adults in whom a better function means an enhanced ability to perform activities of daily living. Anecdotal reports from athletes have claimed that creatine supplementation may induce muscle cramps. However, it appears that muscle cramping might be due to the intensity of exercise rather than creatine supplementation itself. Despite published allegations of detrimental effects of oral creatine supplementation on liver metabolism, studies on humans have not shown any significant increase in plasma urea, nor liver enzyme activity, during 5 years of creatine supplementation. Even if there are no health risks induced by oral creatine supplementation, it is safer to remain cautious when this substance is administered chronically. It has been advised that creatine supplementation should not be used by individuals with pre-existing renal disease or those with a potential risk of renal dysfunction (diabetes, hypertension, reduced glomerular filtration rate). Regular check-ups should be undertaken to monitor potential dysfunction, which could appear with some individuals less prone to compensate any homeostatic imbalance. Great care should also be taken as far as the purity of exogenous creatine supplements is concerned. Analytical tests must prove their unique nutraceutical composition, as safety is not assured in some preparations [10240].

It is known from studies in young athletes that creatine supplements have beneficial effects on muscular functional capacity, so it is being widely used as a performance-enhancing substance in both professional and amateur sports men and women. They are approved and considered relatively safe, but there have been a few case reports of renal dysfunction associated with their use. It was presented a case of a patient who developed acute renal failure and lactic acidosis while using creatine and metformin simultaneously [10241].

It currently is unknown whether creatine supplementation is safe for people with or at risk of kidney disease. It was reported on the short-term effects of creatine supplementation on kidney function in a young man with a single kidney and mildly decreased glomerular
filtration rate (GFR). A 20-year-old man who had undergone unilateral nephrectomy and presented with mildly decreased GFR without kidney damage underwent a trial with 35 days of creatine supplementation (20 g/d for 5 days followed by 5 g/d for the next 30 days) and had his kidney function monitored. After the intervention, $^{51}$Cr-EDTA clearance, proteinuria, and electrolyte levels were unchanged. Albuminuria, serum urea level, and estimated creatinine clearance were decreased, whereas serum creatinine level was slightly increased, falsely suggesting kidney function impairment. This prospective report suggests that short-term creatine supplementation may not affect kidney function in an individual with a single kidney, mild decreased GFR, and ingesting a high-protein diet (i.e. 2.8 g/kg/d). This finding has great relevance considering that creatine-induced kidney disease has been a growing concern, even for healthy people [10242].

It is known from studies in young athletes that creatine supplements have beneficial effects on muscular functional capacity, so it is being widely used as a performance-enhancing substance in both professional and amateur sports men and women. They are approved and considered relatively safe, but there have been a few case reports of renal dysfunction associated with their use. It was presented a case of a patient who developed acute renal failure and lactic acidosis while using creatine and metformin simultaneously [10243].

Coingestion of D-pinitol with creatine (CR) has been reported to enhance creatine uptake. The purpose of this study was to evaluate whether adding D-pinitol to CR affects training adaptations, body composition, whole-body creatine retention, and/or blood safety markers when compared to CR ingestion alone after 4 weeks of resistance training. Twenty-four resistance trained males were randomly assigned in a double-blind manner to creatine + pinitol (CRP) or creatine monohydrate (CR) prior to beginning a supervised 4-week resistance training program. Subjects ingested a typical loading phase (i.e. 20 g/d-1 for 5 days) before ingesting 5 g/d-1 the remaining 23 days. Performance measures were assessed at baseline (T0), week 1 (T1), and week 4 (T2) and included 1 repetition maximum (1RM) bench press (BP), 1RM leg press (LP), isokinetic knee extension, and a 30-second Wingate anaerobic capacity test. Fasting blood and body composition using dual-energy x-ray absorptiometry (DEXA) were determined at T1 and T3. Data were analyzed by repeated measures analysis of variance (ANOVA). Creatine retention significantly increased in both groups as a result of supplementation but was not different between groups. Significant improvements in upper- and lower-body strength and body composition occurred in both groups. However, significantly greater increases in lean mass and fat-free mass occurred in the CR group when compared to CRP. Thus, adding D-pinitol to creatine monohydrate does not appear to facilitate further physiological adaptations while resistance training. Creatine monohydrate supplementation helps to improve strength and body composition while resistance training [09348].

The objective of this study was to determine whether creatine supplementation could improve mechanical power output, and swimming performance in highly trained junior competitive fin swimmers. Sixteen male fin swimmers (age: 16 ± 2 years) were randomly and evenly assigned to either a creatine (4x5 g/day creatine monohydrate for 5 days) or placebo group (same dose of a dextrose-ascorbic acid placebo) in a double-blind research. Before and after creatine the average power output was determined by a Bosco-test and the swimming time was measured in two maximal 100 m fin swims. After five days of creatine the average power of one minute continuous rebound jumps increased by 20 percent. The lactate concentration was significantly less after 5 minutes restitution at the second measurement in both groups. The swimming time was significantly reduced in both first and second sessions of swimming in the creatine group, but remained almost unchanged in the placebo group. The results of this study indicate that five day creatine supplementation enhances the dynamic strength and may increase anaerobic metabolism in the lower
extremity muscles, and improves performance in consecutive maximal swims in highly trained adolescent fin swimmers [09323].

The purpose of the present study was to examine player-movement patterns to determine total distance covered during competitive Rugby League match play using global positioning systems (GPSs) and (b) examine pre, during, and postmatch creatine kinase (CK) and endocrine responses to competitive Rugby League match play. Seventeen elite rugby league players were monitored for a single game. Player movement patterns were recorded using portable GPS units (SPI-Pro, GPSports, Canberra, Australia). Saliva and blood samples were collected 24 hours prematch, 30 minutes prematch, 30 minutes postmatch, and then at 24-hour intervals for a period of 5 days postmatch to determine plasma CK and salivary testosterone, cortisol, and testosterone:cortisol ratio (T:C). The change in the dependent variables at each sample collection time was compared to 24-hour prematch measures. Backs and forwards traveled distances 5,747 ± 1,095 and 4,774 ± 1,186 m, respectively, throughout the match. Cortisol and CK increased significantly from 30 minutes prematch to 30 minutes postmatch. Creatine kinase increased significantly postmatch, with peak CK concentration measured 24 hours postmatch (889 ± 238 UL). Cortisol displayed a clear pattern of response with significant elevations up to 24 hours postmatch, compared with 24 hours prematch. The GPS was able to successfully provide data on player-movement patterns during competitive rugby league match play. The CK and endocrine profile identified acute muscle damage and a catabolic state associated with Rugby League match play. A return to normal T:C within 48 hours postmatch indicates that a minimum period of 48 hours is required for endocrine homeostasis postcompetition. Creatine kinase remained elevated despite 120 hours of recovery postmatch identifying that a prolonged period of at least 5 days modified activity is required to achieve full recovery after muscle damage during competitive Rugby League match play [10495].

The classical role of creatine-phosphate (PCr) is seen as a reservoir of high-energy phosphates defending cellular ATP levels under anaerobic conditions, high rates of energy transfer or rapid fluctuations in energy requirement. Although the high concentration of PCr in glycolytic fast-twitch fibers supports the role of PCr as a buffer of ATP, the primary importance of the creatine kinase (CK) reaction may in fact be to counteract large increases in ADP, which could otherwise inhibit cellular ATPase-mediated systems. A primary role for CK in the maintenance of ADP homeostasis may explain why, in many conditions, there is an inverse relationship between PCr and muscle contractility but not between ATP and muscle contractility. The high rate of ATP hydrolysis during muscle contraction combined with restricted diffusion of ADP supports that ADP concentration increases transiently during the contraction phase (ADP spikes) and that these are synchronized with the contraction. The presence of CK, structurally bound in close vicinity to the sites of ATP utilization, will reduce the amplitude and duration of the ADP spikes through PCr-mediated phosphotransfer. When PCr is reduced, the efficiency of CK as an ATP buffer will be reduced and the changes in ADP will become more prominent. The presence of ADP spikes is supported by the finding that other processes known to be activated by ADP (i.e. AMP deamination and glycolysis) are stimulated during exercise but not during anoxia, despite the same low global energy state. Breakdown of PCr is driven by increases in ADP above that depicted by the CK equilibrium and the current method to calculate ADPfree from the CK reaction in a contracting muscle is therefore questionable [11267].

Supplemental creatine has been promoted for its positive health effects and is best known for its use by athletes to increase muscle mass. In addition to its role in physical performance, creatine supplementation has protective effects on the brain in models of neuronal damage and also alters mood state and cognitive performance. Creatine is found in high protein foods, such as fish or meat, and is also produced endogenously from the biosynthesis of
arginine, glycine, and methionine. Changes in brain creatine levels, as measured using magnetic resonance spectroscopy, are seen in individuals exposed to drugs of abuse and depressed individuals. These changes in brain creatine indicate that energy metabolism differs in these populations relative to healthy individuals. Recent work shows that creatine supplementation has the ability to function in a manner similar to antidepressant drugs and can offset negative consequences of stress. These observations are important in relation to addictive behaviors as addiction is influenced by psychological factors such as psychosocial stress and depression. The significance of altered brain levels of creatine in drug-exposed individuals and the role of creatine supplementation in models of drug abuse have yet to be explored and represent gaps in the current understanding of brain energetics and addiction [11588].

Creatine (N-(aminoiminomethyl)-N-methyl glycine) is an ingredient commonly found in food, mainly in fish and meat, and is sold as a dietary supplement in markets around the world. Creatine crystallizes from water as monoclinic prisms holding one molecule of water of crystallization per molecule of creatine. Continued drying of CM results in a loss of the water of crystallization at around 100°C, yielding anhydrous creatine. Creatine is a weak base with a pkb value of 11.02 at 25°C. As a result, creatine can only form salts with strong acids, having a pka value of less than 3.98. Creatine forms salts by the protonation of its guanidine moiety. In addition to salt formation, creatine is able to act as a complexing agent. Creatinine use as an ergogenic aid and possible treatment for certain neuromuscular disorders is well documented in scientific literature. Numerous studies have found that CM supplementation increases muscle phosphagen levels generally by 10 to 40 percent. Acute and chronic supplementation of CM has been reported to improve performance primarily during high intensity, intermittent activities. The impact on performance has been associated with the magnitude of change in baseline phosphagen levels. Numerous studies have shown that CM supplementation during training promotes greater gains in performance and/or fat-free mass. The only clinically significant side effect reported in literature has been weight gain, which is an attribute desired by many athletes who desire to increase muscle mass as well as clinical populations concerned about muscle wasting. Consequently, CM has proven to be one of the most effective, safe, and well-studied ergogenic aids. In recent years, the popularity of creatine has risen dramatically, especially among athletes. In the USA alone, creatine-containing dietary supplements make up a large portion of the estimated USD 2.7 billion in annual sales of sports nutrition supplements. The form of creatine that has been most extensively studied and commonly used in dietary supplements is creatine monohydrate (CM). Studies have consistently indicated that CM supplementation increases muscle creatine and phosphocreatine concentrations by approximately 15 to 40 percent, enhances anaerobic exercise capacity, and increases training volume leading to greater gains in strength, power, and muscle mass. A number of potential therapeutic benefits have also been suggested in various clinical populations. Studies have indicated that CM is not degraded during normal digestion and that nearly 99 percent of orally ingested CM is either taken up by muscle or excreted in urine. Further, no medically significant side effects have been reported in literature. Nevertheless, supplement manufacturers have continually introduced newer forms of creatine into the marketplace. These newer forms have been purported to have better physical and chemical properties, bioavailability, efficacy, and/or safety profiles than CM. However, there is little to no evidence that any of the newer forms of creatine are more effective and/or safer than CM whether ingested alone and/or in combination with other nutrients. In addition, whereas the safety, efficacy, and regulatory status of CM is clearly defined in almost all global markets; the safety, efficacy, and regulatory status of other forms of creatine present in today's marketplace as a dietary or food supplement is less clear. Accompanying the explosive growth in sales has been the introduction of different forms of creatine. Creatine monohydrate (CM), first marketed in the early 1990s, is the form most commonly found in dietary supplement/food products and most
frequently cited in scientific literature. The introduction into the marketplace of alternate forms of creatine, beginning in the late 1990s, was presumably an attempt to differentiate the multitude of creatine-containing products available to consumers and improve certain attributes such as solubility and efficacy. However, the legal and regulatory status of these various forms of creatine in the USA and other markets around the world is at best uncertain. To date, with the exception of Japan, CM is the only form of creatine to be officially approved or accepted in key markets such as the USA, European Union (EU), Canada and South Korea. The continued presence of other forms of creatine in the marketplace, especially in the US, may be due to a multitude of factors. These include, but may not be limited to, a lack of awareness or understanding on the part of marketers of applicable laws and regulations, intentional noncompliance with the law, and/or inadequate enforcement of the law. The public health implications of widespread distribution and use of these unauthorized forms of creatine is unknown and warrants careful monitoring. New forms of creatine are marketed with claims of improved physical, chemical, and physiological properties in comparison to CM. Claims include improved stability when combined with other ingredients or in liquids, improved solubility in water, improved bioavailability, and even an increase in performance. Creatine salts such as citrate, maleate, fumarate, tartrate, pyruvate, ascorbate, and orotate were first introduced to the marketplace as early as the late 1990s. Creatine and acids with multiple acid moieties such as citric acid can form salts as well as complexation products. In addition to creatine and its salts, derivatives of creatine such as creatine ester or even creatine alcohols are currently marketed as dietary supplements. Both ingredients do not contain creatine as such, since they have been chemically altered. While it is assumed that the human body will transfer those molecules into creatine upon intake, there are no published data available to base firm conclusions. The amount of creatine in different forms of creatine varies. Creatine monohydrate contains 88 percent of creatine, whereas the creatine content in other forms of creatine is lower with the exception of creatine anhydrous. Commercial creatine salts are formed in solution or by mechanical processes such as milling or grinding under the presence of residual water. Complexes are formed by the subsequent replacement of the solvating molecules by the new ligands. Creatine monohydrate powder is very stable showing no signs of degradation over years, even at elevated temperatures. To detect a potential degradation of creatine, one must measure the content of its degradation product, creatinine. The degradation of creatine can be reduced or even halted by either lowering the pH under 2.5 or increasing the pH. A very high pH results in the deprotonation of the amide function of the creatine molecule, thereby preventing the intramolecular cyclization. A very low pH results in the protonation of the amide function of the creatine molecule, thereby preventing the intramolecular [11268].

The uptake of creatine is simplified in a two-step approach: first, uptake into the bloodstream; second, uptake into the target tissue. The term “bioavailability” refers to both the intestinal absorption and the use of a substance by the body’s cells and tissues. First indications of a potential change of creatine bioavailability can be gathered from the amount of creatine taken up into the blood plasma after oral administration. However, a change in the total amount of creatine in the blood plasma cannot be directly extrapolated to a potential increase in desired performance. An increased amount of creatine in the plasma could be the result of decreased uptake into the target tissue resulting in an actual decrease in overall bioavailability. On the other hand, an initial rise in plasma creatine levels, followed by a reduction in plasma levels, is an indication of increased uptake into the target tissue. This has been demonstrated in vivo by combining creatine with insulin-stimulating ingredients such as high amounts of glucose or protein. Conclusive proof of an increase in relevant bioavailability can only be gained by assessing the amount of creatine reaching the target tissue, the muscle, measured by muscle biopsy and/or whole body creatine retention assessed by measuring the difference between creatine intake and urinary excretion. Dietary creatine is presumed to have high bioavailability since intestinal absorption of CM is already
close to 100 percent. However, the response to creatine supplementation is heterogeneous, due in part to some non-responders, which might be overcome by alternative forms of creatine. Over the years, there has been significant commercial interest in determining whether creatine could be delivered in a liquid form. The thought has been since CM is relatively insoluble that development of a liquid or suspended form of creatine may be more convenient to consume, be more readily absorbed into the blood stream, and promote a greater efficiency in transport of creatine to the muscle. Some companies have even claimed that minimal amounts of liquid creatine would need to be ingested because of enhanced efficiency in transport through the blood and into the muscle. A limitation with these theories is that CM is not stable for any substantial length of time in liquid. Consequently, while researchers have been working on ways to suspend creatine within gels and fluids, it has been generally considered to be impractical to develop into a product due to limitations in shelf-life. In addition, while people may prefer the taste of liquid or gel versions of creatine, there is no evidence that these delivery forms provide a superior performance benefit. In analysis of this literature, it is clear that CM supplementation promotes significant increases in muscle creatine levels in most individuals. There is some evidence that co-ingestion of CM with various nutrients (e.g. carbohydrate, protein, d-pinitol) may enhance creatine uptake to a greater degree. However, there is no evidence that effervescent creatine, liquid creatine, and/or CEE promotes greater uptake of creatine to the muscle. Rather, there is some evidence that some of these forms of creatine may be less effective and/or be of greater clinical concern in terms of safety [11268].

Explanations as to why these alternate creatine forms are prevalent in the marketplace despite not having met the legal and regulatory requirements in the various markets are likely two-fold. Legal definitions of and regulatory requirements for “dietary supplements” (USA and Korea), “food supplements” (EU), “natural health products” (Canada), and “non-drug food additives” (Japan) are complex, differ between countries/regions, and can be confusing. Lack of awareness and/or understanding of the given country’s applicable requirements may be one explanation for the lack of compliance on the part of some marketers. To the extent that the laws and regulations are known and understood, inadequate enforcement by regulators can create an environment where noncompliance is perceived to be without consequences, resulting in the forgoing of required registration or notification requirements. In the USA, the increased prevalence of these alternate forms (CEE in particular) in dietary supplement products, with no enforcement action from FDA, has helped to support this misperception. The public health implications of having unsanctioned or unapproved forms of creatine on the market remain to be fully realized. While classical animal toxicity and short- and long-term clinical safety studies have been conducted in humans, the basic safety data on new forms of creatine is lacking. At present, there do not appear to be any imminent or specific safety concerns associated with any of these alternate forms. However, this must be monitored carefully through post-market surveillance and published case reports. As far as the marketplace is concerned, the presence of these newer and typically more expensive forms of creatine in a multitude of consumer products that are often marketed with misleading and/or unsubstantiated claims of greater bioavailability, efficacy, and safety sets a negative precedent. The reality that companies need not fulfill the necessary registration or notification requirements to satisfy regulatory authorities, but still feel free to market their ingredients and products without penalty establishes an “unlevel” playing field among competitors. This undermines any incentive to invest upfront resources to establish ingredients as safe and efficacious prior to reaching consumers. Inevitably, this will result in unintended and unforeseen consequences, which will serve to erode consumer confidence [11268].

It aimed to investigate the possible role of creatine (CR) supplementation in counteracting dexamethasone-induced muscle wasting and insulin resistance in rats. Also, it was examined whether CR intake would modulate molecular pathways involved in muscle remodeling and
insulin signaling. Animals were randomly divided into four groups: (1) dexamethasone (DEX); (2) control pair-fed (CON-PF); (3) dexamethasone plus CR (DEX-CR); and (4) CR pair-fed (CR-PF). Dexamethasone (5 mg/kg/day) and CR (5 g/kg/day) were given via drinking water for 7 days. Plantaris and extensor digitorum longus (EDL) muscles were removed for analysis. Plantaris and EDL muscle mass were significantly reduced in the DEX-CR and DEX groups when compared with the CON-PF and CR-PF groups. Dexamethasone significantly decreased phospho-Ser(473)-Akt protein levels compared to the CON-PF group and CR supplementation aggravated this response. Serum glucose was significantly increased in the DEX group when compared with the CON-PF group. CR supplementation significantly exacerbated hyperglycemia in the dexamethasone-treated animals. Dexamethasone reduced GLUT-4 translocation when compared with the CON-PF and CR-PF groups and this response was aggravated by CR supplementation. In conclusion, supplementation with CR resulted in increased insulin resistance and did not attenuate muscle wasting in rats treated with dexamethasone. Given the contrast with the results of human studies that have shown benefits of CR supplementation on muscle atrophy and insulin sensitivity, it was suggested caution when extrapolating this animal data to human subjects [11269].

Nutritional supplements are very popular especially among athletes although some studies show either controversial or even negative results. However, whey protein and creatine seem to have positive effects on muscle size, strength and athletic performance without major adverse effects and high costs. Most studies have shown that supplementation of whey protein can enhance muscle growth in response to resistance training. Some studies also suggest that whey may enhance recovery from heavy exercise and possibly decrease muscle damage and soreness. Creatine supplementation increases the intracellular pool of phosphocreatine in skeletal muscle. Phosphocreatine provides a reserve of energy to rapidly regenerate ATP, which is consumed as a result of muscle contraction. Creatine has been studied in hundreds of clinical trials and has shown benefits including increased muscle strength, power and size [11270].

It was described a case in which serum creatinine is elevated due to the use of creatine ethyl ester. One week after withdrawal, the plasma creatinine had normalised. There are two types of creatine products available: creatine ethyl ester (CEE) and creatine monohydrate (CM). Plasma creatinine is not elevated in all creatine-using subjects. CEE, but not CM, is converted into creatinine in the gastrointestinal tract. As a result the use of CEE may be associated with elevated plasma creatinine levels. Since plasma creatinine is a widely used marker for renal function, the use of CEE may lead to a false assumption of renal failure [11271].

Creatine monohydrate (Cr), the most diffuse supplement in the sports industry, is receiving greater attention because of its beneficial effects in a wide number of human degenerative diseases and conditions. These effects can be barely explained on the basis of the sole ergogenic role of the Cr/CrP system. Indeed, a wide number of research articles indicate that Cr is capable of exerting multiple, non-energy related, effects on diverse and relevant cellular targets. Among these effects, the antioxidant activity of Cr emerges as an additional mechanism which is likely to play a supportive role in the Cr-cytoprotection paradigm [11272].

Doubtful allegations of adverse effects of creatine supplementation have been released through the press media and through scientific publications. In one review it was tried to separate the wheat from the chaff by looking for the experimental evidence of any such claims. Anecdotal reports from athletes have appeared on muscle cramp and gastrointestinal complaints during creatine supplementation, but the incidence of these is limited and not
necessarily linked to creatine itself. Despite several unproved allegations, liver (enzymes, urea) and kidneys (glomerular filtration urea and albumin excretion rates) show no change in functionality in healthy subjects supplemented with creatine, even during several months, in both young and older populations. The potential effects (production of heterocyclic amines) of mutagenicity and carcinogenicity induced by creatine supplementation have been claimed by a French Sanitary Agency (AFSSA), which might put consumers at risk. Even if there is a slight increase (within the normal range) of urinary methylamine and formaldehyde excretion after a heavy load of creatine (20 g/day) this is without effect on kidney function. The search for the excretion of heterocyclic amines remains a future task to definitively exclude the unproved allegation made by some national agencies. It was advised that high-dose (>3-5 g/day) creatine supplementation should not be used by individuals with pre-existing renal disease or those with a potential risk for renal dysfunction (diabetes, hypertension, reduced glomerular filtration rate). A pre-supplementation investigation of kidney function might be considered for reasons of safety, but in normal healthy subjects appears unnecessary [11273].

The daily oral ingestion of supplementary creatine monohydrate can substantially elevate the creatine content of human skeletal muscle. One chapter aimed to summarize the current knowledge regarding the impact muscle creatine loading can have on exercise performance and rehabilitation. The major part of the elevation of muscle creatine content is already obtained after one week of supplementation, and the response can be further enhanced by a concomitant exercise or insulin stimulus. The elevated muscle creatine content moderately improves contractile performance in sports with repeated high-intensity exercise bouts. More chronic ergogenic effects of creatine are to be expected when combined with several weeks of training. A more pronounced muscle hypertrophy and a faster recovery from atrophy have been demonstrated in humans involved in resistance training. The mechanism behind this anabolic effect of creatine may relate to satellite cell proliferation, myogenic transcription factors and insulin-like growth factor-1 signalling. An additional effect of creatine supplementation, mostly when combined with training, is enhanced muscle glycogen accumulation and glucose transporter (GLUT4) expression. Thus, creatine may also be beneficial in sport competition and training characterized by daily glycogen depletion, as well as provide therapeutic value in the insulin-resistant state [07346].

Studies attributing gains in strength and lean body mass (LBM) to creatine monohydrate (CrM) during resistance exercise (RE) training have not assessed these changes alongside cellular and subcellular adaptations. Additionally, CrM-treated groups have seldom been compared with a group receiving a placebo similar in nitrogen and energy. The purpose of one study was to examine the effects of a CrM-containing protein-carbohydrate (PRO-CHO) supplement in comparison with a supplement containing a similar amount of nitrogen and energy on body composition, muscle strength, fiber-specific hypertrophy, and contractile protein accrual during RE training. In a double-blind, randomized protocol, resistance-trained males were matched for strength and placed into one of three groups: protein (PRO), PRO-CHO, or the same PRO-CHO supplement (1.5 g/kg body weight and day) containing CrM (Cr-PRO-CHO) (0.1 g/kg body weight and day). Assessments were completed the week before and after a 10-wk structured, supervised RE program: strength (1RM, three exercises), body composition (DEXA), and vastus lateralis muscle biopsies for determination of muscle fiber type (I, IIA, IIX), cross-sectional area (CSA), contractile protein, and creatine content. Cr-PRO-CHO provided greater improvements in 1RM strength. At least 40% of the strength improvements could be attributed to hypertrophy of muscle involved in this exercise. Cr-PRO-CHO also resulted in greater increases in LBM, fiber CSA, and contractile protein compared with PRO and PRO-CHO. In RE-trained participants, supplementation with Cr-PRO-CHO provided greater muscle hypertrophy than an equivalent dose of PRO-CHO, and
this response was apparent at three levels of physiology (LBM, fiber CSA, and contractile protein content) [07347].

The effects of short-term oral creatine (Cr) supplementation on exercise performance and on blood pressure and renal function were assessed. Thirty-five healthy, active duty, U.S. Army volunteers (20 men and 15 women; age, 22-36 years) at Fort Sam Houston, Texas, supplemented their diet for 7 days with 20 g/day of either Cr or taurine (as placebo). There was no significant difference in 2-minute push-up counts between the Cr and taurine groups from before to after supplementation. The Cr group demonstrated a significant increase in serum creatinine levels, compared with the taurine group, and this increase could be misinterpreted as impairment of renal function. No adverse changes in blood pressure, body composition, weight, or serum Cr phosphokinase levels were observed. We conclude that short-term creatine supplementation appears to be safe but does not enhance push-up performance [07348].

Creatine supplementation (CS) has been reported to increase body weight and improve performance during high intensity, short duration, exercise tasks. However, none of the published studies has investigated the influence of CS on performance related hydrodynamic variables during swimming. To investigate the effect of oral CS on swimming velocity, body composition and hydrodynamic variables during the period of final preparation of competitive junior female swimmers. In a double blind and randomized manner, 16 female swimmers, were supplemented with 20 g/day of creatine monohydrate (CS group), or a maltodextrin placebo (PL group) for 21 days. Just pre- and post-21 days of supplementation, subjects performed 2x25 swimming bouts at maximum velocity with a 3 min recovery between bouts. The variables measured were 25 m swimming velocity (MSV(25)); active drag force (D(f)); hydrodynamic coefficient (C(x)); power output (P(o)). Body measures were also analysed: body weight (kg), fat-mass (% FAT), body water (% H_2O), and fat free mass (FFM). Significant differences were observed in hydrodynamic values: the CS group showed a significant reduction (approximately 25 %), in D(f), C(x) and P(o) values, when comparing pretest with post-test. No differences were found in variables related to body composition and performance between CS group and PL group, as well as for CS group during the experimental period. These data suggest that 21 days of CS produced significant effects on gross and/or propelling efficiency during swimming in female athletes. However, CS did not influence performance, body weight and body composition [07349].

Rugby union football requires muscular strength and endurance, as well as aerobic endurance. Creatine supplementation may enhance muscular performance, but it is unclear if it would interfere with aerobic endurance during running because of increased body mass. The purpose of one study was to determine if creatine supplementation during 8 weeks of a season of rugby union football can increase muscular performance, without negatively affecting aerobic endurance. Rugby union football players were randomized to receive 0.1 g.kg(-1).d(-1) creatine monohydrate (n=9) or placebo (n=9) during 8 weeks of the rugby season. Players practiced twice per week for approximately 2 h per session and played one 80 min game per week. Before and after the 8 weeks, players were measured for body composition (air displacement plethysmography), muscular endurance (number of repetitions at 75% of one repetition maximum (1 RM) for bench press and leg press), and aerobic endurance (Leger shuttle-run test with 1 min stages of progressively increasing speed). There were time main effects for body mass, fat mass, and a trend for an increase in lean tissue mass, with no differences between groups. The group receiving creatine supplementation had a greater increase in the number of repetitions for combined bench press and leg press tests compared with the placebo group. There were no changes in either group for aerobic endurance. Creatine supplementation during a rugby union football season is effective for increasing muscular endurance, but has no effect on body composition or
aerobic endurance [07350].

It was examined the effects of creatine supplementation on the response to repeated bouts of resistance exercise. Young men (24 ± 5 years) were divided into creatine monohydrate (CM, n=9) and placebo (PL, n=9) groups. On day (D) 1 and D15, subjects performed four sets of bicep curls at 75 percent 1-RM to concentric failure. On D8-D13, subjects consumed either 20 g/d creatine monohydrate or placebo. Muscle soreness and elbow joint range of motion (ROM) were assessed on D1-D5 and D15-D19. Serum creatine kinase activity (CK) was assessed on D1, D3, D5, D15, D17, and D19. The first exercise bout produced increases in muscle soreness and CK, and decreases in ROM in both groups. The second bout produced lesser rises in serum CK, muscle soreness, and a lesser decrease in ROM with greater attenuation of these damage markers in CM than PL. CK levels on D17 were lower muscle soreness from D15-19 was lower, and elbow ROM was decreased in PL, but not CM on D16. It was concluded that creatine supplementation provides an additive effect on blunting the rise of muscle damage markers following a repeated bout of resistance exercise. The mechanism by which creatine augments the repeated bout effect is unknown but is likely due to a combination of creatine's multifaceted functions [13681].

Creatine is an ergogenic supplement that has been used by athletes with the goal of increasing strength gains in the weight room. In the 1990s, creatine became a popular supplement used by athletes to augment resistance training. The popularity of creatine grew as studies began to show some benefits with strength training, particularly with short, high-intensity exercises. A survey of first division athletes in 1999 found that 48 percent of male athletes reported current or prior use of creatine. Creatine also was found to be the most popular supplement used by a cohort of high school athletes in a survey completed in Iowa in 2001. It was found that usage increased in high school by grade, and in the 11th and 12th grade, the usage was about 12 percent. The recent surveys have shown a decrease in popularity of creatine with whey protein being the most popular. Creatine has been one of the more extensively studied dietary supplements. There have been upward of 300 studies evaluating the effects of creatine on resistance training, with 70% reporting increases in strength. Several forms of creatine exist; however creatine monohydrate has been the most extensively studied, and its formulation has shown benefits in short-duration, high-intensity weightlifting and cycling [13682].

Creatine monohydrate is a dietary supplement that increases muscle performance in short-duration, high-intensity resistance exercises, which rely on the phosphocreatine shuttle for adenosine triphosphate. Many situations within athletics and during training for sport require fast and intense muscle contractions. Intense sport activities less than 10 s in duration are dependent on intramuscular stores of adenosine triphosphate (ATP) and phosphocreatine. Several studies have shown increases in intramuscular stores of creatine and phosphocreatine with creatine monohydrate supplementation, and the increases range from 10 to 40 percent. However there is an upper limit of creatine stores that are possible in human muscle, which has been reported as high as 160 g in the human body. Therefore athletes with full stores of creatine in their muscles will not receive benefit from supplementation. People with lower creatine stores in their muscles receive the greatest effect on intramuscular creatine stores when supplemented with oral creatine. Therefore the theory behind creatine supplementation is to increase stores in the muscle to facilitate ATP and phosphocreatine production, delaying the onset of muscle fatigue. The effective dosing for creatine supplementation includes loading with 0.3 g/kg/d for 5 to 7 days, followed by maintenance dosing at 0.03 g/kg/d most commonly for 4 to 6 weeks. However loading doses are not necessary to increase the intramuscular stores of creatine. Creatine monohydrate is the most studied; other forms such as creatine ethyl ester have not shown added benefits. Creatine is a relatively safe supplement with few adverse effects reported. The most
common adverse effect is transient water retention in the early stages of supplementation. When combined with other supplements or taken at higher than recommended doses for several months, there have been cases of liver and renal complications with creatine. Further studies are needed to evaluate the remote and potential future adverse effects from prolonged creatine supplementation [13682].

**Biochemistry**

Creatine is a nitrogenous amine that was discovered in 1832. It is found primarily in skeletal muscle, with 95 percent of the body's creatine stores found within skeletal muscle. The total amount of creatine in the body is equal to the free creatine plus the phosphocreatine, which equals approximately 120 g in a 70-kg person. The exogenous sources of creatine are animal products such as red meat and fish. The normal dietary intake of creatine in an omnivorous diet is around 1 g per day. The liver, kidney, and pancreas form endogenous stores of creatine. The endogenous production of creatine is down-regulated during exogenous creatine supplementation; however, the endogenous production returns to baseline after supplementation is discontinued. The first step in endogenous synthesis of creatine occurs in the kidney and starts with the amino acids glycine and arginine. The product is then transferred to the liver where a methyl group from methionine is added forming creatine. Circulating creatine is brought into skeletal muscle via transporters in the cell membrane. The rate of creatine uptake has been shown to be influenced by exercise, catecholamines, and insulin-like growth factor. Once within the cell, creatine can be phosphorylated to form phosphocreatine in a reversible enzymatic reaction facilitated by creatine kinase. The phosphate group comes from ATP forming adenosine diphosphate (ADP). The reverse reaction occurs when ATP is being used by the cell, and phosphocreatine can shuttle a phosphate group to ADP [13682].

During short-duration, high-intensity exercises, ATP needs are met by both anaerobic glycolysis and phosphocreatine shuttle. Anaerobic glycolysis is the dominant form of ATP production between 10 and 30 s when at maximal effort, while the phosphocreatine shuttle predominates as an ATP source during maximal effort exercises less than 10 s. By increasing stores of phosphocreatine with creatine supplementation, the belief is one can decrease muscle fatigue and increase performance by prolonging the phosphocreatine shuttle. In addition to increasing phosphocreatine stores, there are other proposed mechanisms by which creatine supplementation can improve performance during these exercises. One proposed mechanism is faster resynthesis of phosphocreatine during rest and recovery between bouts of maximal exercises; more creatine in the muscles would equate to more potential phosphocreatine. Conflicting data exist regarding creatine supplementation improving phosphocreatine resynthesis. Other mechanisms include aiding ATP production via glycolysis by increasing phosphofructokinase activity or by buffering hydrogen ions [13682].

**Creatine ethyl ester**

Creatine ethyl ester has received recent attention. In order to increase the intramuscular creatine levels, one of the latest creatine variations is creatine ethyl ester. Creatine ethyl ester is alleged to increase creatine bioavailability. Esterification of creatine decreases its hydrophilicity, and manufacturers of creatine ethyl ester claim that this allows it to bypass the creatine transporter due to enhanced sarcolemmal permeability toward creatine. Studies have shown that creatine ethyl ester is a substrate for creatine kinase. Yet recent studies show that creatine ethyl ester is converted to creatinine, not creatine. Increases in plasma creatinine were found with creatine ethyl ester. In addition, nonenzymatic cleavage of creatine ethyl ester was reported, leading them to report that creatine ethyl ester is a pronutrient for creatinine rather than creatine under all physiological conditions encountered.
during transit through the various tissues; thus no ergogenic effect is to be expected from supplementation. Other forms of creatine, such as a buffered form of creatine, to increase hydrophilic nature of the molecule is a more efficacious and/or safer form of creatine to consume than creatine monohydrate. Creatine ethyl ester has received recent attention. In order to increase the intramuscular creatine levels, one of the latest creatine variations is creatine ethyl ester. Creatine ethyl ester is alleged to increase creatine bioavailability. Esterification of creatine decreases its hydrophilicity, and manufacturers of creatine ethyl ester claim that this allows it to bypass the creatine transporter due to enhanced sarcolemmal permeability toward creatine. Studies have shown that creatine ethyl ester is a substrate for creatine kinase. Yet recent studies show that creatine ethyl ester is converted to creatinine, not creatine. Increases in plasma creatinine were found with creatine ethyl ester. In addition, nonenzymatic cleavage of creatine ethyl ester was reported, leading them to report that creatine ethyl ester is a pronutrient for creatinine rather than creatine under all physiological conditions encountered during transit through the various tissues; thus no ergogenic effect is to be expected from supplementation. Other forms of creatine, such as a buffered form of creatine, to increase hydrophilic nature of the molecule is a more efficacious and/or safer form of creatine to consume than creatine monohydrate [13682].

**Physiological aspects**

Creatine is produced endogenously at an amount of about 1 g/d. Synthesis predominately occurs in the liver, kidneys, and to a lesser extent in the pancreas. The remainder of the creatine available to the body is obtained through the diet at about 1 g/d for an omnivorous diet. Ninety-five percent of the bodies’ creatine stores are found in the skeletal muscle and the remaining 5 percent is distributed in the brain, liver, kidney, and testes. As creatine is predominately present in the diet from meats, vegetarians have lower resting creatine concentrations. The majority of creatine in the human body is in two forms, either the phosphorylated form making up 60 percent of the stores or in the free form which makes up 40 percent of the stores. The average 70 kg young male has a creatine pool of around 120-140 g which varies between individuals depending on the skeletal muscle fiber type and quantity of muscle mass. The endogenous production and dietary intake matches the rate of creatinine production from the degradation of phosphocreatine and creatine at 2.6 percent and 1.1 percent/day respectively. In general, oral creatine supplementation leads to an increase of creatine levels within the body. Creatine can be cleared from the blood by saturation into various organs and cells or by renal filtration. Three amino acids (glycine, arginine and methionine) and three enzymes (L-arginine:glycine amidinotransferase, guanidinoacetate methyltransferase and methionine adenosyltransferase) are required for creatine synthesis. The impact creatine synthesis has on glycine metabolism in adults is low, however the demand is more appreciable on the metabolism of arginine and methionine. Creatine ingested through supplementation is transported into the cells exclusively by CreaT1. However, there is another creatine transporter Crea T2, which is primarily active and present in the testes. Creatine uptake is regulated by various mechanisms, namely phosphorylation and glycosylation as well as extracellular and intracellular levels of creatine. Crea T1 has shown to be highly sensitive to the extracellular and intracellular levels being specifically activated when total creatine content inside the cell decreases. It has also been observed that in addition to cytosolic creatine, the existence of a mitochondrial isoform of Crea T1 allows creatine to be transported into the mitochondria. Indicating another intramitochondrial pool of creatine, which seems to play an essential role in the phosphate-transport system from the mitochondria to the cytosol. Myopathy patients have demonstrated reduced levels of total creatine and phosphocreatine as well as lower levels of CreaT1 protein, which is thought to be a major contributor to these decreased levels [12419].

**Effects of creatine supplementation on physical performance**
Chronic supplementation with creatine monohydrate has been shown to promote increases in total intramuscular creatine, phosphocreatine, skeletal muscle mass, lean body mass and muscle fiber size. Furthermore, there is robust evidence that muscular strength and power will also increase after supplementing with creatine. However, it is not known if the timing of creatine supplementation will affect the adaptive response to exercise. Thus, the purpose of one investigation was to determine the difference between pre versus post exercise supplementation of creatine on measures of body composition and strength. Nineteen healthy recreational male bodybuilders (mean; age 23; height 166 cm; weight: 80 kg) participated in this study. Subjects were randomly assigned to one of the following groups: PRE-SUPP or POST-SUPP workout supplementation of creatine (5 grams). The PRE-SUPP group consumed 5 grams of creatine immediately before exercise. On the other hand, the POST-SUPP group consumed 5 grams immediately after exercise. Subjects trained on average five days per week for four weeks. Subjects consumed the supplement on the two non-training days at their convenience. Body composition (Bod Pod(R)) and 1-RM bench press (BP) were determined. Diet logs were collected and analyzed (one random day per week; four total days analyzed). There was a significant time effect for fat-free mass (FFM) and BP, however, fat mass (FM) and body weight did not reach significance. While there were trends, no significant interactions were found. However, using magnitude-based inference, supplementation with creatine post workout is possibly more beneficial in comparison to pre workout supplementation with regards to FFM, FM and 1-RM BP. Qualitative inference represents the likelihood that the true value will have the observed magnitude. Furthermore, there were no differences in caloric or macronutrient intake between the groups. It was concluded that creatine supplementation plus resistance exercise increases fat-free mass and strength. Based on the magnitude inferences it appears that consuming creatine immediately post-workout is superior to pre-workout vis a vis body composition and strength [13683].

It was examined the effects of creatine supplementation on the response to repeated bouts of resistance exercise. Young men (24 year) were divided into creatine (CM, n=9) and Placebo (PL, n=9) groups. On day (D) 1 and D15, subjects performed four sets of bicep curls at 75% 1-RM to concentric failure. On D8-D13, subjects consumed either 20 g/d creatine monohydrate or placebo. Muscle soreness and elbow joint range of motion (ROM) were assessed on D1-D5 and D15-D19. Serum creatine kinase activity (CK) was assessed on D1, D3, D5, D15, D17, and D19. The first exercise bout produced increases in muscle soreness and CK, and decreases in ROM in both groups. The second bout produced lesser rises in serum CK, muscle soreness, and a lesser decrease in ROM with greater attenuation of these damage markers in CM than PL. CK levels on D17 were lower (+110 % over D15 for CM vs +343 % for PL), muscle soreness from D15-19 was lower (-75 % for CM vs -56 % for PL compared with first bout), and elbow ROM was decreased in PL, but not CM on D16. It was concluded creatine supplementation provides an additive effect on blunting the rise of muscle damage markers following a repeated bout of resistance exercise. The mechanism by which creatine augments the repeated bout effect is unknown but is likely due to a combination of creatine's multifaceted functions [13685].

It was evaluated the effect of Creatine (Cr) supplementation on muscle fatigue and physiological indices after intermittent swimming bouts in trained swimmers. Sixteen healthy non-elite swimmers (19 ± 4 years, 75 ± 12 kg) were randomly assigned into two groups of either Cr supplementation or placebo and performed six repeated sprints swimming bouts of 50-m departing every 120 seconds. The Cr group was supplemented 4 times a day for 6 days. Blood lactate, Creatine Kinase (CK), creatinine, heart rate, best repeated sprint (RSb) and mean repeated sprint (RSm) times, and percentage of speed decrement (%Dec) were measured at the various phases of swimming bouts. Repeated measure ANOVA and
independent t-student tests showed CK and blood lactate concentration increased gradually after the third and sixth swimming bouts. % Dec in Cr group was significantly lower after 3rd swimming bout, also heart rate in Cr group was associated with lower increase in HR mean compared to placebo. These results suggest that Cr supplementation may improve swimming performance and reduce increased blood lactate levels following intermittent sprint swimming bouts. In conclusion Cr supplementation in trained swimmers may improve anaerobic performance and heart rate variations independent of the effect of intensive sprint swimming bouts [13686].

The goal of one study was to evaluate the effects of creatine (Cr) supplementation on oxidative stress and inflammation markers after acute repeated-sprint exercise in humans. Twenty-five players under age 20 years were randomly assigned to two groups: Cr supplemented and placebo. Double-blind controlled supplementation was performed using Cr (0.3 g/kg) or placebo tablets for 7 d. Before and after 7 d of supplementation, the athletes performed two consecutive Running-based Anaerobic Sprint Tests (RAST). RAST consisted of six 35-m sprint runs at maximum speed with 10 sec rest between them. Blood samples were collected just prior to start of test (pre), just after the completion (0 h), and 1 h after completion. Average, maximum, and minimum power values were significantly greater in the Cr-supplemented group compared with placebo. There were significant increases in plasma tumor necrosis factor alpha (TNF-alpha) and C-reactive protein (CRP) up to 1 h after acute sprint exercise in the placebo-supplemented group. Malondialdehyde, lactate dehydrogenase (LDH), catalase, and superoxide dismutase enzymes also were increased after exercise in both groups. Red blood cell glutathione was lower after exercise in both groups. Cr supplementation reversed the increase in TNF-alpha and CRP as well as LDH induced by acute exercise. Controversially, Cr supplementation did not inhibit the rise in oxidative stress markers. Also, antioxidant enzyme activity was not different between placebo and Cr-supplemented groups. Thus, creatinin supplementation inhibited the increase of inflammation markers TNF-alpha and CRP, but not oxidative stress markers, due to acute exercise [13684].

In addition to performance measurements, evidence has supported increases in fat-free mass and type II muscle fiber area. Muscle glycogen levels may also be affected by creatine supplementation, likely as a result of increased cellular water content. Increases in body mass with creatine supplementation have been reported as far back as 1928. However current evidence suggests that the increase in body mass observed with creatine is due to the decreased urine output and water retention during the initial stages of creatine loading. There is no evidence that creatine supplementation affects protein synthesis. Sport-specific performance also has been studied quite extensively to see if the effect of creatine supplementation extended from the weight room to the field of play. Multiple studies have investigated creatine supplementation effect on sprinting, swimming, and agility training and have failed to show an effect [13682].

Creatine monohydrate’s effect on resistance training exercises has been extensively researched. There are numerous controlled studies that have reported increases in performance and muscle strength in short-duration, maximum-intensity exercises. Resistance training has been measured in many ways in the literature, including exercises such as bench press, leg press, biceps curls, leg extensions, jump squats, and bicycle ergometry. The method of measurement of strength and performance in creatine studies includes one repetition maximum, mean power, total force, and number of repetitions. The results regarding creatine supplementation’s ergogenic effect are not unanimous. However, there is a significant body of evidence that creatine increases performance in short-duration, maximum-intensity resistance training. Conflicting evidence exists regarding studies of the effect of creatine supplementation on anaerobic performance. Currently, studies consistently
have observed no effect on aerobic performance with creatine supplementation [13682].

The majority of studies focusing on creatine supplementation report an increase in the body’s creatine pool. There is a positive relationship between muscle creatine uptake and exercise performance. It has been observed a significant increase in strength performance after 12 weeks creatine supplementation with a concurrent periodized heavy resistance training protocol. The creatine supplementation protocol consisted of a week-long loading period of 25 g/d followed by a 5 g maintenance dose for the remainder of the training. These positive effects were attributed to an increased total creatine pool resulting in more rapid adenosine triphosphate (ATP) regeneration between resistance training sets allowing athletes to maintain a higher training intensity and improve the quality of the workouts along the entire training period. It is regularly reported that creatine supplementation, when combined with heavy resistance training leads to enhanced physical performance, fat free mass, and muscle morphology. A 2003 meta-analysis showed individuals ingesting creatine, combined with resistance training, obtain on average +8 and +14 percent more performance on maximum (1RM) or endurance strength (maximal repetitions at a given percent of 1RM) respectively than the placebo groups. However, contradicting studies have reported no effects of creatine supplementation on strength performance. These conflicting results can be explained by the possibility that the supplemented groups were formed by a greater amount of non-responders or even because creatine supplementation was administered on the training days only (3 times a week). This strategy has not been adequately tested as effective in middle aged and older men for maintaining post loading elevated creatine stores. A quantitative, comprehensive scientific summary and view of knowledge up to 2007 on the effects of creatine supplementation in athletes and active people was published in a 100 citation review position paper by the International Society of Sports Nutrition. More recent literature has provided greater insight into the anabolic/performance enhancing mechanisms of creatine supplementation suggesting that these effects may be due to satellite cell proliferation, myogenic transcription factors and insulin-like growth factor-1 signalling. Collectively, in spite of a few controversial results, it seems that creatine supplementation combined with resistance training would amplify performance enhancement on maximum and endurance strength as well muscle hypertrophy [12419].

Effects of creatine on anaerobic exercise

Creatine has demonstrated neuromuscular performance enhancing properties on short duration, predominantly anaerobic, intermittent exercises. It has been observed enhanced neuromuscular function of the elbow flexors in both electrically induced and voluntary contractions but not on endurance performance after 4 loading doses of 5 g creatine plus 15 g maltodextrin for 5/d in young, moderately trained men. Creatine supplementation may facilitate the reuptake of Ca$^{2+}$ into the sacroplasmic reticulum by the action of the Ca$^{2+}$ adenosine triphosphatase pump, which could enable force to be produced more rapidly through the faster detachment of the actomyosin bridges. A previous meta-analysis reported an overall creatine supplementation effect size of 0.24 ± 0.02 for activities lasting ≤30 s. (primarily using the ATP-phosphocreatine energy system). For this short high-intensity exercise, creatine supplementation resulted in a 7.5 ± 0.7 percent increase from base line which was greater than the 4.3 ± 0.6 percent improvement observed for placebo groups. When looking at the individual selected measures for anaerobic performance the greatest effect of creatine supplementation was observed on the number of repetitions which showed an ES of 0.64 ± 0.18. Furthermore, an increase from base line of 45.4 ± 7.2 percent compared to 22.9 ± 7.3 percent for the placebo group was observed. The second greatest ES was on the weight lifted at 0.51 ± 0.16 with an increase from base line of 13.4 ± 2.7 percent for the placebo group and 24.7 ± 3.9 percent for the creatine group. The possible effect of creatine supplementation on multiple high intensity short duration bouts (<30 s) have shown
an effect size not statistically significant from 0. This would indicate that creatine supplementation might be useful to attenuate fatigue symptoms over multiple bouts of high-intensity, short duration exercise. The ES of creatine on anaerobic endurance exercise (>30-150s), primarily using the anaerobic glycolysis energy system, was 0.19 ± 0.05 with an improvement from baseline of 4.9 ± 1.5 percent for creatine and -2.0 ± 0.6 percent for the placebo [12419].

Although creatine supplementation has been shown to be more effective on predominantly anaerobic intermittent exercise, there is some evidence of its positive effects on endurance activities as well. From a meta-analysis, it would appear that the ergogenic potential for creatine supplementation on predominantly aerobic endurance exercise diminishes as the duration of the activity increases over 150s. However it is suggested that creatine supplementation may cause a change in substrate utilization during aerobic activity possibly leading to an increase in steady state endurance performance. Despite that, the effects of creatine supplementation on endurance performance have been questioned by some studies. In addition, of the concern related to the dosage used in many studies, it could be possible that the potential benefits of creatine supplementation on endurance performance were more related to effects of anaerobic threshold localization [12419].

Effects of creatine supplementation on skeletal muscle hypertrophy

It has been observed greater improvements on 1RM, lean body mass, fiber cross sectional area and contractile protein in trained young males when resistance training was combined with a multi-nutrient supplement containing 0.1 g/kg/d of creatine, 1.5 g/kg/d of protein and carbohydrate compared with protein alone or a protein carbohydrate supplement without the creatine. These findings were novel because at the time no other research had noted such improvements in body composition at the cellular and sub cellular level in resistance trained participants supplementing with creatine. When creatine supplementation is combined with heavy resistance training, muscle insulin like growth factor (IGF-1) concentration has been shown to increase. Compared to placebo, creatine groups produced greater increments in IGF-1 (78 % vs 55 %) and body mass (2.2 vs 0.6 kg). Additionally, vegetarians within the supplemented group had the largest increase of lean mass compared to non vegetarian (2.4 and 1.9 kg, respectively). Changes in lean mass were positively correlated to the modifications in intramuscular total creatine stores which were also correlated with the modified levels of intramuscular IGF-1. The authors suggested that the rise in muscle IGF-1 content in the creatine group could be due to the higher metabolic demand created by a more intensely performed training session. These amplifying effects could be caused by the increased total creatine store in working muscles. Even though vegetarians had a greater increase in high energy phosphate content, the IGF-1 levels were similar to the amount observed in the non vegetarian groups. These findings do not support the observed correlation pattern by which a low essential amino acid content of a typical vegetarian diet should reduce IGF-1 production [12419].

The objective of one study was to determine if additional dietary protein improves the lean tissue deposition and carcass merit of pigs supplemented creatine monohydrate in combination with a high glycemic carbohydrate (dextrose). Forty-eight crossbred barrows and gilts (91 kg) were blocked by sex assigned to 1 of 12 pens (4 pigs/pen, 16 pigs/treatment). Treatments included: control (CON; basal diet consisting of a ground corn-soybean base), combination diet (COMBO; basal diet supplemented with 0.92 % creatine monohydrate and 2.75 % dextrose), and a combination high protein diet (COMBOHP; COMBO formulated to contain a minimum of 16 % crude protein). Barrows on the COMBOHP gained the least 10th rib fat and expressed the highest percentage fat-free carcass lean after 28 days on test. No significant treatment differences were noted in the fat
and lean tissue accretion of gilts. Treatments had no affect the meat quality parameters of barrow and gilt carcasses [11400].

**Effect on plasma levels of pro-inflammatory cytokines**

The effect of creatine supplementation upon plasma levels of pro-inflammatory cytokines: interleukin (IL) 1 beta and IL-6, tumor necrosis factor alpha (TNFalpha), and Interferon alpha (INF alpha) and prostaglandin E2 (PGE2) after a half-ironman competition were investigated.

Eleven triathletes, each with at least three years experience of participation in this sport were randomly divided between the control and experimental groups. During 5 days prior to competition, the control group (n=6) was supplemented with carbohydrate (20 g/day) whereas the experimental group (n=5) received creatine (20 g/day) in a double-blind trial. Blood samples were collected 48 h before and 24 and 48 h after competition and were used for the measurement of cytokines and PGE2. Forty-eight hours prior to competition there was no difference between groups in the plasma concentrations (pg/mL) of IL-6, TNFalpha, INF alpha, IL-1 beta, and PGE2. Twenty-four and 48 h after competition plasma levels of TNFalpha, INF alpha, IL-1 beta and PGE2 were significantly increased in both groups. However, the increases in these were markedly reduced following creatine supplementation. An increase in plasma IL-6 was observed only after 24 h and, in this case, there was no difference between the two groups. It was concluded that creatine supplementation before a long distance triathlon competition may reduce the inflammatory response induced by this form of strenuous of exercise [07351].

**Effects of creatine on glycogen stores**

It is suggested that another mechanism for the effect of creatine could be enhanced muscle glycogen accumulation and GLUT4 expression, when creatine supplementation is combined with a glycogen depleting exercise. Whereas it has been observed that creatine supplementation alone does not enhance muscle glycogen storage it was observed positive effects of creatine supplementation for enhancing initial and maintaining a higher level of muscle glycogen during 2 hours of cycling. In general, it is accepted that glycogen depleting exercises, such as high intensity or long duration exercise should combine high carbohydrate diets with creatine supplementation to achieve heightened muscle glycogen stores [12419].

**Effects on blood lactate**

To determine the effects of creatine supplementation on blood lactate during incremental cycling exercise 13 male subjects (23 ± 2 years) performed a maximal, incremental cycling test to exhaustion before (Pre) and after (Post) 6 d of creatine supplementation (4 doses/d of 5 g creatine + 15 g glucose). Blood lactate was measured at the end of each exercise stage during the protocol, and the lactate threshold was determined as the stage before achieving 4 mmol/L. Lactate concentrations during the incremental test were analyzed using a 2 (condition) × 6 (exercise stage) repeated-measures ANOVA. Differences in power at lactate threshold, power at exhaustion, and total exercise time were determined by paired t tests. Lactate concentrations were reduced during exercise after supplementation, demonstrating a significant condition effect. There was a tendency for increased power at the lactate threshold. Total time to fatigue approached significant increases as did maximal power output. The findings demonstrate that creatine supplementation decreases lactate during incremental cycling exercise and tends to raise lactate threshold. Therefore, creatine supplementation could potentially benefit endurance athletes [13687].

**Effects on kidney function**

1928
The aim of one study was to determine the effects of creatine supplementation on kidney function in resistance-trained individuals ingesting a high-protein diet. A randomized, double-blind, placebo-controlled trial was performed. The participants were randomly allocated to receive either creatine (20 g/d for 5 d followed by 5 g/d throughout the trial) or placebo for 12 weeks. All of the participants were engaged in resistance training and consumed a high-protein diet (i.e., ≥ 1.2 g/Kg/d). Subjects were assessed at baseline (Pre) and after 12 weeks (Post). Glomerular filtration rate was measured by $^{51}$Cr-EDTA clearance. Additionally, blood samples and a 24-h urine collection were obtained for other kidney function assessments. No significant differences were observed for $^{51}$Cr-EDTA clearance throughout the trial. Creatinine clearance, serum and urinary urea, electrolytes, proteinuria, and albuminuria remained virtually unchanged. It was concluded that a 12-week creatine supplementation protocol did not affect kidney function in resistance-trained healthy individuals consuming a high-protein diet; thus reinforcing the safety of this dietary supplement [13688].

**Effects on inflammatory markers**

The goal of one study was to evaluate the effects of creatine (Cr) supplementation on oxidative stress and inflammation markers after acute repeated-sprint exercise in humans. Twenty-five players under age 20 years were randomly assigned to two groups: Cr supplemented and placebo. Double-blind controlled supplementation was performed using Cr (0.3 g/kg) or placebo tablets for 7 d. Before and after 7 d of supplementation, the athletes performed two consecutive Running-based Anaerobic Sprint Tests (RAST). RAST consisted of six 35-m sprint runs at maximum speed with 10 sec rest between them. Blood samples were collected just prior to start of test (pre), just after the completion (0 h), and 1 h after completion. Average, maximum, and minimum power values were greater in the Cr-supplemented group compared with placebo. There were significant increases in plasma tumor necrosis factor alpha (TNF-alpha) and C-reactive protein (CRP) up to 1 h after acute sprint exercise in the placebo-supplemented group. Malondialdehyde, lactate dehydrogenase (LDH), catalase, and superoxide dismutase enzymes also were increased after exercise in both groups. Red blood cell glutathione was lower after exercise in both groups. Cr supplementation reversed the increase in TNF-alpha and CRP as well as LDH induced by acute exercise. Controversially, Cr supplementation did not inhibit the rise in oxidative stress markers. Also, antioxidant enzyme activity was not different between placebo and Cr-supplemented groups. It was concluded that Cr supplementation inhibited the increase of inflammation markers TNF-alpha and CRP, but not oxidative stress markers, due to acute exercise [13689].

**Side effects of creatinine**

The International Society of Sports Nutrition’s position statement on creatine monohydrate states that there is no scientific evidence of side effects or adverse effects when creatine is used appropriately. They therefore conclude that if used properly, creatine is an acceptable nutritional supplement and ergogenic aid for young athletes to use. No adverse effects were reported in a study of young healthy individuals after supplementation with creatine from 7 d to 10 weeks. Creatine is excreted by the kidney, which led to the hypothesis that creatine supplementation may be detrimental to renal function. Several studies have looked at serum creatinine levels during creatine loading but have not reported significant increases in serum creatinine in younger healthy populations. Slight increases in serum creatinine levels have been reported with larger doses of creatine during the loading phase, although not statistically significant. However increases in urinary creatinine excretion and decreases in total urine volume output have been reported during creatine loading. The decrease in
urinary output is thought to result in fluid retention and weight gain during the initial phases of creatine supplementation. Creatine has been reported to result in weight gain and water retention during short-term use. In the literature, there is a case report of a 20-year-old man with interstitial nephritis as a result of creatine supplementation. However the individual in the case was taking loading doses of creatine (20 g/d) over a 4-week period instead of the recommended and well-studied loading phase of 5 to 7 d. A longer study of creatine supplementation in nonathletes with other medical comorbidities also did not show evidence of renal problems [13682].

Although there have not been significant increases in serum creatinine with creatine supplementation, there have been other concerns about the effects of creatine loading on the kidney. Creatine can be metabolized to methylamine and subsequently formaldehyde during urinary excretion. Both methylamine and formaldehyde are known cytotoxic substances raising concerns about potential harmful effects to the kidney with long-term use. Studies have shown significant increases in both methylamine and formaldehyde after short-term creatine supplementation at loading doses. Further studies are needed to further evaluate the potential harm to the kidney related to the increases in urinary methylamine and formaldehyde levels, particularly with longer term use and high loading doses of creatine. In the literature, there are case reports of young healthy individuals developing acute liver failure when one of the dietary supplements they were ingesting was creatine. However in these cases, the individuals were taking large doses of creatine in addition to several other dietary supplements for weight training. When creatine has been studied in isolation and at acceptable doses, there have been no significant adverse effects to the liver [13682].

Since creatine does result in a decrease in urinary volume and water retention during supplementation, concerns arose that athletes could develop problems staying hydrated and regulating body temperature. Creatine supplementation increases intracellular volume with increased cellular water volume. A study evaluated lower extremity anterior compartment pressures after heat-stressed exercise, and it did find transient asymptomatic increases in compartment pressures with creatine supplementation compared to placebo. There is a case report of compartment syndrome occurring with large doses of creatine, with subsequent resolution with cessation. In 1998, the University of Tennessee football team had many of their football players develop cramping during a game, after the team instituted a creatine supplementation program. The number of athletes was disproportional to historic values of athletes cramping, which caused the linkage to creatine usage. Further studies have since shown no increase in the incidence of cramping in college football players taking creatine. Several studies also have reported no issues with heat tolerance or hydration status with creatine supplementation [13682].

*Dosing protocols applied in creatine*

A typical creatine supplementation protocol consists of a loading phase of 20 g CM/d or 0.3 g CM/kg/d split into 4 daily intakes of 5 g each, followed by a maintenance phase of 3-5 g CM/d or 0.03 g CM/kg/d for the duration of the supplementation period. Other supplementation protocols are also used such as a daily single dose of around 3-6 g or between 0.03 to 0.1 g/kg/d. However, this method takes longer (between 21 to 28 days) to produce ergogenic effects. It was also found that a moderate protocol consisting of 20 g CM taken in 1g doses (evenly ingested at 30-min intervals) for 5 days resulted in reduced urinary creatine and methylamine excretion, leading to an estimated increase in whole body retention of creatine (+13 %) when compared with a typical loading supplementation protocol of 4 x 5 g/d during 5 days (evenly ingested at 3 hour intervals). This enhancement in creatine retention would lead to a significantly higher weight gain when people follow a moderate
protocol ingestion of several doses of small amounts of CM evenly spread along the day [12419].

Studies have shown that intramuscular stores of total creatine and phosphocreatine can be increased by supplementing with oral creatine monohydrate for 5 to 7 d with a dose of 20 to 25 g/d. The greatest increase of creatine and phosphocreatine is reported to be in the first 2 d of supplementation. The typical dosing in studies that have shown increases in strength performance includes both a loading and maintenance phase. Depending on the study, the loading phase varies from 5 to 7 days at 0.3 g/kg/day. During the loading phase, the daily dose is divided into four equal doses throughout the day dissolved in a liquid. After the 5- to 7-d loading phase, the athlete continues with the maintenance phase at 0.03 g/kg/day. The length of the maintenance phase varies in studies from 28 d to 10 week. When a carbohydrate or protein is added to creatine supplementation, there may be an increase in muscle retention of creatine, particularly in the first few days, resulting in a decreased need for loading. However alternative dosing methods also have been shown to effectively increase creatine stores and have effects on strength gains. Regimens without the creatine loading phase, 3 to 6 g/day for 28 days and 6 g/day for 12 weeks, also have been shown to be effective in increasing creatine stores. The increase in creatine stores occurs more slowly and therefore may take longer to see the strength training effects [13682].

**Commercially available forms of creatine**

There are several different available forms of creatine: creatine anhydrous which is creatine with the water molecule removed in order to increase the concentration of creatine to a greater amount than that found in CM. Creatine has been manufactured in salt form: creatine pyruvate, creatine citrate, creatine malate, creatine phosphate, magnesium creatine, creatine orotate, Kre Alkalyn (creatine with baking soda). Creatine can also be manufactured in an ester form. Creatine ethyl ester (hydrochloride) is an example of this, as is creatine gluconate which is creatine bound to glucose. Another form is creatine effervescent which is creatine citrate or CM with citric acid and bicarbonate. The citric acid and bicarbonate react to produce an effervescent effect. When mixed with water the creatine separates from its carrier leaving a neutrally charged creatine, allowing it to dissolve to a higher degree in water. Manufacturers claim that creatine effervescent has a longer and more stable life in solution. When di-creatine citrate effervescent was studied for stability in solution it was found that the di-creatine citrate dissociates to citric acid and creatine in aqueous solutions which in turn forms CM and eventually crystallises out of the solution due to its low solubility. Some of the creatine may also convert to creatinine. In summary, creatine salts have been show to be less stable than CM. However the addition of carbohydrates could increase their stability. The potential advantages of creatine salts over CM include enhanced aqueous solubility and bioavailability which would reduce their possible gastrointestinal adverse effects. The possibility for new additional formulation such as tablets or capsules is interesting for its therapeutic application due to its attributed better dissolution kinetics and oral absorption compared to CM. However more complete in vivo pharmaceutical analysis of creatine salts are required to fully elucidate their potential advantages/disadvantages over the currently available supplement formulations [12419].

**Monhydrate**

Creatine monohydrate (CrM) has been consistently reported to increase muscle creatine content and improve high-intensity exercise capacity. However, a number of different forms of creatine have been purported to be more efficacious than CrM. The purpose of this study was to determine if a buffered creatine monohydrate (KA) that has been purported to promote greater creatine retention and training adaptations with fewer side effects at lower doses is more efficacious than CrM supplementation in resistance-trained individuals. In a
double-blind manner, 36 resistance-trained participants (20 years, 181 cm, 82 kg, and 15 % body fat) were randomly assigned to supplement their diet with CrM (Creapure® AlzChem AG, Trostberg, Germany) at normal loading (4 x 5 g/d for 7-days) and maintenance (5 g/d for 21-days) doses; KA (Kre-Alkaly®; All American Pharmaceutical, Billings, MT, USA) at manufacturer’s recommended doses (KA-L, 1.5 g/d for 28-days); or, KA with equivalent loading (4 x 5 g/d for 7-days) and maintenance (5 g/d) doses of CrM (KA-H). Participants were asked to maintain their current training programs and record all workouts. Muscle biopsies from the vastus lateralis, fasting blood samples, body weight, DEXA determined body composition, and Wingate Anaerobic Capacity (WAC) tests were performed at 0, 7, and 28-days while 1RM strength tests were performed at 0 and 28-days. Data were analyzed by a repeated measures multivariate analysis of variance (MANOVA) and are presented as mean ± SD changes from baseline after 7 and 28-days, respectively. Muscle free creatine content obtained in a subgroup of 25 participants increased in all groups over time after 7 and 28-days, respectively, with no significant differences among groups. However, while no overall group differences were observed, pairwise comparison between the KA-L and CrM groups revealed that changes in muscle creatine content tended to be greater in the CrM group. Although some significant time effects were observed, no significant group x time interactions were observed in changes in body mass, fat free mass, fat mass, percent body fat, or total body water; bench press and leg press 1RM strength; WAC mean power, peak power, or total work; serum blood lipids, markers of catabolism and bone status, and serum electrolyte status; or, whole blood makers of lymphocytes and red cells. Serum creatinine levels increased in all groups with higher doses of creatine promoting greater increases in serum creatinine but the increases observed (0.1-0.2 mg/dL) were well within normal values for active individuals (i.e. <1.28 ± 0.2 mg/dL). Serum LDL was decreased to a greater degree following ingesting loading doses in the CrM group but returned to baseline during the maintenance phase. No side effects were reported. It was concluded that neither manufacturers recommended doses of KA (1.5 g/d) or KA with equivalent loading (20 g/d for 7-days) and maintenance doses (5 g/d for 21-days) of CrM promoted greater changes in muscle creatine content, body composition, strength, or anaerobic capacity than CrM (20 g/d for 7-days, 5 g/d for 21-days). There was no evidence that supplementing the diet with a buffered form of creatine resulted in fewer side effects than CrM. These findings do not support claims that consuming a buffered form of creatine is a more efficacious and/or safer form of creatine to consume than creatine monohydrate [12421].

Creatine is a hydrophilic polar molecule that consists of a negatively charged carboxyl group and a positively charged functional group. The hydrophilic nature of creatine limits its bioavailability. In an attempt to increase creatines bioavailability creatine has been esterified to reduce the hydrophilicity; this product is known as creatine ethyl ester. Manufacturers of creatine ethyl ester promote their product as being able to by-pass the creatine transporter due to improved sarcolemmal permeability toward creatine. The results of at least one study showed that ethyl ester was not as effective as CM to enhance serum and muscle creatine stores. Furthermore creatine ethyl ester offered no additional benefit for improving body composition, muscle mass, strength, and power. This research did not support the claims of the creatine ethyl ester manufacturers [12419].

Polyethylene glycol is a non-toxic, water-soluble polymer that is capable of enhancing the absorption of creatine and various other substances. Polyethylene glycol can be bound with CM to form polyethylene glycosylated creatine. There are that results seem to indicate that the addition of polyethylene glycol could increase the absorption efficiency of creatine but further research is needed before a definitive recommendation can be reached [12419].

**Polyethylene glycosylated creatine**
The purpose of this study was to examine the effects of 28 days of polyethylene glycosylated
creatine (PEG-creatine) supplementation (1.25 and 2.50 g/d) on anaerobic performance measures (vertical and broad jumps, 40-yard dash, 20-yard shuttle run, 3-cone drill), upper and lower body muscular strength and endurance (bench press and leg extension), and body composition. The study used a randomized, double-blind, placebo-controlled, parallel design. Seventy-seven adult males (mean age 22 years; body mass 82 kg) volunteered to participate and were randomly assigned to a placebo (n=23), 1.25 g/d of PEG-creatine (n=27) or 2.50 g/d of PEG-creatine (n=27) group. The subjects performed anaerobic performance measures, muscular strength (1RM) and endurance (80 % 1RM) tests for bench press and leg extension, and underwater weighing for the determination of body composition at Day 0 (baseline), Day 14, and Day 28. The results indicated there were significant improvements in vertical jump, 20-yard shuttle run, 3-cone drill, muscular endurance for bench press, and body mass for at least one of the PEG-creatine groups without changes for the placebo group. Thus, the present results demonstrated that PEG-creatine supplementation at 1.25 and/or 2.50 g/d had an ergogenic effect on lower body vertical power, agility, change-of-direction ability, upper body muscular endurance, and body mass [13690].

Creatine in combination with other supplements

Although creatine can be bought commercially as a standalone product it is often found in combination with other nutrients. A prime example is the combination of creatine with carbohydrate or protein and carbohydrate for augmenting creatine muscle retention mediated through an insulin response from the pancreas. The addition of 10 g of creatine to 75 g of dextrose, 2 g of taurine, vitamins and minerals, induced a change in cellular osmolarity which in addition to the expected increase in body mass, seems to produce an up regulation of large scale gene expression (mRNA content of genes and protein content of kinases involved in osmosensing and signal transduction, cytoskeleton remodelling, protein and glycogen synthesis regulation, satellite cell proliferation and differentiation, DNA replication and repair, RNA transcription control, and cell survival). Similar findings have also been reported for creatine monohydrate supplementation alone when combined with resistance training. A commercially available pre-workout formula comprised of 2.05 g of caffeine, taurine and glucuronolactone, 7.9 g of L-leucine, L-valine, L-arginine and L-glutamine, 5 g of di-creatine citrate and 2.5 g of beta-alanine mixed with 500 ml of water taken 10 minutes prior to exercise has been shown to enhance time to exhaustion during moderate intensity endurance exercise and to increase feelings of focus, energy and reduce subjective feelings of fatigue before and during endurance exercise due to a synergistic effect of the before mentioned ingredients. The role of creatine in this formulation is to provide a neuroprotective function by enhancing the energy metabolism in the brain tissue, promoting antioxidant activities, improving cerebral vasculature and protecting the brain from hyperosmotic shock by acting as a brain cell osmolyte. Creatine can provide other neuroprotective benefits through stabilisation of mitochondrial membranes, stimulation of glutamate uptake into synaptic vesicles and balance of intracellular calcium homeostasis [12419].

Safety and side effects of creatine

There have been a few reported renal health disorders associated with creatine supplementation. These are isolated reports in which recommended dosages are not followed or there is a history of previous health complaints, such as renal disease or those taking nephrotoxic medication aggravated by creatine supplementation. Specific studies into creatine supplementation, renal function and/or safety conclude that although creatine does slightly raise creatinine levels there is no progressive effect to cause negative consequences to renal function and health in already healthy individuals when proper dosage recommendations are followed. Urinary methylamine and formaldehyde have been shown to increase due to creatine supplementation of 20 g/d; this however did not bring the production
outside of normal healthy range and did not impact on kidney function. A retrospective study, that examined the effects of long lasting (0.8 to 4 years) CM supplementation on health markers and prescribed training benefits, suggested that there is no negative health effects (including muscle cramp or injuries) caused by long term CM consumption. In addition, despite many anecdotal claims, it appears that creatine supplementation would have positive influences on muscle cramps and dehydration. Creatine was found to increase total body water possibly by decreasing the risk of dehydration, reducing sweat rate, lowering core body temperature and exercising heart rate. Furthermore, creatine supplementation does not increase symptoms nor negatively affect hydration or thermoregulation status of athletes exercising in the heat. Additionally, CM ingestion has been shown to reduce the rate of perceived exertion when training in the heat. It is prudent to note that creatine supplementation has been shown to reduce the body’s endogenous production of creatine, however levels return to normal after a brief period of time when supplementation ceases. However, long term effects are unknown, therefore safety cannot be guaranteed. Whilst the long term effects of creatine supplementation remain unclear, no definitive certainty of either a negative or a positive effect upon the body has been determined for many health professionals and national agencies [12419].

**In muscle wasting**

It was aimed to investigate the possible role of creatine (CR) supplementation in counteracting dexamethasone-induced muscle wasting and insulin resistance in rats. Also, it was examined whether CR intake would modulate molecular pathways involved in muscle remodeling and insulin signaling. Animals were randomly divided into four groups: (1) dexamethasone (DEX); (2) control pair-fed (CON-PF); (3) dexamethasone plus CR (DEX-CR); and (4) CR pair-fed (CR-PF). Dexamethasone (5 mg/kg/day) and CR (5 g/kg/day) were given via drinking water for 7 days. Plantaris and extensor digitorum longus (EDL) muscles were removed for analysis. Plantaris and EDL muscle mass were significantly reduced in the DEX-CR and DEX groups when compared with the CON-PF and CR-PF groups. Dexamethasone significantly decreased phospho-Ser473-Akt protein levels compared to the CON-PF group and CR supplementation aggravated this response. Serum glucose was significantly increased in the DEX group when compared with the CON-PF group. CR supplementation significantly exacerbated hyperglycemia in the dexamethasone-treated animals. Dexamethasone reduced GLUT-4 translocation when compared with the CON-PF and CR-PF groups and this response was aggravated by CR supplementation. In conclusion, supplementation with CR resulted in increased insulin resistance and did not attenuate muscle wasting in rats treated with dexamethasone. Given the contrast with the results of human studies that have shown benefits of CR supplementation on muscle atrophy and insulin sensitivity, it was suggested caution when extrapolating this animal data to human subjects [12422].

**Effects of statins**

It was measured the serum levels of myoglobin, total creatine kinase (CK), and the CK myocardial (CK-MB), muscle (CK-MM), and brain (CK-BB) isoenzymes in 37 subjects treated with statins and 43 nonstatin-treated controls running the 2011 Boston Marathon. Venous blood samples were obtained the day before (PRE) and within 1 hour (FINISH) and 24 hours after (POST) the race. The hematocrit and hemoglobin values were used to adjust for changes in the plasma volume. The CK distribution was normalized using log transformation before analysis. The exercise-related increase in CK 24 hours after exercise, adjusted for changes in plasma volume, was greater in the statin users than in the controls. The increase in CK-MB 24 hours after exercise was also greater in the statin users than in the controls. However, the increases in muscle myoglobin did not differ at any point between the 2 groups.
Increases in CK at both FINISH and POST race measurements were directly related to age in the statin users but not in the controls suggesting that susceptibility to exercise-induced muscle injury with statins increases with age. In conclusion, the results show that statins increase exercise-related muscle injury [12423].

**Creatine monohydrate and conjugated linoleic acid**

Aging is associated with lower muscle mass and an increase in body fat. We examined whether creatine monohydrate (CrM) and conjugated linoleic acid (CLA) could enhance strength gains and improve body composition (i.e. increase fat-free mass (FFM); decrease body fat) following resistance exercise training in older adults (>65 y). Men (n=19) and women (n=20) completed six months of resistance exercise training with CrM (5g/d)+CLA (6g/d) or placebo with randomized, double blind, allocation. Outcomes included: strength and muscular endurance, functional tasks, body composition (DEXA scan), blood tests (lipids, liver function, CK, glucose, systemic inflammation markers (IL-6, C-reactive protein)), urinary markers of compliance (creatine/creatinine), oxidative stress (8-OH-2dG, 8-isoP) and bone resorption (Nu-telopeptides). Exercise training improved all measurements of functional capacity and strength, with greater improvement for the CrM+CLA group in most measurements of muscular endurance, isokinetic knee extension strength, FFM, and lower fat mass. Plasma creatinine, but not creatinine clearance, increased for CrM+CLA, with no changes in serum CK activity or liver function tests. Together, this data confirms that supervised resistance exercise training is safe and effective for increasing strength in older adults and that a combination of CrM and CLA can enhance some of the beneficial effects of training over a six-month period [07352].

**Creatine kinase**

Total creatine kinase (CK) levels depend on age, gender, race, muscle mass, physical activity and climatic condition. High levels of serum CK in apparently healthy subjects may be correlated with physical training status, as they depend on sarcomeric damage: strenuous exercise that damages skeletal muscle cells results in increased total serum CK. The highest post-exercise serum enzyme activities are found after prolonged exercise such as ultradistance marathon running or weight-bearing exercises and downhill running, which include eccentric muscular contractions. Total serum CK activity is markedly elevated for 24 h after the exercise bout and, when patients rest, it gradually returns to basal levels. Persistently increased serum CK levels are occasionally encountered in healthy individuals and are also markedly increased in the pre-clinical stages of muscle diseases. Some authors, studying subjects with high levels of CK at rest, observed that, years later, subjects developed muscle weakness and suggested that early myopathy may be asymptomatic. Others demonstrated that, in most of these patients, hyperCKemia probably does not imply disease. In many instances, the diagnosis is not formulated following routine examination with the patients at rest, as symptoms become manifest only after exercise. Some authors think that strength training seems to be safe for patients with myopathy, even though the evidence for routine exercise prescription is still insufficient. Others believe that, in these conditions, intense prolonged exercise may produce negative effects, as it does not induce the physiological muscle adaptations to physical training given the continuous loss of muscle proteins. High CK serum levels in athletes following absolute rest and without any further predisposing factors should prompt a full diagnostic workup with special regards to signs of muscle weakness or other simple signs that, in both athletes and sedentary subjects, are not always promptly evident. These signs may indicate subclinical muscle disease, which training loads may evidence through the onset of profound fatigue. It is probably safe to counsel athletes with suspected myopathy to continue to undertake physical activity at a
lower intensity, so as to prevent muscle damage from high intensity exercise and allow ample recovery to favour adequate recovery. CK values show great variability among individuals. Some athletes are low responders to physical training, with chronically low CK serum levels. Some athletes are high responders, with higher values of enzyme: the relationship among level of training, muscle size, fibre type and CK release after exercise should be investigated further. In addition, more details about hyperCKemia could come from the evaluation of the kinetics of CK after stress in healthy athletes with high levels of CK due to exercise, comparing the results with the ones obtained from athletes with persistent hyperCKemia at rest. Finally, it would be important to quantify the type of exercise more suited to athletes with myopathy and the intensity of exercise not dangerous for the progression of the pathology [07402].

Under such conditions the catalytic concentration of CK in the serum displays a far greater increase than the serum concentration of other muscle proteins. The serum concentration of CK peaks 1-4 days after exercise and remains elevated for several days. Thus, athletes participating in daily training have higher resting values than non-athletes, although this response to training is mitigated by the so-called repeated-bout effect. The serum concentration of creatine kinase (CK) is used widely as an index of skeletal muscle fibre damage in sport and exercise. Since athletes have higher CK values than non-athletes, comparing the values of athletes to the normal values established in non-athletes is pointless. The purpose of this study was to introduce reference intervals for CK in athletes. CK was assayed in serum samples from 483 male athletes and 245 female athletes, aged 7-44. Samples had been obtained throughout the training and competition period. For comparison, CK was also assayed in a smaller number of non-athletes. The reference intervals were 82-1083 U/L (37 degrees C) in male and 47-513 U/L in female athletes. The upper reference limits were twice the limits reported for moderately active non-athletes in the literature or calculated in the non-athletes in this study. The upper limits were up to six times higher than the limits reported for inactive individuals in the literature. When reference intervals were calculated specifically in male football (soccer) players and swimmers, a threefold difference in the upper reference limit was found (1492 vs 523 U/L, respectively), probably resulting from the different training and competition demands of the two sports. It was concluded that sport training and competition have profound effects on the reference intervals for serum CK. Introducing sport-specific reference intervals may help to avoid misinterpretation of high values and to optimise training [07353].

**Influence on glucose metabolism**

Findings have indicated that creatine supplementation may affect glucose metabolism. This study aimed to examine the effects of creatine supplementation, combined with aerobic training, on glucose tolerance in sedentary healthy male. Subjects (n=22) were randomly divided in two groups and were allocated to receive treatment with either creatine (CT) (approximately 10 g/day over three months) or placebo (PT) (dextrose). Administration of treatments was double blind. Both groups underwent moderate aerobic training. An oral glucose tolerance test (OGTT) was performed and both fasting plasma insulin and the homeostasis model assessment (HOMA) index were assessed at the start, and after four, eight and twelve weeks. CT demonstrated significant decrease in OGTT area under the curve compared to PT. There were no differences between groups or over time in fasting insulin or HOMA. The results suggest that creatine supplementation, combined with aerobic training, can improve glucose tolerance but does not affect insulin sensitivity, and may warrant further investigation with diabetic subjects [07354].

**Creatine and whey protein**
Studies that have attributed gains in lean body mass to dietary supplementation during resistance exercise (RE) training have not reported these changes alongside adaptations at the cellular and subcellular levels. Therefore, the purpose of this study was to examine the effects of two popular supplements – whey protein (WP) and creatine monohydrate (CrM) (both separately and in combination) – on body composition, muscle strength, fiber-specific hypertrophy (i.e. type I, IIa, IIx), and contractile protein accrual during RE training. In a double-blind randomized protocol, resistance-trained males were matched for strength and placed into one of four groups: creatine/carbohydrate (CrCHO), creatine/whey protein (CrWP), WP only, or carbohydrate only (CHO) (1.5 g/kg body weight per day). All assessments were completed the week before and after an 11-week structured, supervised RE program. Assessments included strength (1RM, three exercises), body composition (DEXA), and vastus lateralis muscle biopsies for determination of muscle fiber type (I, IIa, IIx), cross-sectional area (CSA), contractile protein, and creatine (Cr) content. Supplementation with CrCHO, WP, and CrWP resulted in significantly greater 1RM strength improvements (three of three assessments) and muscle hypertrophy compared with CHO. Up to 76 percent of the strength improvements in the squat could be attributed to hypertrophy of muscle involved in this exercise. However, the hypertrophy responses within these groups varied at the three levels assessed (i.e. changes in lean mass, fiber-specific hypertrophy, and contractile protein content). Although WP and/or CrM seem to promote greater strength gains and muscle morphology during RE training, the hypertrophy responses within the groups varied. These differences in skeletal muscle morphology may have important implications for various populations and, therefore, warrant further investigation [07355].

Cognitive effects of creatine

The effect of creatine supplementation and sleep deprivation, with intermittent moderate-intensity exercise, on cognitive and psychomotor performance, mood state, effort and salivary concentrations of cortisol and melatonin were examined. Subjects were divided into a creatine supplementation group and a placebo group. They took 5 g of creatine monohydrate or a placebo, dependent on their group, four times a day for 7 days immediately prior to the experiment. They undertook tests examining central executive functioning, short-term memory, choice reaction time, balance, mood state and effort at baseline and following 18-, 24- and 36-h sleep deprivation, with moderate intermittent exercise. Saliva samples were taken prior to each set of tests. A group x time analysis of covariance, with baseline performance the covariate, showed that the creatine group performed significantly better than the placebo group on the central executive task but only at 36 h. The creatine group demonstrated a significant linear improvement in performance of the central executive task throughout the experiment, while the placebo group showed no significant effects. There were no significant differences between the groups for any of the other variables. A significant main effect of time was found for the balance test with a linear improvement being registered. Cortisol concentrations on Day 1 were significantly higher than on Day 2. Mood significantly deteriorated up to 24 h with no change from 24 to 36 h. Effort at baseline was significantly lower than in the other conditions. It was concluded that, during sleep deprivation with moderate-intensity exercise, creatine supplementation only affects performance of complex central executive tasks [07356].

Use in women

Creatine is an amino acid compound produced by the liver, kidneys, and pancreas. It is also found in meat and fish. Ninety-five percent of the body’s creatine stores are in muscle.
Creatine provides a fast, but short, energy burst. Energy stored in adenosine triphosphate is used in the first second of activity, while energy stored in creatine phosphate is used in the next 10 seconds. Creatine supplementation is used to increase myocyte phosphocreatine; it has been found to increase muscle phosphocreatine by 20 percent [07086].

**Prevalence**

Many surveys have found creatine to be the most commonly used supplement. Creatine use is consistently higher in men compared with women. The 2001 NCAA study of substance use habits of college student-athletes showed that 26 percent of athletes had tried creatine at least once in the past year. The same survey repeated in 2005 found an increased prevalence of 40 percent athletes who had tried creatine. The study did not differentiate between women and men. A survey given to high preadolescent and adolescent boys and girls found that 0.4 percent of girls had tried creatine in the past year, compared with 4.3 percent of boys. Further, a study evaluating high school athletes found that significantly more boys than girls had used creatine (21 % vs 3 %) [07086].

**Effects**

Creatine supplementation has been tested in athletes participating in both sprint and endurance activities. Studies have shown benefits in short, repetitive, and high-intensity activities with brief recovery periods. A meta-analysis by Branch supported claims of previous studies that creatine can improve performance during high-intensity exercise lasting shorter than 30 seconds. Smaller benefits were seen in longer durations of 30 to 150 seconds. A benefit has also been found in number of maximum repetitions in weight lifters, power output and force in cyclists, and short-burst sprints in swimming. Evidence for improvement in track and field events has been inconclusive. Several studies have been conducted to analyze creatine’s effect in women. An article compared the effect on size in men and women. Studies in women had a larger relative improvement from baseline compared with men (14 ± 3.8 % vs 5.5 ± 0.7%). A double-blind, randomized, placebo-controlled trial published in 2002 examined creatine’s effect in elite women soccer players under conditions simulating game or practice. They found a significant increase in body mass resulting from creatine supplementation for 6 days, an average increase of 0.8 kg. They also found improved performance in repeated sprint and agility tasks that they calculated might significantly impact game performance [07086].

**Side effects**

However, creatine-related adverse effects include weight gain, gastrointestinal discomfort, and muscle cramps. There have been case reports of renal compromise, but these have been unsubstantiated [07086].

**Effect on myostatin**

Myostatin is a catabolic regulator of skeletal muscle mass. The purpose of one study was to determine the effect of resistance training for 8 weeks in conjunction with creatine supplementation on muscle strength, lean body mass, and serum levels of myostatin and growth and differentiation factor-associated serum protein-1 (GASP-1). In a double-blinded design 27 healthy male subjects were assigned to control (CON), resistance training plus placebo (RT+PL) and resistance training+creatine supplementation (RT+CR) groups. The protocol consisted of 3 days per week of training for 8 weeks, each session including three sets of 8-10 repetitions at 60-70 percent of 1 RM for whole-body exercise. Blood sampling, muscular strength testing and body composition analysis (full body DEXA) were performed at 0, 4th and 8th weeks. Myostatin and GASP-1 was measured. Resistance training caused significant decrease in serum levels of myostatin and increase in that of GASP-1. Creatine supplementation in conjunction with resistance training lead to greater decreases in serum
myostatin, but had not additional effect on GASP-1. The effects of resistance training on serum levels of myostatin and GASP-1, may explain the increased muscle mass that is amplified by creatine supplementation [10244].

**Children**

The safety of creatine in the pediatric and adolescent population is lacking appropriate research. In addition, studies that have shown benefit in resistance training have not included subjects less than 18 years of age. Therefore creatine supplementation in athletes less than 18 years old needs further research before it should be recommended [13682].

**Creatine purity**

Creatine is an unregulated dietary supplement that is readily available to consumers and legal for use in athletic training. The supplement is not banned by The National Collegiate Athletic Association (NCAA) or by the International Olympic Committee (IOC). The NCAA does prohibit individual institutions from distributing creatine supplements. The IOC ruled to allow creatine given the substance is readily found in animal proteins. However creatine is a dietary supplement that falls under the Dietary Supplement Health and Education Act, and is not directly regulated by the Food and Drug Administration. In general, those who participate in athletics under the supervision of institutions such as the NCAA and IOC should use caution when using any dietary supplement due to reported contamination of creatine [13682].

**In taekwondo**

Taekwondo (TKD) is a combat sport, which has also been proposed as a fitness program, with a strong anaerobic component. Creatine (Cr) supplementation is used to improve both anaerobic exercise performance and body composition. Therefore, Cr supplementation could be beneficial in TKD. To determine the effect of Cr supplementation (50 mg/kg body wt) on body composition, anaerobic power and blood chemistry in young male TKD practitioners 10 male TKD practitioners (age 20 ± 2 years) participated in a placebo-controlled, double blind, crossover study. Body composition (DEXA), anaerobic power (Wingate Test), blood lactate and blood chemistry were measured before and after supplementation. Differences between data before and after supplementation were calculated for each treatment (Cr and Placebo) and were compared using the Wilcoxon signed-rank test. Fat mass (kg) decreased after placebo and increased following Cr intake. Serum triglyceride concentration (mg/mL) increased after Cr and decrease with placebo. No changes were found in others parameters. Thus, Cr supplementation may increase fat mass and serum triglycerides concentration in young male TKD practitioners without improvement in anaerobic power. Cr supplementation appears to be safe, but athletes should be careful when they want to loss fat [13691].

**Effect on tennis**

Creatine supplementation is popular among tennis players but it is not clear whether it actually enhances tennis performance. To examine the effects of creatine supplementation on tennis specific performance indices. In a randomised, double blind design, 36 competitive male tennis players (24 creatine, mean age 23 years; 12 placebo, 23 years) were tested at baseline, after six days of creatine loading, and after a maintenance phase of four weeks (14 creatine, 10 placebo). Serving velocity (10 serves), forehand and backhand velocity (three series of 5x8 strokes), arm and leg strength (bench press and leg press), and intermittent running speed (three series of five 20 metre sprints) were measured. Compared with
placebo, neither six days nor five weeks of creatine supplementation had a significant effect on serving velocity; forehand velocity, or backhand velocity. There was also no significant effect of creatine supplementation on repetitive sprint power after 5, 10, and 20 metres, or in the strength of the upper and lower extremities. It was concluded that creatine supplementation is not effective in improving selected factors of tennis specific performance and should not be recommended to tennis players [06270].

*Effect on ice-hockey*

Creatine monohydrate supplementation is beneficial for enhancing high-intensity exercise performance, especially activities that involve repeated sprints. Creatine monohydrate supplementation is common in ice-hockey players. The purpose of one study was to determine the effect of creatine monohydrate supplementation on sprint skating performance in Junior B and collegiate ice-hockey players. Seventeen ice-hockey players were randomly assigned to receive creatine (0.3 g/kg body mass/day for 5 days) or placebo. Before and after supplementation players performed repeated sprints to exhaustion on a skating treadmill (repeated 10-s sprints; 30-s rest between sprints) while blood lactate was simultaneously collected. The time to exhaustion on the treadmill test was calculated as total amount of time, including partial intervals, before the player reached exhaustion. Players were also tested for peak torque and average power during knee extension/flexion (3 sets of 10 reps; 60-s rest between sets) on an isokinetic dynamometer at 60 degrees/s. The change in time to exhaustion from before to after supplementation averaged 21 ± 7 s in the creatine group and 22 ± 13 s in the placebo group, with no differences between groups. Likewise, there were no differences between groups for changes in isokinetic peak torque and average power. There were no differences between groups over time for blood lactate changes during the repeated sprints on the treadmill. It was concluded that creatine was not effective for improving performance in these ice-hockey players [06271].

*Effect on sprint running*

The aim of one study was to examine the effects of short-term creatine monohydrate supplementation on multiple sprint running performance. Using a double-blind research design, 42 physically active men completed a series of 3 indoor multiple sprint running trials (15 x 30 m repeated at 35-second intervals). After the first 2 trials (familiarization and baseline), subjects were matched for fatigue score before being randomly assigned to 5 days of either creatine (4/day x 5 g creatine monohydrate + 1 g maltodextrin) or placebo (4/day x 6 g maltodextrin) supplementation. Sprint times were recorded via twin-beam photocells, and earlobe blood samples were drawn to evaluate posttest lactate concentrations. Relative to placebo, creatine supplementation resulted in a 0.7 kg increase in body mass and a 0.4 percent reduction in body fat. There were no significant between-group differences in multiple sprint measures of fastest time, mean time, fatigue, or posttest blood lactate concentration. Despite widespread use as an ergogenic aid in sport, the results of the study suggest that creatine monohydrate supplementation conveys no benefit to multiple sprint running performance [06272].

*During sleep deprivation*

The effect of creatine supplementation and sleep deprivation, with intermittent moderate-intensity exercise, on cognitive and psychomotor performance, mood state, effort and salivary concentrations of cortisol and melatonin were examined. Subjects were divided into a creatine supplementation group and a placebo group. They took 5 g of creatine monohydrate or a placebo, dependent on their group, four times a day for 7 days
immediately prior to the experiment. They undertook tests examining central executive functioning, short-term memory, choice reaction time, balance, mood state and effort at baseline and following 18-, 24- and 36-h sleep deprivation, with moderate intermittent exercise. Saliva samples were taken prior to each set of tests. A group x time analysis of covariance, with baseline performance the covariate, showed that the creatine group performed significantly better than the placebo group on the central executive task but only at 36 h. The creatine group demonstrated a significant linear improvement in performance of the central executive task throughout the experiment, while the placebo group showed no significant effects. There were no significant differences between the groups for any of the other variables. A significant main effect of time was found for the balance test with a linear improvement being registered. Cortisol concentrations on Day 1 were significantly higher than on Day 2. Mood significantly deteriorated up to 24 h with no change from 24 to 36 h. Effort at baseline was significantly lower than in the other conditions. It was concluded that, during sleep deprivation with moderate-intensity exercise, creatine supplementation only affects performance of complex central executive tasks [06273].

Side effects

Allegations about side effects of creatine supplementation by athletes have been published in the popular media and scientific publications. One of the purported effects of oral creatine supplementation is increased muscle mass. A review of the literature reveals a 1.0 to 2.3 percent increase in body mass, which is attributed to fat-free mass and, more specifically, to skeletal-muscle mass. Although it is unlikely that water retention can completely explain these changes, increase in muscle-protein synthesis has never been observed after creatine supplementation. Indirect evidence based on mRNA analyses suggests that transcription of certain genes is enhanced. Although the effect of creatine on muscle-protein synthesis seems irrefutable according to advertising, this allegation remains under debate in the scientific literature. The kidneys appear to maintain their functionality in healthy subjects who supplement with creatine, even over several months. The authors, however, thought that creatine supplementation should not be used by an individual with preexisting renal disease and that risk should be evaluated before and during any supplementation period. Even if there is a slight increase in mutagenic agents (methyamine and formaldehyde) in urine after a heavy load of creatine (20 g/day), their excretion remains within a normal range. No data are currently available regarding the potential production of heterocyclic amines with creatine supplementation. In summary, the major risk for health is probably associated with the purity of commercially available creatine [06274].

The main aim of one study was to investigate the effects of two different creatine-supplementation protocols on incidence of gastrointestinal (GI) distress in top-level athletes. Data were collected from 59 top-level male soccer players who were allocated in a double-blind design to three randomly assigned trials: ingesting creatine supplement (2 x 5-g doses or 1 x 10-g dose) or placebo for 28 days. In order to assess potential side effects of the supplementation regimen, all subjects were instructed to report any adverse effects of supplementation on their GI system. Survey questions covered perceived side effects on GI system linked with creatine supplementation. In all three treatment groups, the most frequent GI complaints were diarrhoea (39 %), stomach upset (24 %), and belching (17 %). It was not found a significant difference between incidence of GI distress symptoms between 5 gram doses and the placebo group after the survey. Yet, significant differences were found for incidence of diarrhoea between the 5 and 10 gram groups (29 % vs 56 %, respectively). Moreover, diarrhoea was significantly more frequent in the 10 gram group as compared with the placebo group (56 % vs 35 %). There is no reason to believe that short-term oral creatine supplementation for 28 days has any detrimental effect on the GI tract if taken in a recommended amount (10 g per day in two equal doses). The risk of diarrhoea may be
increased, however, following intake of 10 grams of creatine per single serving [08413].

**Safe levels**
Creatine monohydrate (creatine) has become an increasingly popular ingredient in dietary supplements, especially sports nutrition products. A large body of human and animal research suggests that creatine does have a consistent ergogenic effect, particularly with exercises or activities requiring high intensity short bursts of energy. Human data are primarily derived from three types of studies: acute studies, involving high doses (20 g/d) with short duration (<1 week), chronic studies involving lower doses (3-5 g/d) and longer duration (1 year), or a combination of both. Systematic evaluation of the research designs and data do not provide a basis for risk assessment and the usual safe Upper Level of Intake (UL) derived from it unless the newer methods described as the Observed Safe Level (OSL) or Highest Observed Intake (HOI) are utilized. The OSL risk assessment method indicates that the evidence of safety is strong at intakes up to 5 g/d for chronic supplementation, and this level is identified as the OSL. Although much higher levels have been tested under acute conditions without adverse effects and may be safe, the data for intakes above 5 g/d are not sufficient for a confident conclusion of long-term safety [06275].

**Creatine and bicarbonate**
Creatine and sodium bicarbonate supplementation independently increase exercise performance, but it remains unclear whether combining these 2 supplements is more beneficial on exercise performance. The purpose of one study was to evaluate the impact of combining creatine monohydrate and sodium bicarbonate supplementation on exercise performance. Thirteen healthy, trained men (21) completed 3 conditions in a double-blinded, crossover fashion: (a) Placebo (Pl; 20 g maltodextrin), (b) Creatine (Cr; 20 g), and (c) Creatine plus sodium bicarbonate (Cr + Sb). Each condition consisted of supplementation for 2 days followed by a 3-week washout. Peak power, mean power, relative peak power, and bicarbonate concentrations were assessed during six 10-second repeated Wingate sprint tests on a cycle ergometer with a 60-second rest period between each sprint. Compared with Pl, relative peak power was significantly higher in Cr (4 %) and Cr + Sb (7 %). Relative peak power was significantly lower in sprints 4-6, compared with that in sprint 1, in both Pl and Cr. However, in Cr + Sb, sprint 6 was the only sprint significantly lower compared with sprint 1. Pre-Wingate bicarbonate concentrations were significantly higher in Cr + Sb (10 %), compared with in Pl and Cr, and mean concentrations remained higher after sprint 6, although not significantly. Combining creatine and sodium bicarbonate supplementation increased peak and mean power and had the greatest attenuation of decline in relative peak power over the 6 repeated sprints. These data suggest that combining these 2 supplements may be advantageous for athletes participating in high-intensity, intermittent exercise [13693].

**Experimental**
Thirty-six male rats were used; divided into 6 groups (n=6): saline; creatine (Cr); eccentric exercise (EE) plus saline 24 h (saline + 24 h); eccentric exercise plus Cr 24 h (Cr + 24 h); eccentric exercise plus saline 48 h (saline + 48 h); and eccentric exercise plus Cr 48 h (Cr + 48 h). Cr supplementation was administered as a solution of 300 mg/kg body weight/day in 1 mL water, for two weeks, before the eccentric exercise. The animals were submitted to one downhill run session at 1.0 km/h until exhaustion. Twenty-four and forty-eight hours after the exercise, the animals were killed, and the quadriceps were removed. Creatine kinase levels, superoxide production, thiobarbituric acid reactive substances (TBARS) level, carbonyl content, total thiol content, superoxide dismutase, catalase, glutathione peroxidase,
interleukin-1beta (IL-1beta), nuclear factor kappa B (NF-kb), and tumour necrosis factor (TNF) were analysed. Cr supplementation neither decreases Cr kinase, superoxide production, lipoperoxidation, carbonylation, total thiol, IL-1beta, NF-kb, or TNF nor alters the enzyme activity of superoxide dismutase, catalase, and glutathione peroxides in relation to the saline group, respectively. There are positive correlations between Cr kinase and TBARS and TNF-alpha 48 hours after eccentric exercise. The present study suggests that Cr supplementation does not decrease oxidative stress and inflammation after eccentric contraction [13692].

**Carnitine**

The purpose of one study was to investigate the effects of acute L-Carnitine intake on badminton players' metabolic and blood lactate values. A total of 16 Turkish national badminton players (8 male, 8 female) were voluntarily participated. No significant differences were found between first (without L-Carnitine intake) and second (with L-Carnitine intake) measurements of female participants as regards to all measured parameters. There was a significant difference in EMHR (exercise maximum heart rate) of males between two measurements. However the differences in other parameters were not significant. Anaerobic threshold values of female subjects were not significant difference. Respiratory exchange ratio of males was significantly different at anaerobic threshold. These results show that L-carnitine intake one hour prior to the exercise has no effect on the metabolic and blood lactate values of badminton players [08414].

Ninety-five per cent of the body’s carnitine (3-hydroxy-4-N,N,N-trimethylaminobutyric acid; C7H15NO3) store (about 25 g) exists within skeletal muscle where it plays a central role in fat and carbohydrate oxidation, particularly during exercise. The recommended upper limit of L-carnitine supplementation is 2 g/day, but no adverse effects were reported following feeding up to 6 g/day for 1 year. The main food source of carnitine is meat. Non-vegetarians ingest 1 mg/kg of dietary carnitine per day, whereas strict vegetarians ingest around 0.01 mg/kg. Research has been directed towards supplementing dietary L-carnitine to improve exercise performance. However, neither oral (2-6 g/day for 1 day to 4 months) nor intravenous (up to 65 mg/kg) L-carnitine administration has been found to alter fuel metabolism during exercise or, more importantly, increase muscle carnitine content in humans. Despite this, L-carnitine feeding as a tool to promote apparent fat loss remains the foundation of a multimillion dollar dietary supplement industry in the present day. More recently, intravenous infusion of L-carnitine along with insulin (to stimulate Na+ dependent muscle carnitine transport) has been found to increase muscle total carnitine content by about 15 percent in healthy volunteers, and to have a measurable effect on muscle fuel metabolism at rest. This stimulatory effect on muscle carnitine accumulation occurred in the physiological range for serum insulin concentration (50-90 mU/l). Furthermore, feeding L-carnitine (3 g/d) together with carbohydrate (500 ml solution containing 94 g of simple sugars) for 2 weeks increased whole-body carnitine retention compared with ingestion of L-carnitine alone. However, because orally administered L-carnitine has a poor bioavailability (<15 % for a 2-6 g dose), it is likely that this supplementation regimen would take about 100 days to increase muscle carnitine content by about 10 percent. Further research is required to determine the impact that an insulin-mediated increase in muscle carnitine content has on muscle fuel metabolism and performance during exercise [09344].

Carnosine is found in high concentrations in skeletal muscles, where it is involved in several physiological functions. The muscle carnosine content measured within a population can vary by a factor 4. The aim of one study was to further characterize suggested determinants
of the muscle carnosine content (diet, gender and age) and to identify new determinants (plasma carnosinase activity and testosterone). It was investigated a group of 149 healthy subjects, which consisted of 94 men (12 vegetarians) and 55 women. Muscle carnosine was quantified in M. soleus, gastrocnemius and tibialis anterior using magnetic resonance proton spectroscopy and blood samples were collected to determine CNDP1 genotype, plasma carnosinase activity and testosterone concentration. Compared to women, men have 36, 28 and 82 percent higher carnosine concentrations in M. soleus, gastrocnemius and tibialis anterior muscle, respectively, whereas circulating testosterone concentrations were unrelated to muscle carnosine levels in healthy men. The carnosine content of the M. soleus is negatively related to the subjects’ age. Vegetarians have a lower carnosine content of 26 percent in gastrocnemius compared to omnivores. In contrast, there is no difference in muscle carnosine content between omnivores with a high or low ingestion of beta-alanine. Muscle carnosine levels are not related to the polymorphism of the CNDP1 gene or to the enzymatic activity of the plasma carnosinase. In conclusion, neither CNDP1 genotype nor the normal variation in circulating testosterone levels affects the muscular carnosine content, whereas vegetarianism, female gender and increasing age are the factors associated with reduced muscle carnosine stores [10410].

Twenty nonvegetarian active males were pair-matched and randomly assigned to receive 2 g of L-carnitine L-tartrate (LC) or placebo per day for 2 weeks. Participants exercised for 90 min at 70 percent VO₂ max after 2 days of a prescribed diet (13.6 ± 1.6 MJ, 57 % carbohydrate, 15 % protein, 26 % fat, 2 % alcohol) before and after supplementation. Results indicated no change in carbohydrate oxidation, nitrogen excretion, branched-chain amino acid oxidation, or plasma urea during exercise between the baseline and end of supplementation in either group. After 2 weeks of carnitine supplementation the plasma ammonia response to exercise tended to be suppressed, with no change in the placebo group. The data indicate that 2 weeks of carnitine supplementation does not affect fat, carbohydrate, and protein contribution to metabolism during prolonged moderate-intensity cycling exercise. The tendency toward suppressed ammonia accumulation, however, indicates that oral carnitine supplementation might have the potential to reduce the metabolic stress of exercise or alter ammonia production or removal, which warrants further investigation [09349].

Infusion of carnitine has been observed to increase non-oxidative glucose disposal in several studies, but the effect of oral carnitine on glucose disposal in non-diabetic lean versus overweight/obese humans has not been examined. This study examined the effects of 14 days of L-carnitine L-tartrate oral supplementation (LC) on blood glucose, insulin, non-esterified fatty acids (NEFA) and GLP-1 responses to an oral glucose tolerance test (OGTT). Sixteen male participants were recruited (lean, n=8, and overweight/obese, n=8). After completing a submaximal predictive exercise test, participants were asked to attend three experimental sessions. These three visits were conducted in the morning to obtain fasting blood samples and to conduct 2 h OGTTs. The first visit was a familiarisation trial and the final two visits were conducted 2 weeks apart following 14 days of ingestion of placebo (PL, 3 g glucose/day) and then LC (3 g LC/day) ingested as two capsules 3x/day with meals. On each visit, blood was drawn at rest, at intervals during the OGTT for analysis of glucose, insulin, non-esterified fatty acids (NEFA) and total glucagon-like peptide-1 (GLP-1). Data obtained were used for determination of usual insulin sensitivity indices (HOMA-IR, AUC glucose, AUC insulin, 1st phase and 2nd phase β-cell function, estimated insulin sensitivity index and estimated metabolic clearance rate). Data were analysed using RMANOVA and post hoc comparisons where appropriate. There was a significant difference between groups for body mass, percent fat and BMI with no significant difference in age and height. Mean (SEM) plasma glucose concentration at 30 min was significantly lower in the lean group on the LC trial compared with PL. Conversely, plasma glucose concentration was not different at
30 min, but was significantly higher at 90 min in the overweight/obese group on the LC trial LC; mmol/L). Estimated first phase and second phase beta-cell function both tended to be greater following LC in the lean group only. No effects of LC were observed on NEFA or total GLP-1 response to OGGT. It is concluded that LC supplementation induces changes in blood glucose handling/disposal during an OGTT, which is not influenced by GLP-1. The glucose handling/disposal response to oral LC is different between lean and overweight/obese suggesting that further investigation is required. LC effects on gastric emptying and/or direct ‘insulin-like’ actions on tissues should be examined in larger samples of overweight/obese and lean participants, respectively [11401].

 Ninety-five percent of the body carnitine pool resides in skeletal muscle where it plays a vital role in fuel metabolism. However, vegetarians obtain negligible amounts of carnitine from their diet. It was tested the hypothesis that muscle carnitine uptake is elevated in vegetarians compared with that in nonvegetarians to maintain a normal tissue carnitine content. Forty-one young (aged 22 years) vegetarian and nonvegetarian volunteers participated in 2 studies. The first study consisted of a 5-h intravenous infusion of L-carnitine while circulating insulin was maintained at a physiologically high concentration (170 mU/L; to stimulate muscle carnitine uptake) or at a fasting concentration (6 mU/L). The second study consisted of oral ingestion of 3 g L-carnitine. Basal plasma total carnitine concentration, 24-h urinary total carnitine excretion, muscle total carnitine content, and muscle carnitine transporter (organic cation transporter 2, OCTN2) messenger RNA and protein expressions were 16 percent, 58 percent, 17 percent, 33 percent and 37 percent lower, respectively, in vegetarian volunteers. However, although nonvegetarians showed a 15 percent increase in muscle TC during L-carnitine infusion with hyperinsulinemia, L-carnitine infusion in the presence or absence of hyperinsulinemia had no effect on muscle TC content in vegetarians. Nevertheless, 24-h urinary total carnitine excretion was 55 percent less in vegetarians after L-carnitine ingestion. It was concluded that vegetarians have a lower muscle total carnitine and reduced capacity to transport carnitine into muscle than do nonvegetarians, possibly because of reduced muscle OCTN2 content. Thus, the greater whole-body carnitine retention observed after a single dose of L-carnitine in vegetarians was not attributable to increased muscle carnitine storage [11402].

The effects of 15 d of supplementation with L-carnitine L-tartrate (LC) on metabolic responses to graded-intensity exercise under conditions of altered substrate availability were examined. Fifteen endurance-trained male athletes undertook exercise trials after a 2-d high-carbohydrate diet (60 % CHO, 25 % fat) at baseline (D0), on Day 14 (D14), and after a single day of high fat intake (15 % CHO, 70 % fat) on Day 15 (D15) in a double-blind, placebo-controlled, pair-matched design. Treatment consisted of 3 g LC (2 g L-carnitine/d; n=8) or placebo (P, n=7) for 15 d. Exercise trials consisted of 80 min of continuous cycling comprising 20-min periods at each of 20, 40, 60, and 80 percent of VO2peak. There was no significant difference between whole-body rates of CHO and fat oxidation at any workload between D0 and D14 trials for either the P or LC group. Both groups displayed increased fat and reduced carbohydrate oxidation between the D14 and D15 trials. During the D15 trial, heart rate and blood glucose concentration were lower during exercise in the LC group than in P. These responses suggest that LC may induce subtle changes in substrate handling in metabolically active tissues when fatty-acid availability is increased, but it does not affect whole-body substrate utilization during short-duration exercise at the intensities studied [11534].

The enhancement of fat oxidation during exercise is an aim for both recreational exercising individuals and endurance athletes. Nutritional status may explain a large part of the variation in maximal rates of fat oxidation during exercise. One review revealed novel insights into nutritional manipulation of substrate selection during exercise, explaining putative
mechanisms of action and evaluating the current evidence. Lowering the glycaemic index of the pre-exercise meal can enhance lipid utilisation by up to 100 percent through reduced insulin concentrations, although its application may be restricted to specific training sessions rather than competition. Chronic effects of dietary glycaemic index are less clear and warrant future study before firm recommendations can be made. A flurry of recent advances has overthrown the conventional view of l-carnitine supplementation, with skeletal muscle uptake possible under certain dietary conditions and providing a strategy to influence energy metabolism in an exercise intensity-dependent manner. Use of non-carbohydrate nutrients to stimulate muscle l-carnitine uptake may prove more beneficial for optimising lipid utilisation, but this requires more research. Studies investigating fish oil supplementation on fat oxidation during exercise are conflicting. In spite of some strong putative mechanisms, the only crossover trial showed no significant effect on lipid use during exercise. Carnitine may increase NEFA availability although it is not clear whether these effects occur. Carnitine and caffeine can increase NEFA availability under certain circumstances which could theoretically enhance fat oxidation, yet strong experimental evidence for this effect during exercise is lacking. Co-administration of nutrients to maximise their effectiveness needs further investigation.

The effects of 15 days of supplementation with L-carnitine L-tartrate (LC) on metabolic responses to graded-intensity exercise under conditions of altered substrate availability were examined. Fifteen endurance-trained male athletes undertook exercise trials after a 2-d high-carbohydrate diet (60 % CHO, 25 % fat) at baseline (D0), on day 14 (D14), and after a single day of high fat intake (15 % CHO, 70 % fat) on day 15 (D15) in a double-blind, placebo-controlled, pair-matched design. Treatment consisted of 3 g LC (2 g L-carnitine/d; n=8) or placebo (P, n=7) for 15 days. Exercise trials consisted of 80 min of continuous cycling comprising 20-min periods at each of 20, 40, 60, and 80 percent VO2peak. There was no significant difference between whole-body rates of CHO and fat oxidation at any workload between D0 and D14 trials for either the P or LC group. Both groups displayed increased fat and reduced carbohydrate oxidation between the D14 and D15 trials. During the D15 trial, heart rate and blood glucose concentration were lower during exercise in the LC group than in P. These responses suggest that LC may induce subtle changes in substrate handling in metabolically active tissues when fatty-acid availability is increased, but it does not affect whole-body substrate utilization during short-duration exercise at the intensities studied.

This study examined the effect of acute L-carnitine loading on the endurance performance of footballers. Measurements were performed on 26 candidate professional footballers who volunteered to take part in the study. Athletes were given a glass of fruit juice one hour before applying L-carnitine with the double blind method. Then 12 participants were given 3 gr of L-carnitine (LK-3) and the remaining 14 were given 4 gr (LK-4). Athletes began the exercise test at a running speed of 8 km/h, and then continued at 10km/h. The speed was increased 1 km/h every three minutes and the test continued until the subject chose to quit. Heart rate was registered using a portable telemetric heart rate monitor during the test. Blood samples were taken from the eardrums of the footballers both before the test and before the speed increase (during the 1-minute intervals), and the lactate (La) concentration was measured enzymatically. The test was repeated after one week as a group of placebos (P-3 and P-4). The result showed that the running speeds corresponding to specific La concentrations were increased and La and heart rate responses to the running speeds were decreased in both supplemented groups compared to placebos. A significant reduction in heart rate was found in LK-4 and P-4. When the Borg responses to the running speeds were analyzed, a significant difference was found in both supplemented groups. The results show that 3 or 4 gr L-carnitine taken before physical exercise prolonged exhaustion.
A huge range of supplements claims to enhance sports performance by affecting body composition – either by increasing muscle mass and/or reducing body fat. Supplements in the broad “weight loss” category include caffeine, l-carnitine, chromium picolinate, CLA (conjugated linoleic acid), dairy/calcium supplements, HMB (beta-hydroxy-beta-methylbutyrate), hydroxycut, leucine, phenylalanine, protein supplements and tyrosine. L-carnitine is synthesised from the amino acids lysine and methionine, and is found naturally in the human diet, particularly in red meat and dairy products. It has been suggested that L-carnitine supplementation can increase fatty acid transport into mitochondria, leading to an increase in fatty acid oxidation, and hence the proposed potential benefit for weight management [13678].

**Effect on post-resistance-exercise (RE)**

The purpose of one investigation was to determine the effects of 3 weeks of L-carnitine L-tartrate (LCLT) supplementation and post-resistance-exercise (RE) feeding on hormonal and androgen receptor (AR) responses. Ten resistance-trained men (mean age, 22) supplemented with LCLT (equivalent to 2 g of L-carnitine per day) or placebo (PL) for 21 d, provided muscle biopsies for AR determinations, then performed two RE protocols: one followed by water intake, and one followed by feeding (8 kcal.kg body mass, consisting of 56 % carbohydrate, 16 % protein, and 28 % fat). RE protocols were randomized and included serial blood draws and a 1-h post-RE biopsy. After a 7-d washout period, subjects crossed over, and all experimental procedures were repeated. LCLT supplementation upregulated preexercise AR content compared with PL. RE increased significantly AR content compared with pre-RE values in the PL trial only. Post-RE feeding significantly increased AR content compared with baseline and water trials for both LCLT and PL. Serum total testosterone concentrations were suppressed significantly during feeding trials with respect to corresponding water and pre-RE values. Luteinizing hormone demonstrated subtle, yet significant changes in response to feeding and LCLT. In summary, the data demonstrated that feeding after RE increased AR content, which may result in increased testosterone uptake, and thus enhanced luteinizing hormone secretion via feedback mechanisms; and LCLT supplementation upregulated AR content, which may promote recovery from RE [06281].

**Side effects**

Carnitine is a conditionally essential amino acid-like compound involved in the transport of long-chain fatty acids into the mitochondria during the beta-oxidation process. Carnitine has become an increasingly popular ingredient in dietary supplements, especially weight loss and some sports nutrition products. A number of clinical trials have been conducted examining the effect of carnitine supplementation on weight loss and energy balance. Regarding safety, systematic evaluation of the research designs and data do not provide a basis for risk assessment and the usual safe upper level of intake (UL) derived from it unless the newer methods described as the observed safe level (OSL) or highest observed intake (HOI) are utilized. The OSL risk assessment method indicates that the evidence of safety is strong at intakes up to 2000mg/day L-carnitine equivalents for chronic supplementation, and this level is identified as the OSL. Although much higher levels have been tested without adverse effects and may be safe, the data for intakes above 2000mg/day are not sufficient for a confident conclusion of long-term safety [06282].

Intestinal microbiota metabolism of choline and phosphatidylcholine produces trimethylamine (TMA), which is further metabolized to a proatherogenic species, trimethylamine-N-oxide (TMAO). It was demonstrated that metabolism by intestinal microbiota of dietary L-carnitine,
a trimethylamine abundant in red meat, also produces TMAO and accelerates atherosclerosis in mice. Omnivorous human subjects produced more TMAO than did vegans or vegetarians following ingestion of L-carnitine through a microbiota-dependent mechanism. The presence of specific bacterial taxa in human feces was associated with both plasma TMAO concentration and dietary status. Plasma L-carnitine levels in subjects undergoing cardiac evaluation (n=2,595) predicted increased risks for both prevalent cardiovascular disease (CVD) and incident major adverse cardiac events (myocardial infarction, stroke or death), but only among subjects with concurrently high TMAO levels. Chronic dietary L-carnitine supplementation in mice altered cecal microbial composition, markedly enhanced synthesis of TMA and TMAO, and increased atherosclerosis, but this did not occur if intestinal microbiota was concurrently suppressed. In mice with an intact intestinal microbiota, dietary supplementation with TMAO or either carnitine or choline reduced in vivo reverse cholesterol transport. Intestinal microbiota may thus contribute to the well-established link between high levels of red meat consumption and CVD risk [13679].

Methylhexaneamine (DMAA)

1,3 dimethylamylamine or methylexaneamine (DMAA) is a synthetic pharmaceutical patented in the 1940s as a nasal decongestant which can be used as a recreational stimulant. Alleged to occur in nature, DMAA has become a widely used ingredient in sports food supplements, despite its status as a doping agent and concerns over its safety. There is now some doubt as to whether it can be sourced naturally or whether it actually occurs naturally at all. The presence of DMAA was investigated by high performance liquid chromatography (HPLC) in extracts of the leaves and stems of four geranium species and of three well-known cultivars. The amounts of DMAA in commercial geranium (Pelargonium graveolens) oil and the leading sports supplement which uses the ingredient were also measured. DMAA was not found in any of the leaves or stems or in the commercial geranium oil included in this study. Approximately 30 mg per daily dose was found in the food supplement. Therefore, the amount of DMAA found in the supplement is most unlikely to have been sourced in nature, and it must be concluded that synthetic DMAA, known to be capable of causing severe adverse physiological effects, has been added [12436].

A number of supplements which are advertised for weight loss contain compounds which can be classified as stimulants or are related to known stimulants. Stimulants have been used as anorectic substances to aid in appetite suppression by the pharmaceutical industry for some time with compounds such as phentermine, sibutramine, benfluorex, diethylpropion, amphetamine, benzphetamine, and phenethylamine being available and included on the World Anti-Doping Agency (WADA) Prohibited List. Other stimulants such as benzylpiperazine, phenpromethamine, synephrine, and phenylethylamines have also been sold as ‘supplements’ or components of supplements and have caused athletes to return an adverse analytical finding with subsequent sanctions. This seems to be a trend that has recently surfaced where weak stimulants are added to supplements to allow them to be advertised as slimming agents. Recently the use of 2-amino-4-methylhexane (1,3-dimethylpentylamine, methylhexaneamine) has become widespread in such supplements resulting in a plethora of adverse findings since its detection has been published and implemented by anti-doping laboratories. Invariably the athletes have stated that they were unaware of the material's presence in the preparation and this plea has considerable weight, considering the numerous variations in the nomenclature used to describe this component. WADA's Explanatory Notes on the 2011 Prohibited List state. Many contain methylhexaneamine under one of many pseudonyms including Geranamine, geranium oil or extract, or a number of chemical names such as 1,3-dimethylpentylamine. This has resulted in many athletes returning an adverse finding and having sanctions imposed. Other stimulants such
as caffeine, phenpromethamine, synephrine, and phenethylamines are also to be found in supplements. One communication shows that geranium oils do not contain methylhexaneamine and that products labelled as containing geranium oil but which contain methylhexaneamine can only arise from the addition of synthetic material. Since the usual dose of methylhexaneamine is large, the drug is excreted at relatively high amounts for more than 29 h, the time for which the excretion was studied. One of the attempts to try to conceal the presence of this compound is to refer to its presence under the name Geranamine and imply that it comes from geranium oil. The concern that geranium oil may contain methylhexaneamine has also been raised and so any product listing geranium oil could be used as a defence. Geranium oils appear to be mainly obtained from Pelargonium graveolens which is a popular species of the geranium plant used in the production of geranium oil. Extraction of the oil can be performed by steam distillation or a cold-pressed process. A previous study of the chemical components in geranium oil by showed the presence of methylhexaneamine in geranium oil with a relative abundance of 0.66 percent. This is the only such reference and is often quoted when referring to methylhexaneamine. The aim of one paper was to determine the presence/absence of methylhexaneamine in a selection of geranium oils by GC-MS compared to authentic standard. Geranium oils from companies which had been extracted from various species by cold-pressing or steam distillation procedures were purchased. It should be noted that methylhexaneamine is a volatile substance and is likely to have been lost through a steam distillation extraction, so cold-pressed material had the best chance of retaining this compound. Methylhexaneamine was not found in any of the geranium oils described. The spectrum of methylhexaneamine itself consists mainly of one ion m/z 44. Since very many substances possess a spectrum similar to this and this ion is of poor diagnostic value, it is reasonable to assert that the assignment was incorrect. The paper also performs quantification based on the TIC integration without calibration to any standards so their quantitative values will also have a very large uncertainty. Four supplements – Adrenaline, Bang, Lipo 6 Black, and Phenadrine – were analyzed and the stimulant ingredients were identified as methylhexaneamine (in Adrenaline), caffeine (in Bang), alpha- and beta-phenethylamine (in Lipo 6 Black), and methylhexaneamine and phenpromethamine (in Phenadrine). This is interesting, considering that the labels indicated they had mixtures of all these ingredients! The excretion study for the supplement Phenadrine was undertaken to provide an excretion urine set for methylhexaneamine. Methylhexaneamine is easily detected as the parent compound in the urine. The concentration of methylhexaneamine is not corrected for specific gravity but the data shows that it remains very high for more than 29 h (>3 ug/mL) which would allow it to be detected for several days if values above 50 ng/mL are reported [11590].

1,3 dimethylamylamine or methylexaneamine (DMAA) is a synthetic pharmaceutical patented in the 1940s as a nasal decongestant which can be used as a recreational stimulant. Alleged to occur in nature, DMAA has become a widely used ingredient in sports food supplements, despite its status as a doping agent and concerns over its safety. There is now some doubt as to whether it can be sourced naturally or whether it actually occurs naturally at all. The presence of DMAA was investigated by high performance liquid chromatography (HPLC) in extracts of the leaves and stems of four geranium species and of three well-known cultivars. The amounts of DMAA in commercial geranium (Pelargonium graveolens) oil and the leading sports supplement which uses the ingredient were also measured. DMAA was not found in any of the leaves or stems or in the commercial geranium oil included in this study. Approximately 30 mg per daily dose was found in the food supplement. Therefore, the amount of DMAA found in the supplement is most unlikely to have been sourced in nature, and it must be concluded that synthetic DMAA, known to be capable of causing severe adverse physiological effects, has been added [13698].

Methylhexaneamine (MHA) is a stimulant that is added to dietary supplements and its safety...
is an on-going debate, prompting the World Anti-Doping Agency to add it to the 2010 prohibited list. Gas chromatography-low resolution mass spectrometry (GC-MS) with electron ionization (EI) requires derivatization to convert MHA into a less volatile compound, and a 2-3 min solvent delay to prevent filament damage. Without derivatization, the EI mass spectrum of MHA, which exhibits an abundant immonium ion at m/z 44 and no other fragment ions with relative intensity >10 percent, is very similar to the EI mass spectra of 2-aminoheptane, 1,4-dimethylamylamine, and n-hexylmethylamine. When using derivatization with trifluoroacetic anhydride (TFAA) and GC-high resolution time-of-flight mass spectrometry with soft ionization, the derivatized MHA diastereoisomers can be distinguished from the trifluoroacetyl-derivatives of 1-aminoheptane, 2-aminoheptane, 1,4-dimethylamylamine (1,4-DMAA) and n-hexylmethylamine. Several nutritional supplements were analysed for MHA by this technique and the results of the measurements were presented [13699].

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Within the class of stimulating agents, particularly methylhexaneamine (1,3-dimethylamylamine, DMAA) has been subject of much discussion and research recently, mostly concerning the question as to its natural or non-natural origin and the more than 300 AAFs as recorded in 2012. It was hence a major goal to identify whether the substance is produced by plants and, if so, to which extent. Since health issues as well as deaths were put into context with DMAA (ab)use, studies on its pharmacokinetics and the detection of this compound in doping control as well as nutritional supplements has become a priority in different fields of analytical chemistry. While most analytical methodologies used in the aforementioned studies were conventional GC- or LC-MS/(MS) approaches, the utility of a GC-interfaced soft ionization source (referred to as microplasma photoionization, MPPI) with TOF analyzer for detecting and characterizing DMAA was presented. Employing trifluoroacetylation, the stereoisomers of DMAA were baseline separated on a commonly used HP5MS GC column (30 x 0.25 mm, film thickness 0.25 microm) and the respective Ar-MPPI spectra yielded diagnostic fragment ions with accurate masses allowing to identify the substance in five different nutritional supplements [13009].

Dimethylamylamine (DMAA) is a sympathomimetic amine found in weight-loss/workout supplements or used as an appetite suppressant. DMAA is a stimulant that is banned by the World Anti-Doping Agency (WADA). Adverse health effects as well as fatalities have been implicated with its use. Direct analysis in real time mass spectrometry (DART-MS) is an ambient ionization method that was employed to rapidly identify the presence of DMAA in various samples without any extraction or preparations whatsoever. DMAA was first identified in supplements, sampled directly in their solid forms. Furthermore, DMAA was detected directly in urine over 48 h as a means of indicating recent abuse of the substance. DART-MS analysis is instantaneous, and coupled with the high mass accuracy associated
with the time-of-flight mass analyzer, results in unequivocal identification of the presence of DMAA. These features demonstrate DART-MS as an attractive potential alternative screening method for the presence of drugs and medications or for toxicological investigations [13701].

1,3-dimethylamylamine (DMAA) has been a component of dietary supplements and is also used within "party pills," often in conjunction with alcohol and other drugs. Ingestion of higher than recommended doses results in untoward effects including cerebral hemorrhage. To our knowledge, no studies have been conducted to determine both the pharmacokinetic profile and physiologic responses of DMAA. Eight men reported to the lab in the morning following an overnight fast and received a single 25 mg oral dose of DMAA. Blood samples were collected before and through 24 hours post-DMAA ingestion and analyzed for plasma DMAA concentration using high-performance liquid chromatography-mass spectrometry. Resting heart rate, blood pressure, and body temperature was also measured. One subject was excluded from the data analysis due to abnormal DMAA levels. Analysis of the remaining seven participants showed DMAA had an oral clearance of 20 ± 5 L/hr, an oral volume of distribution of 236 ± 38 L, and terminal half-life of 8.5 ± 1.9 hr. Lag time, the delay in appearance of DMAA in the circulation following extravascular administration, varied among participants but averaged approximately 8 minutes (0.14 ± 0.13 hr). The peak DMAA concentration for all subjects was observed within 3-5 hours following ingestion and was very similar across subjects, with a mean of about 70 ng/mL. Heart rate, blood pressure, and body temperature were largely unaffected by DMAA treatment. These are the first data to characterize the oral pharmacokinetic profile of DMAA. These findings indicate a consistent pattern of increase across subjects with regards to peak DMAA concentration, with peak values approximately 15-30 times lower than those reported in case studies linking DMAA intake with adverse events. Finally, a single 25 mg dose of DMAA does not meaningfully impact resting heart rate, blood pressure, or body temperature [13702].

1,3-Dimethylamylamine (DMAA) is a substance with amphetamine-like effects found in bodybuilding and weight-loss products and recreational drugs (also marketed as methylhexanamine, dimethylpentylamine and geranium). DMAA was recently banned in Australia and other countries owing to toxicity, lack of health benefits, and concerns about long-term safety and potential for misuse [13703].

**Gluthatione**

Halogenated organic compounds, such as 1-bromopentane (1-BPT), are used as cleaning agents, synthesis agents, or extraction solvents in the workplace. In one study, glutathione (GSH) conjugation and hepatotoxicity induced by 1-BPT were investigated in female mice. S-Bromopentyl GSH, S-bromopentyl cysteine, and mono-hydroxypentyl mercapturic acid were identified in liver by liquid chromatography-electrospray ionization tandem mass spectrometry. Oral treatment of mice with 1-BPT at 1500 mg/kg produced maximum GSH conjugates at 6 h after treatment. For hepatotoxicity tests, the animals were treated orally with 1-BPT at 375, 750, or 1500 mg/kg in corn oil once for a dose response study or at 1500 mg/kg for 6, 12, 24, or 48 h for a time course study. 1-BPT dose-dependently increased serum activity of ALT and AST and decreased hepatic GSH levels, peaking at 6 and 12 h after treatment. 1-BPT (750 and 1500 mg/kg) also significantly increased the hepatic content of malondialdehyde. Thus, 1-BPT could cause hepatotoxicity and depletion of GSH content by forming GSH conjugates, presenting a toxicity mechanism and potential biomarkers for low molecular weight haloalkanes [08421].
Choline bitartrate plus acetylcholine

Choline is widely distributed in food fats, especially liver, egg yolk, peanuts, dairy products, human milk and phospholipids. The body synthesises choline, and a deficiency due to dietary inadequacy is unlikely. Choline is a precursor for the neurotransmitter, acetylcholine (which generates muscle contractions), and is a donor of methyl groups (lowering homocysteine levels, reducing heart disease risk). Lecithin is a natural source of choline. Choline is promoted to athletes to improve physical endurance by increasing fat lipolysis and acetylcholine production to increase muscle contractions and delay fatigue. Choline supplements are thought to stimulate brain function (improving memory, intelligence and mood state in rats). Cyclists taking 2.34 g of choline showed improved mood state but no improvement in performance. Evidence does not support claims that choline has a role in reducing body adipose tissue, or that high doses elevate fat metabolism in humans. It was reported decreased plasma choline concentrations (9-40 %) during prolonged exercise (>2 h) in elite and well-trained endurance athletes (marathon runners, cyclists, triathletes, military personnel) and possible links to reduced performance or early fatigue have fuelled interest in supplementation. Decreased choline levels and insufficient acetylcholine availability may contribute to fatigue; increasing exogenous choline may enhance acetylcholine availability for neuromuscular transmission. Two well-designed studies, involving exhaustive exercise protocols in well-trained cyclists and soldiers, provided 2.4-8.4 g of choline citrate and bitartrate in beverages consumed prior to exercise. Although supplementation increased plasma choline, it failed to delay fatigue or show performance benefits in these studies and one other. Free serum choline concentrations vary with dietary choline intake, peaking within hours of ingestion. Choline supplements, as capsules or powders, may cause gastrointestinal side effects including breakdown to trimethylamine, which leads to fishy body odours. While small supplemental doses are not currently considered harmful, athletes with gout should avoid choline supplementation. There is no clear evidence that decreased plasma choline is associated with acetylcholine depletion and fatigue. Despite studies showing elevated choline concentrations due to choline supplementation, there is no evidence that this translates into benefits in exercise performance or reductions in fatigue [10253].

Chondroitin/glucosamine

Glucosamine is a primary building block for proteoglycans. Glucosamine is available in an oral preparation with approximately 90 percent gastrointestinal absorption. Oral administration results in absorption by several tissues including bone and articular cartilage. Chondroitin is a large molecule that is absorbed from the gastrointestinal tract but not as readily as glucosamine. Theoretically, it enhances proteoglycan synthesis and prevents cartilage degradation, whether due to damage or disease. In athletes with degenerative joint disease, non-steroidal anti-inflammatories (NSAIDs) are commonly overused, and this may lead to an increased risk of adverse events. There is therefore interest in the use of glucosamine and chondroitin as both treatment and disease-modifying agents for cartilage damage in athletes. Much of the interest for the disease or damage-modifying aspects of glucosamine and chondroitin is derived from animal studies. Studies on rabbits suggest that there may be a role for glucosamine use in injury to articular cartilage. Both glucosamine and chondroitin have been studied to evaluate their effect on response to joint stress in animal chondrocytes. Results suggest that cartilage subjected to various stresses (i.e. enzyme-induced matrix depletion, heat stress, mechanical compression and cytokine stress) have an enhanced protective metabolic response of the chondrocyte when treated with glucosamine.
and chondroitin. These studies have led to the marketing of glucosamine sulphate and chondroitin to athletes for use in the modification of acute cartilage damage after an acute injury or cartilage damage due to repetitive load in athletics. This is based on the hypothesis that glucosamine sulphate and chondroitin may stimulate chondrocytes to repair damaged cartilage more efficiently and completely. There are no current studies in athletes of any age for either supplement to suggest that these effects occur. There is conflicting evidence for the use of glucosamine and chondroitin as a symptomatic treatment of osteoarthritis. There are studies that suggest both benefit and no benefit for the symptomatic use of both glucosamine and chondroitin in the treatment of knee pain. A recent large-scale Glucosamine/Chondroitin Arthritis Trial leaves doubt as to the effectiveness of glucosamine and chondroitin in the treatment of knee osteoarthritis. There are data to support a combination of glucosamine and chondroitin as effective in those patients with moderate to severe knee pain. However, no benefit was observed with individual use of these agents in this population with respect to knee pain. Neither the combination nor individual use showed any benefit in those patients classified with mild knee pain compared with placebo. A systematic, clinical review determined that there are inconsistent results regarding the effectiveness of glucosamine and chondroitin in improving pain and joint function in knee osteoarthritis. Both supplements have an excellent safety profile. Therefore, if an athlete plans to use these products, it may be suggested that clinicians should discuss whether or not there is potential for benefit in athletes with knee degenerative joint disease, as an alternative to chronic NSAID or analgesic use [10253].

Lecithin

Lecithin is a phospholipid that occurs naturally in foods of animal and plant origin such as egg yolk, soybeans and wheat germ. Lecithin is naturally high in phosphatidylcholine, which is required for normal cellular structure and function. It has been suggested that strenuous exercise results in decreased plasma choline concentrations, which is associated with decreased acetylcholine and delayed muscle contraction. Lecithin supplementation is purported to possess ergogenic properties due either to its phosphate or choline content. Very few studies have investigated the effects of lecithin supplementation on athletic performance. Trained triathletes were given a placebo and acute lecithin supplementation at a dose of 0.2 g/kg 1 h before exercise. The placebo condition decreased plasma choline concentrations by 17 percent; however, when lecithin supplementation was given, average plasma choline levels remained unchanged. In a similar study, 12 accomplished marathon runners were given either 2.2 g of lecithin or a placebo 1 day before running a marathon. Runners given the lecithin supplementation maintained normal plasma-free choline levels compared with the placebo condition, however, there was no effect on performance. Another study found that 14 days of soya lecithin supplementation had no ergogenic effect on grip strength. Acute lecithin supplementation at doses of 2.2 g prior to exercise has been administered without any known side effects. Although lecithin supplementation appears to prevent the decrease in choline levels after exercise, there is no clear evidence of any performance benefits [11279].
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**Linoleic acid**

Linoleic acid, the simplest member of the omega-6 polyunsaturated fatty acid family, is an essential fatty acid. It has a particular role in maintaining the integrity of the skin, so preventing water loss, as a result of its presence within the structure of skin-specific lipids. Linoleic acid is different in structure from conjugated linoleic acid. Linoleic acid is the substrate for biosynthesis of arachidonic acid, the principal precursor of eicosanoids involved in a variety of physiological and pathophysiological responses. Linoleic acid is synthesised in plants. Consequently, plant tissues, seeds and nuts; their oils (e.g. corn oil, sunflower oil, soybean oil); and foodstuffs produced from these oils (e.g. margarines) have a high content of linoleic acid (often > 50 % of the fatty acids are present). Inclusion of seeds, nuts, grains, vegetable oils and margarines in a variety of foods and the presence of linoleic acid in animal-derived foods as a result of animal foraging and modern farming practices (e.g. feeding on grains) mean that linoleic acid is widespread in foods and in the diet. The human requirement for linoleic acid is estimated to be 0.5 percent of energy, which in a typical Western adult equates to an intake of about 1 g/day. The UK National Diet and Nutrition Survey indicated that the average intake of omega-6 fatty acids, most of which would be linoleic acid, among adults aged 19 to 64 years is about 5.4 percent of energy and that 98 percent of the population in this age range consume more than 2.7 percent of energy as omega-6 fatty acids (mainly linoleic acid). Average intakes in g/day were 12 in men and 9 in women, with 98 percent of men consuming more than 4.9 g/day and 98 percent of women consuming more than 4 g/day. Thus, intake of linoleic acid among adults in the UK is greatly in excess of the estimated requirement. A high intake of linoleic acid can competitively inhibit the biosynthesis of the omega-3 fatty acid eicosapentaenoic acid from its precursor α-linolenic acid. Given that linoleic acid is widespread in foods, there does not seem to be a case for its supplementation among athletes [11279].

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**gamma-Linolenic acid**

gamma-Linolenic acid (GLA) is the desaturated derivative of linoleic acid. It is a omega-6 fatty acid. GLA is found in certain unusual plant oils like evening primrose oil and borage oil (sometimes called starflower oil) and is typically rare in the diet. Experimental studies suggest an anti-inflammatory action mediated via prostaglandin E1 and analgesic effects. Low-dose GLA supplementation during exercise had a therapeutic benefit on claudication in patients undertaking standing leg exercises. In a large rehabilitation study in patients with compressive radiculopathy syndrome from disc-nerve root conflict, a daily supplement of 360 mg GLA + 600 mg alpha-lipoic acid improved neuropathic symptoms. Despite a lack of strong evidence from research, a wide range of GLA doses (15-700 mg/day) has been advocated as suitable for athletes wanting to increase performance and reduce inflammation. High doses are thought to impair performance and have an adverse effect on mood, perhaps because GLA is the precursor of arachidonic acid. There are claims that GLA supplementation will help bodybuilders to lose fat, but there does not appear to be strong evidence to support this [11279].

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**Conjugated linoleic acid**

The aim of one study was to measure the effects of 12 weeks of conjugated linoleic acid (CLA) supplementation on body composition, RER, RMR, blood lipid profiles, insulin sensitivity and appetite in exercising, normal-weight persons. In a double-blind, randomised,
controlled trial, sixty-two non-obese subjects (twenty-five men, thirty-seven women) received either 3.9 g/d CLA or 3.9 g high-oleic acid sunflower oil for 12 weeks. Prior to and after 12 weeks of supplementation, oral glucose tolerance, blood lipid concentrations, body composition (dual-energy X-ray absorptiometry and computerised tomography scans), RMR, resting and exercising RER and appetite were measured. There were no significant effects of CLA on body composition or distribution, RMR, RER or appetite. During the oral glucose tolerance tests, mean plasma insulin concentrations (0, 30, 120 min) were significantly lower in women who supplemented with CLA compared to high-oleic acid sunflower oil control. Serum NEFA levels in response to oral glucose were attenuated in both men and women in the CLA compared to control group. However, serum total cholesterol and LDL-cholesterol concentrations decreased in both groups and HDL-cholesterol concentrations decreased in women over 12 weeks. In conclusion, mixed-isomer CLA supplementation had a favourable effect on serum insulin and NEFA response to oral glucose in non-obese, regularly exercising women, but there were no CLA-specific effects on body composition, energy expenditure or appetite [07368].

Conjugated linoleic acid (CLA) is a term for a series of structural and geometric isomers of linoleic acid. The two double bonds in the acyl chain of linoleic acid are on carbons 9 and 12 (counting from the C-terminal carbon), separated by two single carbon-to-carbon bonds, and both are in the cis conformation. In CLA, the double bonds are separated by only a single bond (i.e. the double bonds are conjugated) and each may be in either the cis or trans conformation. Thus there are many possible forms of CLA. The major naturally occurring form in the human diet is cis-9, trans-11 CLA. This is produced as a result of rumen biohydrogenation and is found in ruminant milks, milk products and meats. These foods also contain several other CLA isomers. CLA in supplements is mainly produced by chemical treatment of sunflower oil and typically contains an equal mixture of cis-9, trans-11 and trans-10, cis-12 CLA and, often, smaller amounts of other CLA isomers. Biological effects of CLA have been demonstrated in many animal models and, in some studies, in healthy human volunteers: these effects appear to be isomer specific. Findings of CLA studies conducted among individuals who regularly exercise or in body-builders have been inconsistent. Some studies report decreased fat mass and increased fat-free (lean) mass with CLA supplementation (1.8–6 g/day of mixed isomers but predominantly an equal mix of cis-9, trans-11 and trans-10, cis-12) for about 3 months, although this is not seen in all studies. CLA has been combined with creatine and creatine and whey protein in other studies reporting reduced fat mass and increased fat-free mass. CLA may or may not increase muscle strength. It is likely that the level and duration of intake of specific biologically active CLA isomers is important, and differences in these factors may explain the contradictory findings in the literature. It is currently not possible to make a firm statement about the role of specific CLA isomers in athletic training and performance or to recommend a specific isomer or intake [10251].

Conjugated linoleic acid (CLA) is a term for a series of structural and geometric isomers of linoleic acid. Studies investigating the role of CLA supplements in decreasing fat mass and/or increasing lean mass have been inconsistent [13012].

Fat supplements are nutritional ergogenic aids used by elite and recreational athletes; many sport magazines advise that their intake can improve endurance capacity, increase VO2max, reduce fat body mass, increase lean body mass, reduce muscle glycogen breakdown, improve metabolism, and prevent or reduce muscle damage and inflammatory responses. These supplements include long-chain triacylglycerols, medium-chain triacylglycerols, fish oil, and conjugated linoleic acid (CLA). In the scientific community, a new role of dietary fat, particularly fat supplements, has been delineated in steroidogenesis, although the molecular mechanism has not yet been elucidated. CLA, one of the commercially available fat
supplements, refers to a group of positional and geometrical isomers of linoleic acid (cis-9, cis-12-octadecadienoic acid), an omega-6 essential fatty acid that exhibits various physiological effects including anti-adipogenic, anti-carcinogenic, and immune modulation effects. The main isomers of CLA are cis-9, trans-11, trans-10, and cis-12 octadecenoic acids (c9, t11-CLA and t10, c12-CLA). It has previously been shown that CLA treatment stimulates testosterone biosynthesis in the rat Leydig tumour cell line (R2C), although the testosterone pathway stimulated by the CLA treatment was not identified. High levels of testosterone may enhance muscle mass, which is important in sport science to increase maximal voluntary strength and in cachexia and anti-aging therapies to stop muscle wasting. Moreover, high levels of testosterone may improve endurance performance by increasing haemoglobin concentrations and haematocrit as well as by increasing lactate transport through the enhancement of monocarboxylate transporter 1 and 4 in skeletal muscle. Currently only two pathways have been shown to be responsible for the possible effect of CLA on testosterone biosynthesis. First, in adipocytes, perilipin and hormone-sensitive lipase (HSL) create a protective coat on the lipid droplet surface. Under stimulation, both proteins become hyperphosphorylated, and perilipin is displaced from the lipid droplet, allowing HSL to convert cholesteryl ester to free cholesterol. In Leydig cells, the same pathway may stimulate testosterone production under CLA treatment. Second, CLA may alter steroidogenesis by up-regulating specific genes encoding enzymes and transport proteins involved in the synthesis of testosterone, such as 17alpha-hydroxylase/17,20-lyase (CYP17A1), which converts progesterone into androstenedione. It has been demonstrated that a change in CYP17A1 expression may directly affect the level of testosterone. The aims of one study were to identify the pathway stimulated by CLA supplementation using a cell culture model and to determine whether this same pathway is also stimulated in vivo by CLA supplementation associated with exercise. In vitro, Leydig tumour rat cells (R2C) supplemented with different concentrations of CLA exhibited increasing testosterone biosynthesis accompanied by increasing levels of CYP17A1 mRNA and protein. In vivo, trained mice showed an increase in free plasma testosterone and an up-regulation of CYP17A1 mRNA and protein. The effect of training on CYP17A1 expression and testosterone biosynthesis was significantly higher in the trained mice supplemented with CLA compared to the placebo. The results of the present study demonstrated that CLA stimulates testosterone biosynthesis via CYP17A1, and endurance training led to the synthesis of testosterone in vivo by inducing the overexpression of CYP17A1 mRNA and protein in the Leydig cells of the testis. This effect was enhanced by CLA supplementation. Therefore, CLA-associated physical activity may be used for its steroidogenic property in different fields, such as alimentary industry, human reproductive medicine, sport science, and anti-muscle wasting [13708].

**Leptin**

Leptin is a hormone secreted primarily by adipocytes from the white adipose tissue in direct proportion to the amount of body fat present. Leptin plays a crucial role in the regulation of appetite, body fat mass, basal metabolic rate and gonadal function. Congenital deficiency of leptin is rare, but causes morbid obesity which is normalised following leptin treatment. Circulating leptin levels change acutely in accordance with energy balance; leptin levels increase with food ingestion and reduce with prolonged exercise and fasting. When there is a severe acute negative energy balance, serum leptin levels dramatically reduce by 60-80 percent, despite small changes in total fat mass. Preventing this reduction in leptin levels could attenuate hunger in dieting athletes, facilitating the adjustment of body mass to specific targets. Nevertheless, there is no account of leptin misuse by athletes for this purpose. Leptin receptors are densely expressed in the cerebellum, even more so than in the hypothalamus where leptin is supposed to exert its main action. Leptin-related changes
owing to physical activity levels may promote structural changes in the cerebellum, which is strongly implicated in motor control and learning. Leptin receptors are also expressed in human skeletal muscle, and more abundantly in women than men. Here, the main action of leptin is believed to be the stimulation of fatty acid oxidation via several pathways. Interestingly, these pathways are also activated 30 min after sprint exercise and, like sprint exercise, leptin induces PGC1alpha expression and mitochondrial biogenesis. It is known that exercise reduces leptin resistance in obese rodents or in rodents fed with high fat diets (which causes skeletal muscle insulin and leptin resistance). However, little is known about the influence of exercise on the regulation of leptin receptors and leptin signalling in human skeletal muscle. In obese humans, leptin receptors are reduced in the vastus lateralis, perhaps by a mechanism related to reduced physical activity. By contrast, 12 weeks’ of weightlifting combined with endurance training does not seem to induce changes in the number of leptin receptors in the vastus lateralis of healthy men. However, professional tennis players have increased expression of leptin receptors in the triceps brachii of the dominant arm compared with the non-dominant arm. This suggests that chronic loading may regulate the expression of leptin receptors in human skeletal muscle. An increased expression of leptin receptors in overloaded skeletal muscle may facilitate muscle growth by a mechanism involving leptin signalling, either by leptin itself or by insulin like growth factor 1. In fact, leptin administration in mice with a congenital leptin deficiency (ob/ob mice) promotes muscle hypertrophy. Thus, hypothetically, athletes could think of using leptin or leptin agonists to facilitate a reduction of fat mass, control hunger, promote muscle signalling similar to that induced by sprint training and stimulate mitochondrial biogenesis, as well as using it as an anabolic agent when combined with strength training. Therefore, the concerned authorities should keep track of potential misuse of leptin or leptin agonists [11393].

Inosine

Inosine is a nucleoside formed when hypoxanthine is attached to a ribose ring (also known as a ribofuranose) via a beta-N9-glycosidic bond. Inosine plays a role in several metabolic functions including increasing red blood cell concentrations of 2,3-diphosphoglycerate (2,3-DPG), which is involved in oxygen transport. It may also potentiate the action of endogenously formed adenosine and inhibit its uptake and clearance. It has been proposed to enhance exercise performance via the effects on 2,3-DPG or by an increase in ATP concentrations. Little scientific investigation has been conducted on inosine as an ergogenic aid and few peer-reviewed papers could be found. The available studies involving trained individuals investigated chronic intakes of large doses of inosine (e.g. 5-10 000 mg/day), but these protocols did not provide an ergogenic effect for endurance or sprint activities. It is suggested that high-dose inosine, a uric acid precursor, combined with high urinary excretion, could be detrimental and result in kidney stones or acute renal failure. In summary, there appears to be little support for the use of inosine as an ergogenic aid though it is still used in some over-the-counter fitness products. There are continuing clinical trials that may indicate a future therapeutic effect as an antioxidant for exercise [11241].

Glucosamine

The main aim of one study was to examine the effects of 4 weeks of glucosamine administration on the functional ability and the degree of pain intensity in competitive male athletes after acute knee injury. This study was a randomized, double-blind parallel trial of glucosamine (1500 mg per day) or a placebo for 28 days, utilising 106 patients with an acute
knee injury. Pain and functional ability were evaluated at the beginning of the study and at 7, 14, 21, and 28 days after starting treatment. Pain intensity at rest and while walking was assessed using a visual analog scale. Passive knee flexibility (flexion and extension) of the injured limb was measured using a modified goniometer, and the degree of knee swelling was measured and compared with the noninjured limb. No significant difference was found between the glucosamine, and placebo group in mean pain intensity scores for resting and walking, and degree of knee swelling at the 7-day, 14-day, 21-day, and 28-day assessment. There was no significant difference between passive knee flexibility at the 7-day, 14-day, and 21-day assessment. After 28 days of treatment the patients from the glucosamine group demonstrated significant improvement in knee flexion and extension as compared with the placebo group [07358].

Glucosamine is commonly used for the treatment of osteoarthritis. It is available as an over the counter preparation and also as a prescription pharmaceutical. There is concern from animal experiments that glucosamine may alter glucose metabolism through the hexosamine biosynthetic pathway. The objective of one systematic review was to determine if exogenous glucosamine adversely affects glucose metabolism in humans. The review does not separate out the effects on glucose metabolism of the various glucosamine preparations. An English-language literature search of MEDLINE, EMBASE and EBM Reviews (1950-February 2009) was conducted. The bibliographies of selected papers were manually searched for additional references. Two reviewers independently analyzed studies for quality and content using a standardized data extraction form. Eleven studies were included. Six studies were randomized controlled trials and the remaining five were prospective studies with or without controls. Four of the studies found decreased insulin sensitivity or increased fasting glucose in subjects taking glucosamine. Three of these were clinical studies using oral glucosamine. Studies that included subjects with baseline impaired glucose tolerance or insulin resistance were more likely to detect an effect on glucose metabolism than studies without such subjects. It was concluded that clinical studies, including three using oral glucosamine, have provided mixed evidence about the effect of exogenous glucosamine on glucose metabolism in humans. Therefore, more studies are needed, particularly including subjects at high risk for impairments in glucose homeostasis, before a definite conclusion can be made [11295].

“Fat burners”

The term “fat burner” is used to describe nutrition supplements that are claimed to acutely increase fat metabolism or energy expenditure, impair fat absorption, increase weight loss, increase fat oxidation during exercise, or somehow cause long-term adaptations that promote fat metabolism. Often, these supplements contain a number of ingredients, each with its own controlled mechanism of action and it is often claimed that the combination of these substances will have additive effects. The list of supplements that are claimed to increase or improve fat metabolism is long; the most popular supplements include caffeine, creatine, green tea, conjugated linoleic acid, forskolin, chromium, kelp and fucoxanthin. In this review the evidence for some of these supplements is briefly summarized. Based on the available literature, caffeine and green tea have data to back up its fat metabolism-enhancing properties. For many other supplements, although some show some promise, evidence is lacking. The list of supplements is industry-driven and is likely to grow at a rate that is not matched by a similar increase in scientific underpinning [11540].

L-arginine alpha-ketoglutarate (AAKG)
L-arginine alpha-ketoglutarate (AAKG) is marketed as a body building supplement. The components are intermediates in the metabolism of nitric oxides, but no reputable scientific evidence shows any benefits from taking AAKG as a dietary supplement. The substance was evaluated the pharmacokinetics, safety, and efficacy of L-arginine alpha-ketoglutarate in trained adult men. Subjects participated in two studies that employed a randomized, double-blind, controlled design. In study 1, 10 healthy men (30-50 year old) fasted for 8 h and then ingested 4 g of time-released or non-timed-released AAKG. Blood samples were taken for 8 h after AAKG ingestion to assess the pharmacokinetic profile of L-arginine. After 1 wk the alternative supplement was ingested. In study 2, which was placebo controlled, 35 resistance-trained adult men (30-50 year old) were randomly assigned to ingest 4 g of AAKG (three times a day, i.e. 12 g daily, n=20) or placebo (n=15). Participants performed 4 d of periodized resistance training per week for 8 weeks. At 0, 4, and 8 wk of supplementation the following tests were performed: clinical blood markers, one repetition maximum bench press, isokinetic quadriceps muscle endurance, anaerobic power, aerobic capacity, total body water, body composition, and psychometric parameters tests. Data were analyzed by repeated measures analysis of variance. In study 1, significant differences were observed in plasma arginine levels in subjects taking non-timed-release and timed-release AAKG. In study 2, significant differences were observed in the AAKG group for 1RM bench press, Wingate peak power, blood glucose, and plasma arginine. No significant differences were observed between groups in body composition, total body water, isokinetic quadriceps muscle endurance, or aerobic capacity. It was concluded that AAKG supplementation appeared to be safe and well tolerated, and positively influenced 1RM bench press and Wingate peak power performance. AAKG did not influence body composition or aerobic capacity [06293].

Blend supplements

The authors aimed to examine the acute hormonal and performance responses to resistance exercise with and without prior consumption of an amino acid/creatine/energy supplement. Eight men performed a resistance-exercise protocol at baseline, 20 min after consuming a supplement consisting of essential amino acids, creatine, taurine, caffeine, and glucuronolactone or a maltodextrin placebo. Venous blood samples were obtained before and immediately after, 15 min, and 30 min after each protocol. Area under the curve of resistance-exercise volume revealed that baseline was significantly less than supplement (10 %) and placebo (9 %). For fatigue rate, only supplement (18 % ± 12 %) was significantly lower than baseline (33 % ± 8 %). Total testosterone and growth hormone were significantly elevated at immediately after and 15 min after in all conditions. The growth hormone response was significantly lower, however, in supplement and placebo than in baseline. The total testosterone and growth hormone responses did not differ between supplement and placebo. These results indicated that a supplement consisting of amino acids, creatine, taurine, caffeine, and glucuronolactone can modestly improve high-intensity endurance; however, the anabolic-hormonal response was not augmented [08422].

It was hypothesized that triphasic multinutrient supplementation during acute resistance exercise would enhance muscular performance, produce a more favorable anabolic profile, and reduce biochemical markers of muscle damage in strength-trained athletes. Fifteen male strength-trained athletes completed two acute lower-body resistance exercise sessions to fatigue 7 days apart. After a 4-hour fast, participants consumed either a multinutrient supplement (Musashi 1-2-3 Step System) (SUPP) or placebo (PLA) beverage preexercise (PRE), during (DUR), and immediately postexercise (IP). Session volume loads were calculated as kilograms × repetitions. Lower-body peak power was measured using unloaded repeated countermovement jumps, and blood samples were collected to assess...
biochemistry, serum hormones, and muscle damage markers at PRE, DUR, IP, 30 minutes postexercise (P30), and 24 hours postexercise (P24h). The SUPP demonstrated increased glucose concentrations at DUR and IP compared with at PRE, whereas PLA demonstrated higher glucose at P30 compared with at PRE. Session volume load was higher for SUPP compared with PLA. Cortisol increased at DUR, IP, and P30 compared with at PRE in both treatments; however, SUPP also displayed lower cortisol at P24h compared with at PRE and PLA. The total testosterone response to exercise was higher for PLA compared with SUPP; however, total creatine kinase and C-reactive protein responses to exercise were lower for SUPP compared with PLA. These data indicate that although triphasic multinutrient supplementation did not produce a more favorable anabolic profile, it improved acute resistance exercise performance while attenuating muscle damage in strength-trained athletes [113743].

Nutritional support to maintain proper immune status during intense training

Prolonged exercise and heavy training are associated with depressed immune function which can increase the risk of picking up minor infections. To maintain robust immunity, athletes should eat a well-balanced diet sufficient to meet their energy, carbohydrate, protein, and micronutrient requirements. Dietary deficiencies of protein and specific micronutrients have long been associated with immune dysfunction and an adequate intake of iron, zinc, and vitamins A, D, E, B6 and B12 is particularly important in the maintenance of immune function. Consuming carbohydrate during prolonged strenuous exercise attenuates rises in stress hormones and appears to limit the degree of exercise-induced immune depression. Similar effects can be seen with daily ingestion of high-dose antioxidant vitamin supplements, though concerns have been expressed that excessive antioxidant intake may impair exercise training adaptations. It is safe to say with reasonable confidence that individual amino acids, colostrum, Echinacea, and zinc are unlikely to boost immunity or reduce infection risk in athletes. The ingestion of carbohydrate during exercise and daily consumption of probiotic and plant polyphenol (e.g. quercetin)-containing supplements or foodstuffs (e.g. non-alcoholic beer) currently offer the best chance of success. This approach is likely to be most effective for individuals who are particularly prone to illness [13744].

Electrolytes

Electrolytes are negatively (anions) or positively (cations) charged substances that, when in solution, conduct an electric current. Major physiological electrolytes include Na⁺, K⁺, Cl⁻ and HCO₃⁻, while other electrolytes such as Ca²⁺, Mg²⁺ and trace elements are also found in the body in significant amounts. Na⁺, K⁺, Cl⁻ and HCO₃⁻ are primarily responsible for normal water distribution and homeostasis throughout the body via their effect on osmotic pressure. These major electrolytes also play an essential role in regulating heart and muscle function, maintaining pH and a number of other important biochemical reactions. An athlete's demand for electrolytes increases with exercise and heat stress, as extensive sweating can mean both large water and electrolyte losses in addition to related changes in extra- and intracellular water distribution. Dehydration and an increase in plasma osmolality, which is primarily driven by Na⁺, will also stimulate osmoreceptors prompting an athlete to drink to maintain further or defend plasma volume. The primary electrolytes in sweat are Na⁺ (20-70 mmol/L) and Cl⁻, with comparatively much lower levels of K⁺ (about 5 mmol/L) and even less Ca²⁺ (about 1 mmol/L) and Mg²⁺ (about 0.8 mmol/L). As the sweating rate increases, the concentration of Na⁺ in sweat increases correspondingly, even with the lower sweat Na⁺ concentrations observed after heat acclimation. With Na⁺ being the major cation of extracellular fluid, copious sweating in particular can lead to a sizeable sweat-induced whole-
body exchangeable Na\(^+\) deficit. It is worth noting that such a deficit is not usually indicated by lower plasma osmolality or Na\(^+\) levels. A significant sodium deficit (e.g. 20-30 % of the exchangeable Na\(^+\) pool), combined with a loss in plasma volume prompting water to shift to the intravascular space, can lead to a contracted interstitial fluid compartment and possible widespread skeletal muscle cramping. Athletes affected with exertional muscle cramping related to a significant water or sodium deficit can be effectively treated either with an oral high-salt solution or intravenously. The major source of electrolytes comes from the diets. Although these (especially in the case of sodium) are frequently consumed well in excess of requirements, athletes often look for supplementary forms of electrolytes to replace those lost in sweat. This is sometimes justified when the timing and amount of necessary electrolyte replacement during or after an exercise session cannot be provided easily by food sources. The body normally conserves sufficient extracellular Na\(^+\) and Cl\(^-\) through reabsorption by the kidneys as is needed to maintain whole-body Na\(^+\) and water balance. However, an accumulating sodium deficit often needs to be offset with supplemental dietary NaCl intake during and after exercise or other physical activity when sweat losses are great. Being a predominantly intracellular cation, losses of K\(^+\) in sweat are generally low enough to be adequately met by a normal diet. The presence of modest amounts of sodium (and other electrolytes) in a sports drink, or the addition of electrolyte supplements to other beverages, can increase voluntary intake of fluid and enhance the retention of fluids consumed to restore hydration status more completely after exercise or other dehydrating activities. Accordingly, the value of simultaneous replacement of electrolytes with fluid intake in situations requiring restoration of moderate to large fluid deficits is well accepted. Excessive intake of water or other low sodium fluids can readily lead to water overload, a condition known as hyponatraemia. Exercise-related hyponatraemia typically indicates an excess of total body water compared with total body exchangeable Na\(^+\) and sometimes, in a much lesser contributing way, a measurable sweat-induced exchangeable Na\(^+\) deficit. Severe hyponatraemia can prompt serious consequences. This is because a significant decrease in plasma Na\(^+\) concentration and consequent osmotic gradient can cause brain swelling and altered mental status, seizure, respiratory distress, coma or even death. But less severe exercise-related hyponatraemia (when the plasma Na\(^+\) concentration is 125-130 mmol/l) resulting from overconsumption of fluid during physical activity in excess of sweat and urinary water losses can still prompt nausea, vomiting and headache. Its prevalence is increasingly recognised at endurance events in slow and inexperienced athletes who have low sweat rates and generous opportunities to consume fluid. This clinical situation often needs to be corrected by intravenous saline administration after being verified by a plasma or serum Na\(^+\) measurement [10391].

**Dimethylglycine**

Dimethylglycine (N,N-dimethylglycine, DMG) is a dimethylated derivative of the amino acid glycine. Found naturally in animal and plants cells, it is an intermediate in glycine synthesis from the degradation of choline. Indirectly, it is involved in a wide range of metabolic pathways through transmethylation. DMG is also an active ingredient in pangamic acid (also referred to as vitamin B15), which is another supplement with purported performance benefits. It is proposed that DMG may enhance metabolic variables of aerobic performance in humans and, historically, supplementation has been used by Soviet athletes and American Football players. Studies using trained subjects found no difference in running endurance, or heart rate and VO\(_{2}\max\) after a 21-day treatment period (200 mg/day), 24 or when 135 mg DMG was administered acutely before a treadmill test to exhaustion. Cardiorespiratory function and lactate production were also unaltered in exercising horses. Potential immunomodulating capacities of DMG in humans may have been masked by the study
design. Animal studies have failed to demonstrate an enhancement to specific or non-specific immunity. There is currently insufficient evidence to support DMG supplementation in athletic populations [10347].

**Dietary nitrates**

Nitric oxide (NO), synthesized from L-arginine by NO synthases, plays a role in adaptation to physical exercise by modulating blood flow, muscular contraction and glucose uptake and in the control of cellular respiration. Recent studies show that NO can be formed in vivo also from the reduction of inorganic nitrate (NO$_3^-$ and nitrite NO$_2^-$). The diet constitutes a major source of nitrate, and vegetables are particularly rich in this anion. The aim of one study was to investigate if dietary nitrate had any effect on metabolic and circulatory parameters during exercise. In a randomized double-blind placebo-controlled crossover study, we tested the effect of dietary nitrate on physiological and metabolic parameters during exercise. Nine healthy young well-trained men performed submaximal and maximal work tests on a cycle ergometer after two separate 3-day periods of dietary supplementation with sodium nitrate (0.1 mmol/kg and day) or an equal amount of sodium chloride (placebo). The oxygen cost at submaximal exercise was reduced after nitrate supplementation compared with placebo. On an average VO$_2$ decreased during NIT over the four lowest submaximal work rates. Gross efficiency increased during NIT over the four lowest work rates. There was no difference in heart rate, lactate, ventilation (VE), VE/VO$_2$ or respiratory exchange ratio between nitrate and placebo during any of the submaximal work rates. It was concluded that dietary nitrate supplementation, in an amount achievable through a diet rich in vegetables, results in a lower oxygen demand during submaximal work. This highly surprising effect occurred without an accompanying increase in lactate concentration, indicating that the energy production had become more efficient. The mechanism of action needs to be clarified but a likely first step is the in vivo reduction of dietary nitrate into bioactive nitrogen oxides including nitrite and NO [07377].

Nitric oxide (NO) is an important physiological signalling molecule that can modulate skeletal muscle function through its role in the regulation of blood flow, muscle contractility, glucose and calcium homeostasis and mitochondrial biogenesis and respiration. Until quite recently, it was considered that NO was generated solely through the oxidation of the amino acid L-arginine in a reaction catalysed by nitric oxide synthase (NOS). It is now appreciated, however, that NO may also be produced by the reduction of nitrate to nitrite and subsequently of nitrite to NO. This pathway may be particularly important in hypoxia. Nitrate and nitrite are present in the body as products of NO production through NOS and are also modulated through the diet. Nitrate in foods (particularly green leafy vegetables) can be reduced to nitrite by oral bacteria, leading to an increased plasma nitrite concentration that serves as a circulating “reservoir” for NO production. Several recent studies have addressed the extent to which dietary nitrate supplementation might affect the physiological responses to exercise. It was shown that 3 days of sodium nitrate supplementation (0.1 mmol/kg/day) reduced resting blood pressure and the O$_2$ cost of submaximal cycle exercise. Subsequently, we have reported that enhancing NO bioavailability through supplementation of the diet with a natural foodstuff (nitrate-rich beetroot juice) reduces resting blood pressure and the O$_2$ cost of exercise and improves exercise performance. In the first study, it was found that 4-6 days of dietary nitrate supplementation (0.5 litres of beetroot juice per day containing about 6 mmol nitrate) reduced the “steady-state” O$_2$ cost of submaximal cycle exercise by 5 percent and extended the time to exhaustion during high-intensity cycling by 16 percent. In a follow-up study, it was used $^{31}$P-magnetic resonance spectroscopy to investigate the mechanistic bases of this phenomenon. Dietary nitrate supplementation resulted in both a reduced
pulmonary $O_2$ uptake and reduced muscle metabolic perturbation, enabling high-intensity knee-extension exercise to be tolerated for a greater period of time. These data imply that the reduced $O_2$ cost of exercise following dietary nitrate supplementation is related to a reduced ATP cost of muscle force production, perhaps consequent to reduced cross-bridge cycling or sarcoplasmic reticulum $Ca^{2+}$-ATPase activity. It is also possible, however, that nitrate supplementation enhances mitochondrial efficiency. Larsen et al have reported that sodium nitrate reduced proton leakage and increased the mitochondrial P/O ratio. The positive effects of nitrate supplementation on the $O_2$ cost of submaximal exercise can be manifest acutely (ie, 2.5 h following a 6 mmol nitrate "bolus") and this effect can be maintained for at least 15 days if supplementation at the same daily dose is continued.

Because beetroot juice contains compounds other than nitrate that might also be bioactive, it has been developed a nitrate-depleted beetroot juice as a placebo. Nitrate-depleted beetroot juice had no physiological effects relative to a control condition, whereas nitrate-rich beetroot juice reduced the $O_2$ cost of both walking and running and extended the time to exhaustion by 15 percent. Most recently, it has been investigated the influence of acute dietary nitrate supplementation on 4 and 16.1 km time trial (TT) performance in competitive cyclists. It has been found that cyclists were able to produce a greater power output for the same rate of pulmonary $O_2$ uptake, resulting in a 2.7 percent reduction in the time to complete both the TT distances. Collectively, recent studies indicate that dietary nitrate supplementation reduces the $O_2$ cost of physical activity and might enhance exercise performance. While these early findings are clearly of considerable interest to athletes, it is possible that clinical populations and older people may also benefit if dietary nitrate intake can be shown to reduce the $O_2$ cost of the "activities of daily living" [11532].

Humans can reduce inorganic nitrate ($NO_3^-$) to nitrite ($NO_2^-$), nitric oxide (NO), and other bioactive nitrogen oxides. The purpose of this study was to test the hypothesis that a single dose of inorganic nitrate before exercise might enhance the tolerance of endurance athletes to high intensity exercise. Eleven cyclists (age = 34 ± 5 years, $VO_{2peak} = 65 ± 6 \text{ mL/kg/min}$) participated in this randomized, double-blind, crossover study. Subjects received dietary supplementation with nitrate ($NaNO_3$ 10 mg/kg of body mass) or a placebo ($NaCl$) 3 h before exercise. They then performed a cycle ergometer test that consisted of four 6-min submaximal workloads, corresponding to 2.0, 2.5, 3.0, and 3.5 W/kg of body mass, interspersed with 3 min of passive recovery. After a 5-min recovery period, subjects performed one incremental exercise test until exhaustion. Plasma nitrate and nitrite were significantly higher 3 h after supplementation than after the placebo at resting conditions. Nitrate supplementation significantly reduced $VO_{2peak}$ and the ratio between $VO_2$ and power at maximal intensity. This reduction of $VO_2$ occurred without changes in the time to exhaustion (nitrate = 416 ± 32 s, placebo = 409 ± 27 s) or in the maximal power (nitrate = 416 ± 29 W, placebo = 410 ± 28 W). It was concluded that a single oral dose of inorganic nitrate acutely reduces $VO_{2peak}$ without compromising the maximal exercise performance [11533].

Acute dietary nitrate ($NO_3^-$) supplementation has been reported to lower resting blood pressure, reduce the oxygen ($O_2$) cost of sub-maximal exercise, and improve exercise tolerance. Given the proposed effects of $NO_3^-$ on tissue oxygenation and metabolic rate, it is possible that $NO_3^-$ supplementation might enhance the duration of resting apnea. If so, this might have important applications both in medicine and sport. It was investigated the effects of acute $NO_3^-$ supplementation on pre-apnea blood pressure, apneic duration, and the heart rate (HR) and arterial $O_2$ saturation (SaO$_2$) responses to sub-maximal and maximal apneas in twelve well-trained apnea divers. Subjects were assigned in a randomized, double blind, crossover design to receive 70 ml of beetroot juice (BR; containing 5.0 mmol of nitrate) and placebo juice (PL: 0.003 mmol of nitrate) treatments. At 2.5 h post-ingestion, the subjects completed a series of two 2-min (sub-maximal) static apneas separated by 3 min of rest,
followed by a maximal effort apnea. Relative to PL, BR significantly reduced resting mean arterial pressure by 2 percent. The mean nadir for SaO₂ after the two sub-maximal apneas was 97.2 ± 1.6 percent in PL and 98.5 ± 0.9% in BR while the reduction in HR from baseline was not significantly different between PL and BR. Importantly, BR increased maximal apneic duration by 11 percent. In the longer maximal apneas in BR, the magnitude of the reductions in HR and SaO₂ were greater than in PL. The results suggest that acute dietary NO₃⁻ supplementation may increase apneic duration by reducing metabolic costs [12454].

Six days of dietary nitrate supplementation in the form of beetroot juice (0.5 L/d) has been reported to reduce pulmonary oxygen uptake (VO₂) during submaximal exercise and increase tolerance of high-intensity work rates, suggesting that nitrate can be a potent ergogenic aid. Limited data are available regarding the effect of nitrate ingestion on athletic performance, and no study has investigated the potential ergogenic effects of a small-volume, concentrated dose of beetroot juice. The authors tested the hypothesis that 6 d of nitrate ingestion would improve time-trial performance in trained cyclists. Using a double-blind, repeated-measures crossover design, 12 male cyclists (31 years, VO₂peak 58 mL/kg/min, maximal power 342 ± 10 W) ingested 140 ml/d of concentrated beetroot (8 mmol/d nitrate) juice (BEET) or a placebo (nitrate-deplete beetroot juice; PLAC) for 6 d, separated by a 14-d washout. After supplementation on day 6, subjects performed 60 min of submaximal cycling (2×30 min at 45 % and 65 % maximal power, respectively), followed by a 10-km time trial. Time-trial performance (953 ±1 8 vs 965 ± 18 s) and power output (294 ± 12 vs 288 ± 12 W) improved after BEET compared with PLAC supplementation. Submaximal VO₂ was lower after BEET (45 % maximal power1.92 ± 0.06 vs 2.02 ± 0.09 L/min, 65 % maximal power 2.94 ± 0.12 vs 3.11 ± 0.12 L/min) than with PLAC (main effect). Whole-body fuel selection and plasma lactate, glucose, and insulin concentrations did not differ between treatments. Six days of nitrate supplementation reduced VO₂ during submaximal exercise and improved time-trial performance in trained cyclists [12455].

**Dihydroxyacetone phosphate and pyruvate**

Dihydroxyacetone phosphate (DHAP) and pyruvate are three-carbon metabolites in the glycolytic pathway. In skeletal muscle, the glycolytic pathway metabolises glucose from the blood and stored glycogen. DHAP is formed at an intermediate step, and pyruvate is produced in the final step of glycolysis. During aerobic exercise, pyruvate enters the mitochondrion and is oxidised to produce ATP. It is not immediately clear how ingesting oral doses of DHAP/pyruvate could influence athletic performance, as it is unlikely that the ingested compounds could reach skeletal muscle and have a direct effect on metabolism. Pyruvate undergoes acid hydrolysis in the stomach and gut with the liberation of carbon dioxide gas, and ingestion of large amounts results in gastrointestinal distress. In addition, the pyruvate that is absorbed into the blood could be taken up and stored by the liver, as even the highest tolerable acute dose of pyruvate (about 25 g) represents a small amount of glucose (about 12.5 g). Only three studies have examined the potential for DHAP/pyruvate to improve exercise capacity. Despite no plausible ergogenic mechanism, two studies reported an improvement in exercise time to exhaustion (20 %). The authors attributed the improvement to increased skeletal muscle glucose utilisation. However, these studies used untrained subjects who were not blinded to the treatments. A later study examined the effects of pyruvate only and had well-trained subjects ingest 7 g/day pyruvate or placebo for 1 week followed by cycling to exhaustion at 75-80 percent of VO₂max. There was no difference in cycling endurance (90 min) between the trials, and the subjects were not able to identify the supplement they received. On the basis of these few reports and no apparent mechanism to alter metabolism, several reviews have concluded that oral pyruvate is not
ergogenic. In summary, there is currently no scientific basis for the use of DHAP/pyruvate as an ergogenic aid [10347].

**Methylsulphonymethane**

MSM is an organic sulphur compound. It is a metabolite of dimethyl sulphoxide (DMSO). MSM occurs naturally in certain foods including green vegetables, grains and milk. For over 30 years, MSM has been used as an oral daily nutritional supplement for various conditions including osteoarthritis, seasonal allergic rhinitis and autoimmune diseases, topically via a throat spray for the treatment of snoring, while athletes occasionally use MSM acutely for muscle soreness. High-quality randomised controlled trials (RCT) are lacking for all claims, but some evidence exists for an effect in osteoarthritic symptom relief. Two small RCT (MSM groups n=25 and 26) have identified statistically significant improvement in osteoarthritic pain scores with MSM compared with placebo. However, the clinical significance of the improved scores is unclear. These two RCTs of efficacy used between 3 and 6 g orally per day in divided doses. The treatment duration in both trials was 12 weeks. No studies on dose ranges have been conducted. It has been performed a meta-analysis of RCT using MSM or DMSO for symptomatic pain relief in osteoarthritis. Only 15 percent of DMSO is converted to MSM in the body and other metabolites are also produced. Combining the studies may, therefore, not be appropriate. There is no long-term safety data from studies in humans. One RCT found the rate of adverse event reporting with MSM to be equal to placebo in the short term (12 weeks). Reported adverse events were minor and mainly gastrointestinal. There are no published trials to support the use of MSM by athletes or the general population for delayed-onset muscle soreness (DOMS) or acute soft tissue injuries. In summary, there is a lack of high-quality evidence for many of the beneficial claims for MSM as a nutritional supplement. There is some limited evidence for the potential benefit of MSM in the symptomatic relief of osteoarthritis, but no definite recommendations can be made on current evidence [11359].

**Melamine**

Melamine (2,4,6-triamino-1,3,5-triazine; tripolycyanimide) is used in the production of plastics and adhesives. It has no documented ergogenic capacity. Given its high nitrogen content, melamine has been illegally used to elevate food protein quantity (such as protein supplements). Melamine and derivatives (e.g. cyanuric acid) are toxic to humans even at low quantities, with strong effects on the kidneys. Tolerable daily intake amounts vary by country and consumer age, but a typical adult level is 0.5 mg/kg/d. Shortly after the 2008 Beijing Olympics, it was discovered that protein products from multiple Chinese manufacturers contained melamine and derivatives, and that those products were distributed internationally. Although screening indicated that Olympic athletes were not exposed, other populations (particularly Chinese infants) were clinically affected. Athletes should be aware of possible melamine contamination in protein-rich foodstuffs [11359].

**Nootkatone**

Nootkatone in spray form has been shown as an effective repellent/insecticide against deer ticks and lone star ticks. It is also an effective repellent/insecticide against mosquitoes, and may repel bed bugs, head lice and other insects. It is environmentally friendly insecticide, because it is a volatile essential oil that does not persist in the environment. It is nontoxic to
humans, is an approved food additive, and "is commonly used in foods, cosmetics, and pharmaceuticals". Nootkatone (4,4a,5,6,7,8-hexahydro-6-isopropenyl-4,4a-dimethyl-2(3H)-naphthalenone), a kind of sesquiterpenoid, was first isolated from the heartwood of Alaska yellow cedar (Callitropsis nootkatensis). Traceable amounts of nootkatone are found in major Citrus species such as grapefruit (Citrus paradisi), and a whole grapefruit contains approximately 100 mg of nootkatone, mainly in the peel. Nootkatone is now available through technological advances such as chemical synthesis, biosynthesis and biotransformation. Nootkatone has a grapefruit-like flavour, tastes slightly bitter and has an odour threshold of about 1 mg/L water. It is used predominantly as a flavouring compound.

A recent animal study demonstrated that 0.2 percent (wt/wt) nootkatone feeding for 10 weeks improved swimming endurance (i.e. swimming time to fatigue) and that long-term intake of diets supplemented with 0.1-0.3 percent nootkatone significantly reduced high-fat and high-sucrose diet-induced body weight gain, abdominal fat accumulation and the development of hyperglycaemia, hyperinsulinaemia and hyperleptinaemia. These beneficial effects of nootkatone might be due in part to enhanced energy metabolism through the activation of AMP-activated protein kinase in the muscle and liver. These findings indicate that nootkatone is a potential candidate for being an ergogenic and antiobesity compound. A previous study indicated that consumption of a whole grapefruit or grapefruit juice for 12 weeks reduces body weight and improves insulin resistance in metabolic syndrome patients compared with placebo. On the other hand, there are no reports on the effects of grapefruit, including nootkatone, on physical performance in humans. Therefore, further studies are required to clarify the efficacy of nootkatone as an ergogenic compound, especially for athletes.

Octacosanol and policosanol

Octacosanol (CH3(CH2)26CH2O14) a high-molecular weight, primary aliphatic alcohol, is typically found in the natural wax extract of various plants and is commonly found in fruits, barks, leaves and whole seeds. Octacosanol supplementation has been purported to achieve several health benefits including lowering cholesterol, cytoprotective effects and antiaggregatory properties. The majority of the supplementation studies have used policosanol (wheat-germ oil extract), which contains a natural mixture of primary alcohols isolated from sugar cane wax, of which octacosanol is the main component. It has been suggested that policosanol reduces cholesterol by downregulating the cellular expression of hydroxymethylglutamyl coenzyme A reductase, although the exact mechanism remains to be elucidated. A meta-analysis has reviewed the efficacy of plant sterols and stanols as well as policosanol in the treatment of coronary heart disease, as measured by a reduction in low-density lipoprotein cholesterol levels (LDL). It was found that policosanol was more effective than both plant sterols and stanols in reducing level LDL levels and more favourably altered the lipid profile. In the 29 eligible studies, 12 mg/day (range 5-40 mg/day in 1528 participants) was found to reduce significantly the LDL levels and the LDL:high-density lipoprotein (HDL) ratio. Moreover, policosanol was more effective in reducing the total cholesterol, increasing the HDL cholesterol and lowering the triglyceride levels when compared with a placebo, plant sterols and stanols. Despite these positive findings, it should be noted that, after this meta-analysis was published, several other studies found that policosanol was ineffective in the treatment of hypercholesterolaemia. Fewer studies have examined the effects of octacosanol and policosanol on athletic performance. An early study found that 1000 microg of octacosanol significantly improved grip strength and reaction time in response to a visual stimulus. Another study found that octacosanol supplementation reduced body fat in athletes. However, limitations of this study included the lack of dietary control, lack of blinding of treatments and the involvement of athletes from different sporting backgrounds in the placebo and supplement groups. As a result, these findings should be
interpreted with caution. In summary, research into the effects of octacosanol and policosanol supplementation on athletic performance is lacking and remains unclear [11532].

**Superoxygenated water**

Recently, superoxygenated-water beverages have emerged as a new purported ergogenic substance. One study aimed to determine the effects of superoxygenated water on submaximal endurance performance. Eleven active male subjects completed a 45-min cycle-ergometry exercise test at 70 percent of their previously predicted maximal power output with a 10-min rest period, followed by a 15-min time trial. Thirty min before the exercise test subjects consumed 15 mL of either superoxygenated water or placebo. Subjects then completed the test again a week later for the other condition (double-blind, randomized). There were no significant differences in VO$_2$, VCO$_2$, RER, VE, lactate, PO$_2$, and heart rate during the exercise tests. Neither were there any significant improvements in the total distance covered or the average power output during the 15-min TT. Based on these results the authors concluded that consuming 15 mL of superoxygenated water does not enhance submaximal or maximal time trial cycling performance [09372].

Superoxygenated-water beverages have emerged as a purported ergogenic substance. One study aimed to determine the effects of superoxygenated water on submaximal endurance performance. Eleven active male subjects completed a 45-min cycle-ergometry exercise test at 70 percent of their previously predicted maximal power output with a 10-min rest period, followed by a 15-min time trial (TT). Thirty min before the exercise test subjects consumed 15 mL of either superoxygenated water (E) or placebo (P; water mixed with low-chlorine solution). Subjects then completed the test again a week later for the other condition (double-blind, randomized). The physiological variables measured during exercise were VO$_2$, VCO$_2$, respiratory-exchange ratio (RER), VE, PO$_2$, PCO$_2$, blood lactate (bLa-), and heart rate (HR). Mean distance covered and the average power output for the 15-min TT were also measured as performance indicators. There were no significant differences in VO$_2$, VCO$_2$, RER, VE, bLa-, PO$_2$, and HR during the exercise tests. Neither were there any significant improvements in the total distance covered or the average power output during the 15-min TT. It was concluded that based on these results the authors conclude that consuming 15 mL of superoxygenated water does not enhance submaximal or maximal TT cycling performance [07394].

**Non-pharmacological therapy**

Athletes dedicate their lives to improving their sporting skills and fitness to be better in their sport. It is acceptable (and logical) to enhance performance by physical training without adding substances to the athlete's body. The use of varied training programs and orthotic devices by athletes (e.g. fins for swimming training) during out-of-competition training is standard practice for most sports and one of the fundamentals of training paradigms. Most such performance-enhancing devices are, however, banned during competition [12015].

**Acupuncture**

Acupuncture (and the field of traditional Chinese medicine, TCM) is arguably an archetype of what is considered CAM in Westernised societies. Acupuncture is also increasingly being integrated into conventional medicine in these communities and is one of the most extensively scientifically studied forms of CAM. While there is still debate on the actual
physiological mechanism of how acupuncture works, many scientists believe that several mechanisms are possible. Because these proposed mechanisms are different from the underlying ethos and philosophy that govern acupuncture and TCM – the presence of the life energy (or “Qi”) flowing through channels (or “meridians”) in the human body – and how disease occur, direct comparisons with the scientific paradigms of biomedicine are not always possible. Also, individuals may use CAM and TCM due to their beliefs in the underlying traditional philosophies rather than any scientific motivation. There are several ways in which acupuncture may work. Local anaesthesia at needle insertion sites may block the analgesic effects of acupuncture which suggests that acupuncture is dependent upon neural innervation. Acupuncture may also cause the release of endogenous opioids in brainstem, subcortical, and limbic structures or induce the secretion of adrenocorticotropic hormone and cortisol from the pituitary gland thereby creating a systemic anti-inflammatory response. Indeed, functional MRI studies in humans show that acupuncture modulates limbic and basal forebrain areas involved in pain processing, while PET (positron-emission tomography) scans have shown that acupuncture is able to increase the opioid binding potential in the brain for several days. Other proposed mechanisms of how acupuncture works are through its ability to mechanically stimulate connective tissues, release adenosine at the site of needle stimulation, or increase local blood flow [12015].

Cryostimulation

Tournament season can provoke overreaching syndrome in professional tennis players, which may lead to deteriorated performance. Thus, appropriate recovery methods are crucial for athletes in order to sustain high-level performance and avoid injuries. It was hypothesized that whole-body cryostimulation could be applied to support the recovery process. To assess the effects of 5 days of whole-body cryostimulation combined with moderate-intensity training on immunologic, hormonal, and hematologic responses; resting metabolic rate; and tennis performance in a posttournament season and controlled laboratory study was performed. Twelve high-ranking professional tennis players followed a moderate-intensity training program. A subgroup was treated with the 5-day whole-body cryostimulation (-120°C) applied twice a day. The control subgroup participated in the training only. Pretreatment and posttreatment blood samples were collected and analyzed for tumor necrosis factor α, interleukin 6, testosterone, cortisol, and creatine kinase. Resting metabolic rate and performance of a tennis drill were also assessed. Proinflammatory cytokine (tumor necrosis factor alpha) decreased and pleiotropic cytokine (interleukin 6) and cortisol increased in the group exposed to cryostimulation. In the same group, greater stroke effectiveness during the tennis drill and faster recovery were observed. Neither the training program nor cryostimulation affected resting metabolic rate [12363].

Cooling

Cold water immersion has become a popular means of enhancing recovery from various forms of exercise. However, there is minimal scientific information on the physiological effects of this following cycling in the heat. Eleven male endurance trained cyclists completed two simulated approximately 40-min time trials at 34.3 ± 1.1 degrees C. It was fond that cold water immersion did not result in hypothermia and can be considered safe following high intensity cycling in the heat, using the above protocol. Cold water immersion significantly reduced heart rate and core temperature; however, all other metabolic and endocrine markers were not affected [09373].

Sublingual, ergogenic spray
Nutrients, chemicals, and drugs may be applied sublingually to provide faster absorption. Sublingual absorption occurs when a substance comes in contact with the buccal mucosa, where it diffuses through a membrane of the dense capillaries. The purpose of one study was to assess the effectiveness of a sublingual, ergogenic product containing vitamins, minerals, amino acids, and a coenzyme on muscle performance. National Collegiate Athletic Association Division I linemen (n=23) voluntarily participated in the study. All participants were tested on 102.1 kg (225 lb) bench press repetitions, vertical jump, and grip strength. One week later, participants were either a placebo or the experimental treatment before they were tested again. Repeated-measures analyses of variance (ANOVAs) yielded a significant gain for the bench press. A Newman-Keuls post hoc test revealed a significant change in the treatment group but not in the placebo group. While the treatment group demonstrated greater improvement over the placebo group for each of the remaining variables, none were significant: vertical jump and grip strength. The inconsistency of the results may be due to several factors. First, the spray may not be an ergogenic agent; second, the standardized dose may be too small for those weighing >290 lb and should be administered based on weight. Furthermore, the coenzyme and amino acids may not possess the molecular size, solubility, chemical stability, or hydrophilic character to be easily absorbed. Lastly, the data were generated by field tests and may not be sensitive enough to elicit subtle responses [09374].
Herbs

The use of herbs as ergogenic aids in exercise and sport is not novel. Ginseng, caffeine, ma huang (also called "Chinese ephedra"), ephedrine and a combination of both caffeine and ephedrine are the most popular herbs used in exercise and sports. It is believed that these herbs have an ergogenic effect and thus help to improve physical performance. Numerous studies have been conducted to investigate the effects of these herbs on exercise performance. Recently, researchers have also investigated the effects of Eurycoma longifolia Jack on endurance cycling and running performance. These investigators have reported no significant improvement in either cycling or running endurance after supplementation with this herb. As the number of studies in this area is still small, more studies should be conducted to evaluate and substantiate the effects of this herb on sports and exercise performance. For instance, future research on any herbs should take the following factors into consideration: dosage, supplementation period and a larger sample size [13745].

Ergogenic theory

Plants provide us with most nutrients essential for life. Other than essential nutrients, plant foods contain naturally occurring substances, referred to respectively as phytochemicals. Herbals, which are derived from leaves, bark, berries, roots, gums, seeds, stems or flowers of plants, also contain numerous phytochemicals thought to have nutritive or medicinal value. Herbs have been used as medicine throughout history. It was reported the earliest evidence of human use of plants for healing dates back to the Neanderthal period, and today various modern medicines may be classified as herbals. Thus, herbals are regulated as medicine in some countries, such as Germany, but as dietary supplements in others. Currently in the United States most herbals are regulated by the Dietary Supplement Health and Education Act (DSHEA), more like food ingredients than drugs. However, given the pharmacological effect of various herbals, some health professionals are emphasizing the need for regulations standardizing herbal therapy. Herbals are popular dietary supplements. In the most recent NHANES report, approximately 7 percent of the US population takes herbal or botanical dietary supplements. Athletes also take herbal supplements. For example, it was reported that 17 percent of female collegiate athletes used herbal/botanical supplements. Herbal dietary supplements are marketed to physically active individuals for a variety of reasons, including increasing energy, inducing weight loss, promoting muscle growth, or inducing other physiological or metabolic responses that may enhance exercise performance. For example, the product SportPharm contains multiple herbals, including Thermadrene, Ma Huang, Guarana, Caffeine, Purple Willow Bark, Cayenne pepper, and Ginger root, and is designed to increase mental alertness, stimulate fat-burning metabolism, and help enhance physical performance. Some sports drinks and sports bars contain herbals as well [06297].

Herbal weight loss supplements

Many weight loss supplements used by athletes contain herbs as active agents which can be vexing given their botanical, chemical and clinical diversity. Here, we offer a system for conceptualising herbal weight loss supplements by putative mechanisms of activity based on
each supplement's presumed bioactive compounds, which are mainly secondary metabolites (SM) and can be categorised into six main groups: alkaloids, flavonoids, polysaccharides, phenolics, terpenoids and other molecules. Most herbs contain compounds from multiple SM categories. Alkaloids are nitrogen-rich molecules derived from amino acid precursors: caffeine is a well-known example. Caffeine and similar molecules increase metabolism and consequently achieve a small increase in the body's rate of energy (calorie, kilojoule) expenditure. Although the physiological relevance of this effect is debated, such herbal weight loss aids are often described as thermogenic. Examples of caffeine-rich herbs include coffee, green tea, guarana and yerba mate. Other alkaloid-rich herbs used for weight loss include bitter orange, black pepper, cayenne pepper, ephedra, ginger, and Indian coleus. Not all plants that contain alkaloids are considered weight loss aids (e.g. chocolate, which contains theobromine and theophylline). Reviews have concluded that, of these, bitter orange, caffeine, ephedra, ginger and green tea supplements may be efficacious in weight loss, but isolated supplements of caffeine, ephedra and guarana may carry serious adverse event risks in the general population. Indeed, the sale of ephedra was banned by the FDA in 2004. Furthermore, it was included on the List of Banned Substances by the International Olympic Committee years prior to that and is currently prohibited for use in competition according to the WADA Banned List. Research reports have suggested that multicomponent green tea/black pepper/cayenne pepper and yerba mate supplements may promote weight loss, but yerba mate supplements may carry adverse effects. Terpenoids, also called isoprenoids, are lipids derived from five-carbon isoprene structures and include carotenoids, steroids and saponins. Terpenoids could conceivably work through several different mechanisms, though anorexiant and diuretic effects are most commonly reported. Weight loss herbs that presumably work through terpenoids include bearberry (kinnikinnick, uva ursi), bitter melon, dandelion, ginseng, gymnema, hoodia, Indian coleus, rhodiola, sarsaparilla, Siberian ginseng and veldt grape (Cissus quadrangularis, a frequent component of Hydroxycut). Recent reviews indicate bitter melon, ginseng, Siberian ginseng and veldt grape may be efficacious or have no effect on weight loss in the general population, but none have been flagged for adverse events. The supplement Hydroxycut, which contains multiple herbal and other products and varies in composition, is not recommended for use by athletes due to safety and efficacy concerns. Hoodia has no evidence for or against it as a weight loss agent. One study in overweight women concluded that Indian coleus supplementation does not directly promote weight loss, but may help in weight management. In obese rodents, gymnema supplements stimulated weight loss, whereas in diabetic rodents, bearberry supplementation maintained body weight, but decreased appetite and thirst, and dandelion had no effect. No studies could be located regarding sarsaparilla and weight loss. Flavonoids are derived from phenylalanine precursors and include anthocyanins and quercetin, and are found in herbs such as dandelion and elderberry. A mechanism by which they might assist weight loss is by reducing lipogenesis and/or increasing lipolysis. Elderberry, along with milk thistle, white willow, coffee and tea, also contain phenols (characterised by having both aromatic hydrocarbon and hydroxyl groups) such as tannins. From little information, reviews indicate elderberry, milk thistle and white willow have not convincingly demonstrated weight loss properties, yet none have perceived risk. Some weight loss aids presumably work through fibre (roughage). Fibre is comprised of polysaccharides that stimulate peristalsis through their laxative effects, may satiate appetite and may block fat absorption, thus contributing to weight loss. Fibre-rich weight loss herbs include guar bean, konjac and psyllium. Primary data conflict regarding their safety and efficacy. Two recent reviews concluded konjac and psyllium may be effective but all carry some risk of adverse effects. Literature regarding herbal supplements and weight loss is patchy. However, it is important to note that many of these conclusions are based on isolated studies or small samples; the majority is conducted on obese (e.g. non-athletic) populations; and mechanisms are often speculative. On the other hand, some may confer additional health benefits – for example, polyphenols found in green tea and milk thistle may have
anticancer, anti-inflammatory and antioxidant effects, while elderberry may be an immunomodulator capable of binding and incapacitating the influenza virus. Additional herbs not covered in this review, but used for weight loss, include bladder wrack (Fucus vesiculosus), blood orange (Citrus sinensis), bromeliad (Bromelia spp), celery seed (Apium graveolens), fenugreek (Trigonella foenum-graecum), horsetail (Equisetum spp), passionflower (Passiflora spp), red grape (Vitis vinifera) and xanthan gum. As is the case with phaseolamin and hydroxycitric acid, other compounds from herbal sources have been used for weight loss such as gamma-oryzanol, nootkatone and yohimbine. Thus, there is a vast array of herbs found in supplements including, but not limited to, those marketed for weight loss. The diversity of individual herbs as well as their simultaneous presence in some products, sometimes more than ten in one product, has become a minefield that both athletes and their sports medicine and science support staff find difficult to navigate. It is worrying how prolific these supplements have become, despite their tenuous, if any, link to a mechanism for weight loss and the lack of supporting evidence. The novel system devised here helps demystify and clarify the rationale, albeit frequently not supported by scientific evidence nor linked to weight loss, behind the inclusion of these herbs in many supplements. In terms of weight loss, it is advisable to make informed dietary changes prior to reaching for supplements. As always, and particularly in the case of some of the herbs discussed here where the evidence is scant, athletes are strongly recommended to seek advice from a sports nutrition professional before considering using any supplements. Furthermore, as stressed in previous reviews, it is essential to reiterate that any athlete who competes under the WADA (World Anti-Doping Agency, http://www.wada-ama.org) code needs to be extremely cautious about using supplements and always work with a qualified professional on risk minimisation of supplement use. The ethical/legal issues of sport can be contravened either by the deliberate use of over-the-counter compounds that are prohibited by such codes (e.g. pro-hormones and stimulants) or by inadvertent intake of these products due to contamination, fake or doping issues, with banned stimulants, such as ephedrine or sibutramine, being frequently found in weight loss supplements [13678].

Prebiotics

Prebiotics are non-digestible polysaccharides and other substances that selectively stimulate the growth or activities of one or more species of gut bacteria and which confer a health benefit on the host. These supplements purportedly yield beneficial health effects via the symbiotic relationship that exists between bacteria inhabiting the GI tract, known as the microbiota and their human host. Prebiotics increase the abundance of bacteria indigenous to the gut by providing nutrients as a source of fuel. Naturally occurring food sources with a prebiotic effect include barley, banana, oats, wheat, soya bean, asparagus, leek, chicory, garlic, artichoke and onion. Resistant starch, found in high amylose maize starch and formed through the heating and cooling of starch foods (rice and pasta), is also receiving growing recognition as a potential prebiotic. Various studies have shown that a prebiotic dose above 2.5 g, which is far higher than that occurring in natural foods, increases the abundance of lactic acid and butyrate-producing bacteria. However, all prebiotics are not equal, with clinical outcomes being dose-dependent. The most prevalent areas of clinical research with positive outcomes have been with galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS). The fermentation of these starches by GI bacteria releases metabolic byproducts, including short-chain fatty acids (butyrate, propionate and acetate), vitamins and lipid metabolites. These fermentation byproducts modulate various aspects of the immune system and host metabolism. Non-digestible polysaccharides are thus an important dietary nutrient that warrants more scrutiny from clinicians and researchers. The potential benefits of supplementation with prebiotics on athletic performance are most likely indirect: they may be associated with the maintenance of gut health and perhaps a reduced risk of some illnesses
which might enhance the athlete's ability to train and compete. Research shows that athletes experiencing higher illness rates over a competition season tend to perform more poorly than their healthier peers. Preventing illness during training and competition is therefore a high priority for athletes. Without any direct clinical studies on athletes, select findings from closely related population cohorts provide a glimpse of the potential benefits of prebiotic supplementation in athletes. Prebiotic supplements have had beneficial effects in reducing GI and respiratory illness symptoms. Altering GI microbiota through the use of prebiotics may also favourably influence host metabolism, which may also have implications for athletes. Consumption of 16 g per day of FOS by 10 healthy adults in a randomised, double-blind placebo-controlled trial found that prebiotic consumption reduced appetite and increased plasma gut peptide concentration. In conclusion, prebiotic supplements may be of benefit to athletes by limiting illness impacting on competitive performance. Prebiotics such as GOS and FOS can modify the composition of the GI microbiota and appear to be associated with lower rates of GI and respiratory illness in healthy adults. Although there are no specific studies showing benefits of prebiotics on the health of athletes, there is accumulating evidence from studies of probiotics that alterations in gut microflora can lead to such valuable outcomes. Further research is required in various athlete populations on the various types of prebiotic supplements, dosage, timing (pre-exercise or postexercise) and underlying mechanisms before definitive guidelines on supplementation can be issued [12458].

Probiotics

Probiotic bacteria are defined as live food ingredients that are beneficial to the health of the host. Probiotics occur naturally in fermented food products such as yogurt, kefir, sauerkraut, cabbage kimchee, and soybean-based miso and natto. Numerous health benefits have been attributed to probiotics, including effects on gastrointestinal tract function and diseases, immune function, hyperlipidemia, hypertension, and allergic conditions. A systematic review of the medical literature failed to identify any studies that directly investigated the potential ergogenic effects of probiotics on athletic performance. Two published articles suggest that probiotics may enhance the immune responses of fatigued athletes. In summary, although scientific evidence for an ergogenic effect of probiotics is lacking, probiotics may provide athletes with secondary health benefits that could positively affect athletic performance through enhanced recovery from fatigue, improved immune function, and maintenance of healthy gastrointestinal tract function [07388].

It was studied effects of probiotics and training volume on oxidized LDL lipids (ox-LDL), serum antioxidant potential (s-TRAP) and serum antioxidants (s-alpha-tocopherol, s-gamma-tocopherol, s-retinol, s-beta-carotene and s-ubiquinone-10) in marathon runners during 3-months training period, 6-days preparation period and marathon run. Runners (n=127) were recruited for a randomized, double-blind intervention during which they received either Lactobacillus rhamnosus GG (LGG, probiotic group) or placebo drink (placebo group) during whole study. During the preparation period, subjects decreased training and increased carbohydrate intake. Blood samples were taken at baseline, before 6-days preparation, before and immediately after the marathon. Probiotics did not have any effect on ox-LDL, s-TRAP or serum antioxidants levels during the study. Interestingly, ox-LDL increased by 28 percent and 33 percent during the preparation period and decreased by 16 percent and 19 percent during the marathon run in the placebo and probiotic groups, respectively. No changes were seen in s-TRAP before marathon, but during run s-TRAP raised by 16 percent in both groups. The increase of ox-LDL level during the preparative period after several months' training suggests that aerobic training may reduce the concentration of ox-LDL and
that decrease of training together with increased energy intake, mainly carbohydrate, before marathon is capable of increasing the level of ox-LDL [12461].

Probiotics are food supplements that contain live microorganisms which when administered in adequate amounts can confer a health benefit on the host. There is now a reasonable body of evidence that regular consumption of probiotic strains that are proven to survive gut transit can modify the population of the gut-dwelling bacteria (microbiota) and influence immune function, though it should be noted that such effects are dose- and strain-dependent. Probiotics modify the intestinal microbiota such that the numbers of beneficial bacteria increase and usually numbers of species considered harmful are decreased. Such changes have been associated with a range of potential benefits to the health and functioning of the digestive system, as well as modulation of immune function. Probiotics have several mechanisms of action. By their growth and metabolism, they help inhibit the growth and reduce potentially harmful effects of other bacteria, antigens, toxins and carcinogens in the gut. In addition, probiotics are known to interact with the gut-associated lymphoid tissue, leading to positive effects on the innate and even the acquired immune system. Studies have shown that probiotic intake can improve rates of recovery from rotavirus diarrhoea, increase resistance to enteric pathogens and promote antitumour activity. Some evidence suggests that probiotics may be effective in alleviating some allergic and respiratory disorders in young children. In recent years, it has become evident that some probiotics, particularly Lactobacillus strains, when ingested on a daily basis, can reduce upper respiratory tract infection (URTI) incidence in adults [12462].

**Probiotic intervention studies in athletes**

Although there are few published studies of the effectiveness of probiotic use in athletes, there is growing interest in examining their potential to help maintain overall general health, enhance immune function or reduce URTI incidence and symptom severity/duration. A L. rhamnosus GG supplement was investigated in 141 marathon runners who were recruited for a randomised, double-blind intervention study in which they received probiotic or placebo daily for a 3-month training period and then participated in a marathon race with a 2-week follow-up of illness symptoms. Although there were no differences in the number of URTI or gastrointestinal symptom episodes, the duration of gastrointestinal symptom episodes in the probiotic group was shorter than in the placebo group during the training period (2.9 vs 4.3 days) and during the 2 weeks after the marathon (1.0 vs 2.3 days). In a double-blind, placebo-controlled, cross-over trial in which healthy elite distance runners received the probiotic L fermentum or placebo daily for 28 days with a 28-day washout period between the initial and the second treatment, athletes (n=20) had fewer days of respiratory illness and lower severity of respiratory illness symptoms when taking the daily probiotic. The probiotic treatment also elicited a twofold greater change in whole-blood culture interferon (IFN) gamma production compared with placebo, which may be one mechanism underpinning the positive clinical outcomes. In another study of athletes who presented with fatigue, impaired performance and a deficit in blood CD4 (T-helper) cell IFN-gamma production compared with healthy control athletes, this apparent T cell impairment was reversed following a 1-month course of daily probiotic (L acidophilus) ingestion. In a study on the effect of a L casei probiotic supplement on URTI and immune and hormonal changes in soldiers participating in 3 weeks of commando training followed by a 5-day combat course, no difference in infection incidence between groups receiving daily probiotic or placebo was reported. Among the immune parameters investigated, the major finding was a significant decrease in salivary immunoglobulin A (IgA) concentration after the combat course in the placebo group, with no change over time in the probiotic group. A recent randomised, placebo-controlled trial in 64 university athletes reported a lower incidence of URTI episodes during a 4-month winter training period in subjects receiving daily probiotic (L casei Shirota) compared with placebo:
this study also reported better maintenance of salivary IgA in the probiotic group. Importantly, in athlete and non-athlete populations, falls in salivary IgA have been associated with increased URTI incidence. Another recent study using L fermentum reported reduced URTI incidence among male but not among female athletes during 11 weeks of training. From the research reviewed, one cannot reach a solid conclusion of probiotic benefit for sports people [12462].

**Blueberries**

The improvement of insulin sensitivity by exercise has been shown to be inhibited by supplementation of vitamins acting as antioxidants. To examine effects of exercise with or without blueberries, containing natural antioxidants, on cardio-metabolic risk factors 15 healthy men and 17 women, 28 ± 7 years old, were recruited, and 26 completed a randomized cross-over trial with 4 weeks of exercise by running/jogging 5 km five times/week and 4 weeks of minimal physical activity. Participants were also randomized to consume 150 g of blueberries, or not, on exercise days. Laboratory variables were measured before and after a 5 km running-race at maximal speed at the beginning and end of each period, i.e. there were four maximal running-races and eight samplings in total for each participant. Insulin and triglyceride levels were reduced while HDL-cholesterol increased by exercise compared with minimal physical activity. Participants randomized to consume blueberries showed an increase in fasting glucose levels compared with controls, during the exercise period (blueberries: from 5.12 ± 0.49 mmol/L to 5.32 ± 0.29 mmol/L; controls: from 5.24 ± 0.27 mmol/L to 5.17 ± 0.23 mmol/L). Triglyceride levels fell in the control group (from 1.1 ± 0.49 mmol/L to 0.93 ± 0.31 mmol/L), while HDL-cholesterol increased in the blueberry group (from 1.51 ± 0.29 mmol/L to 1.64 ± 0.33 mmol/L). It was concluded that ingestion of blueberries induced differential effects on cardio-metabolic risk factors, including increased levels of both fasting glucose and HDL-cholesterol. However, since it is possible that indirect effects on food intake were induced, other than consumption of blueberries, further studies are needed to confirm the findings [13741].

**Wolfberry (goji berry)**

Wolfberry and goji berry are interchangeable terms for the red fruits of either of the two boxthorn plants in the potato family, *Lycium barbarum* (syn. *Lycium halimifolium*) and *Lycium chinense*. Both are important herbal components of traditional Chinese medicine, where they are often referred to as gou qi. The berries (ie, fructus barbarum, fructus lycii) are used for both food and medicine whereas root bark (cortex lycii radicis) is used solely for medicine. These plants should not be confused with *Solanum lycocarpum*, variously known as wolf's apple, wolf's fruit, or fruit-of-the-wolf and containing toxic alkaloids. Wolfberry fruits are the plant component used most often in sports supplements and contain several purported bioactive molecules including carotenoids, flavonoids, vitamins including plentiful vitamin C, sterols and polysaccharides. Bioavailability studies suggest that, when berries are extracted in milk as in traditional Chinese medicine, zeaxanthins (a subclass of carotenoids) are found in the bloodstream, peaking at 6 h postingestion. Many claims are made for the benefits of wolfberries with respect to health, including improved immune function, fertility, skin and eye health and even antiaging. However, good peer-reviewed evidence for many of these effects is hard to find. With respect to health, there do appear to be some reported benefits for preventing diabetic retinopathy and inhibiting prostate cancer progression. Reports of allergy to wolf berries suggest the risk of sensitivity in some individuals, even the risk of anaphylaxis and the potential for skin photosensitivity. In conclusion, there is some evidence that there
may be a few health benefits in using wolfberries (goji berries) but potential side effects are possible. There is, however, little scientific evidence to justify commercial claims that wolfberry (gogi berry) is a ‘super food’ and there is no evidence that it is of any benefit as an ergogenic aid to sports performance [13694].

Green tea

To evaluate the effect of acute ingestion of green tea polyphenols (GTP) on blood markers of oxidative stress and muscle damage in soccer players exposed to intense exercise a randomized, double-blinded study was conducted on 16 players during a general preparation period, when all athletes participated in a strength-training program focused on the development of strength endurance. After ingestion of a single dose of GTP (640 mg) or placebo, all athletes performed an intense muscle-endurance test consisting of 3 sets of 2 strength exercises (bench press, back squat) performed to exhaustion, with a load at 60 percent 1-repetition maximum and 1-min rests between sets. Blood samples were collected preexercise, 5 min after the muscle-endurance test, and after 24 hr of recovery. Blood plasma was analyzed for the concentrations of thiobarbituric acid-reacting substances (TBARS), uric acid (UA), total catechins, total antioxidant status (TAS), and activity of creatine kinase (CK); at the same time, erythrocytes were assayed for the activity of superoxide dismutase (SOD). In both groups, plasma TBARS, UA, and TAS increased significantly postexercise and remained elevated after a 24-hr recovery period. SOD activity in erythrocytes did not change significantly in response to the muscle-endurance test, whereas in both groups plasma CK activity increased significantly after 24 hr of recovery. Acute intake of GTP caused a slight but significant increase in total plasma catechins. However, GTP was found not to exert a significant effect on measured parameters. It was concluded that acute ingestion of GTP (640 mg) does not attenuate exercise-induced oxidative stress and muscle damage [12464].

The effect of green tea beverage and green tea extract on lipid changes is controversial. It was aimed to identify and quantify the effect of green tea and its extract on total cholesterol (TC), LDL cholesterol, and HDL cholesterol. It was performed a comprehensive literature search to identify relevant trials of green tea beverages and extracts on lipid profiles in adults. Weighted mean differences were calculated for net changes in lipid concentrations by using fixed-effects or random-effects models. Study quality was assessed by using the Jadad score, and a meta-analysis was conducted. Fourteen eligible randomized controlled trials with 1136 subjects were enrolled in our current meta-analysis. Green tea consumption significantly lowered the TC concentration by 7.20 mg/dL and significantly lowered the LDL-cholesterol concentration by 2.19 mg/dL. The mean change in blood HDL-cholesterol concentration was not significant. Subgroup and sensitivity analyses showed that these changes were not influenced by the type of intervention, treatment dose of green tea catechins, study duration, individual health status, or quality of the study. Overall, no significant heterogeneity was detected for TC, LDL cholesterol, and HDL cholesterol; and results were reported on the basis of fixed-effects models. The analysis of eligible studies showed that the administration of green tea beverages or extracts resulted in significant reductions in serum TC and LDL-cholesterol concentrations, but no effect on HDL cholesterol was observed [11406].

The aim of one study was to evaluate the systemic effects of an isotonic sports drink on the metabolic status of athletes of the Italian Olympic rowing team during recovery after strenuous and prolonged physical exercise by means of nuclear magnetic resonance (NMR)-based metabolomics analysis on plasma and urine. Forty-four male athletes of the Italian
Olympic rowing team were enrolled in a double-blind crossover study. All subjects underwent 2 evaluations at 1-week intervals. The evaluation was performed on a rowing ergometer after strenuous physical exercise to produce a state of dehydration. Afterward, the athletes were rehydrated either with a green tea-based carbohydrate-hydroelectrolyte drink or with oligomineral water. Three blood samples were drawn for each subject: at rest, after the exercise, and following rehydration, while 2 urine samples were collected: at rest and after the rehydration period. Biofluid samples were analyzed by high-resolution 1H NMR metabolic profiling combined with multilevel simultaneous data-analysis (MSCA) and partial-least squares-discriminant analysis (PLS-DA). The between-subject variations, as evaluated by MSCA, reflected the variations of lactate levels induced by the physical exercise. Analysis of the within-individual variance using multilevel PLS-DA models of plasma and urine metabolic profiles showed an effect of the green tea-based sports drink on glucose, citrate, and lactate levels in plasma and on acetone, 3-OH-butyrate, and lactate levels in urine. The increase of caffeine and hippuric acid levels in urine indicated the absorption of green tea extract components. NMR-based metabolomics allowed the complex effects of a green tea extract-based carbohydrate/hydroelectrolyte beverage on the energy metabolism of athletes during recovery by postexercise rehydration to be evaluated [09353].

Researchers have long been investigating strategies that can increase athletes' ability to oxidize fatty acids and spare carbohydrate, thus potentially improving endurance capacity. Green-tea extract (epigallocatechin-3-gallate; EGCG) has been shown to improve endurance capacity in mice. If a green-tea extract can stimulate fat oxidation and as a result spare glycogen stores, then athletes may benefit through improved endurance performance. Eight male cyclists completed a study incorporating a 3-way crossover, randomized, placebo-controlled, double-blinded, diet-controlled research design. All participants received 3 different treatments (placebo 270 mg, EGCG 270 mg, and placebo 270 mg + caffeine 3 mg/kg) over a 6-day period and 1 hr before exercise testing. Each participant completed 3 exercise trials consisting of 60 min of cycling at 60 percent maximum oxygen uptake (VO2max) immediately followed by a self-paced 40-km cycling time trial. The study found little benefit in consuming green-tea extract on fat oxidation or cycling performance, unlike caffeine, which did benefit cycling performance. The physiological responses observed during submaximal cycling after caffeine ingestion were similar to those reported previously, including an increase in heart rate, glucose at the 40-min exercise time point, and resting plasma free fatty acids and no change in the amount of carbohydrate and fat being oxidized. Therefore, it was concluded that green-tea extract offers no additional benefit to cyclists over and above those achieved by using caffeine [30954].

A subclass of flavonoids called catechins (and specifically epigallocatechin-3-gallate (EGCG)) from green tea (Camellia sinensis from eastern Asia; same source as white tea, black tea, etc) leaves have demonstrated cardiovascular benefits in non-exercise studies, and may also mitigate some deleterious effects of exercise. Most studies of green tea ergogenic properties have been performed in endurance-trained male cyclists with doses ranging from 135 to 270 mg/day EGCG or total catechins. Studies have shown no difference in time trial or endurance performance following green tea supplementation. The influence of green tea extract on metabolism is less clear, with one study showing it promotes lower respiratory exchange ratio values during exercise, thus indicating a higher percentage fat utilisation, but other studies showing no metabolic differences [11297].

The purpose of one study was to evaluate the effects of a long-term (4-week) green tea extract (GTE) supplementation in combination with strength training on selected blood markers of oxidative stress and muscular damage after a short-term exercise in previously untrained men. We hypothesized that GTE supplementation would elevate antioxidant potential and attenuate exercise-induced oxidative stress and muscular damage. Thirty-five
male students were exposed to 4 weeks of strength training and received (in a randomized, double-blind design) GTE (n=17; 640 mg polyphenols/d) or placebo (P; n=18). Before (term I) and after 4 weeks of strength training and supplementation (term II), students performed a short-term muscular endurance test. Blood samples were collected at rest, 5 minutes after the muscular endurance test, and after 24 hours of recovery. Supplementation with GTE enhanced plasma total polyphenols at rest and 5 minutes after the muscular endurance test. Supplementation also contributed to the rise of resting total antioxidant status in plasma. Throughout the experiment (terms I and II), a reduction in plasma lipid hydroxyperoxides was observed 24 hours after the muscular endurance test. Four weeks of strength training resulted in an increase in plasma lipid hydroxyperoxides at rest, but only in the P group. In term I, the muscular endurance test induced an increase in activity of creatine kinase in plasma after 24 hours of recovery in both the P and GTE groups. In term II, plasma creatine kinase activity after 24 hours of recovery was elevated only in the P group. In conclusion, in previously untrained men, dietary supplementation with GTE (in combination with strength training) enhances the antioxidant defense system in plasma at rest and, in turn, may give protection against oxidative damage induced by both short-term muscular endurance test and long-term strength training [11541].

Green tea catechins (GTCs) have been studied in randomized control trials for their lipid-lowering effects. Studies, however, have been small and demonstrated conflicting results. The objective of this study was to perform a systematic review and meta-analysis of randomized controlled trials evaluating the relationship between GTCs and serum lipid levels, including total, low-density lipoprotein (LDL), high-density lipoprotein (HDL) cholesterol, and triglycerides. A systematic literature search of MEDLINE, EMBASE, Cochrane CENTRAL, and the Natural Medicines Comprehensive Database was conducted through March 2010. Randomized controlled trials evaluating GTCs vs control in human beings and reporting efficacy data on at least one of the aforementioned serum lipid endpoints were included. Weighted mean differences for changes from baseline for lipid endpoints were calculated using random-effects models. Twenty trials (n=1,415) met all inclusion criteria. Upon meta-analysis, GTCs at doses ranging from 145 to 3,000 mg/day taken for 3 to 24 weeks reduced total and LDL cholesterol compared to control. GTCs did not significantly alter HDL cholesterol or triglyceride levels. The consumption of GTCs is associated with a statistically significant reduction in total and LDL cholesterol levels; however, there was no significant effect on HDL cholesterol or triglyceride levels [11542].

Green tea catechins have been hypothesized to increase energy expenditure and fat oxidation by inhibiting catechol-O-methyltransferase (COMT) and thus promoting more sustained adrenergic stimulation. Metabolomics may help to clarify the mechanisms underlying their putative physiological effects. The study investigated the effects of 7-day ingestion of green tea extract (GTE) on the plasma metabolite profile at rest and during exercise. In a placebo-controlled, double-blind, randomized, parallel study, 27 healthy physically active males consumed either GTE (n=13, 1200 mg catechins, 240 mg caffeine/day) or placebo (n=14, PLA) drinks for 7 days. After consuming a final drink (day 8), they rested for 2 h and then completed 60 min of moderate-intensity cycling exercise. Blood samples were collected before and during exercise. Plasma was analyzed using untargeted four-phase metabolite profiling and targeted profiling of catecholamines. Using the metabolomic approach, it was observed that GTE did not enhance adrenergic stimulation (adrenaline and noradrenaline) during rest or exercise. At rest, GTE led to changes in metabolite concentrations related to fat metabolism (3-beta-hydroxybutyrate), lipolysis (glycerol) and tricarboxylic acid cycle (TCA) cycle intermediates (citrate) when compared to PLA. GTE during exercise caused reductions in 3-beta-hydroxybutyrate concentrations as well as increases in pyruvate, lactate and alanine concentrations when compared to PLA. It was concluded that GTE supplementation resulted in marked metabolic differences during rest
and exercise. Yet these metabolic differences were not related to the adrenergic system, which questions the in vivo relevance of the COMT inhibition mechanism of action for GTE [13742].

Black tea

There is a belief that caffeinated drinks, such as tea, may adversely affect hydration. This was investigated in a randomised controlled trial. Healthy resting males (n=21) were recruited from the general population. Following 24 h of abstention from caffeine, alcohol and vigorous physical activity, including a 10 h overnight fast, all men underwent four separate test days in a counter-balanced order with a 5 d washout in between. The test beverages, provided at regular intervals, were 4 × 240 ml black (i.e. regular) tea and 6 × 240 ml black tea, providing 168 or 252 mg of caffeine. The controls were identical amounts of boiled water. The tea was prepared in a standardised way from tea bags and included 20 ml of semi-skimmed milk. All food taken during the 12 h intervention period was controlled, and subjects remained at rest. No other beverages were offered. Blood was sampled at 0, 1, 2, 4, 8 and 12 h, and a 24 h urine sample was collected. Outcome variables were whole blood cell count, Na, K, bicarbonate, total protein, urea, creatinine and osmolality for blood; and total volume, colour, Na, K, creatinine and osmolality for urine. Although data for all twenty-one participants were included in the analysis (mean age 36 years and mean BMI 26 kg/m²), nineteen men completed all conditions. Statistical analysis, using a factorial ANOVA approach within PROC MIXED, revealed no significant differences between tea and water for any of the mean blood or urine measurements. It was concluded that black tea, in the amounts studied, offered similar hydrating properties to water [11407].

To consider whether consumption of black tea has a positive or negative impact on health databases were searched for relevant epidemiological and clinical studies published between 1990 and 2004. Clear evidence was found for coronary heart disease (CHD), where an intake of 3 cups per day related to risk reduction. The mechanism could involve the antioxidant action of tea polyphenols. While experimental models have suggested that flavonoids attenuated cancer risk, epidemiological studies failed to demonstrate a clear effect for tea, although there is moderate evidence for a slightly positive or no effect of black tea consumption on colorectal cancer. Studies on cancer were limited by sample sizes and insufficient control of confounders. There is moderate evidence suggestive of a positive effect of black tea consumption on bone mineral density although studies were few. There is little evidence to support the effect of tea on dental plaque inhibition but evidence to support the contribution of tea to fluoride intakes and thus theoretical protection against caries. There was no credible evidence that black tea (in amounts typically consumed) was harmful. Normal hydration was consistent with tea consumption when the caffeine content was < 250 mg per cup. A moderate caffeine intake from tea appeared to improve mental performance, although sample sizes were small. There was no evidence that iron status could be harmed by tea drinking unless populations were already at risk from anaemia. Thus, there was sufficient evidence to show risk reduction for CHD at intakes of 3 cups per day and for improved antioxidant status at intakes of one to six cups per day. A maximum intake of eight cups per day would minimise any risk relating to excess caffeine consumption. Black tea generally had a positive effect on health [06290].

Ginseng
Ginseng is one of the most popular herbal dietary supplements worldwide. Sales in the United States have been reported to be over USD 300 million annually. Ginseng consists of several species belonging to the plant family Araliaceae. The two major forms are Chinese, Korean or Asian ginseng which belong to the genus Panax, and Siberian or Russian ginseng which belongs to the genus Eleutherococcus. The biologically active constituents in Panax ginseng are a complex mixture of triterpene saponins known as ginsenosides. Siberian, or Russian, ginseng consists of the dried roots and rhizome of Eleutherococcus senticosus, and contains phenolics, polysaccharides, and eleutherosides. In China, Eleutherococcus senticosus is known as wujiaseng or Ciwujia, and the proposed active ingredients are ciwujianosides. The ergogenic effect of ginseng is attributed to the ginsenosides, eleutherosides, and ciwujianosides. Ginseng is theorized to influence the hypothalamic-pituitary-adrenal cortex axis, possibly mitigating the catabolic effects of the stress hormone cortisol. Given these theorized anti-stress effects, one theory of ginseng supplementation is to enhance sports performance by allowing athletes to train more intensely or to induce an antifatiguing effect and increase stamina during competition. Other theories have been proposed to explain the potential ergonomic effect on aerobic endurance capacity, including favorable metabolic, hematologic, and cardiovascular functions. Given these postulates, much of the research involving the ergogenicity of ginseng supplementation has focused on cardiovascular or aerobic endurance performance, with some emphasis on psychomotor performance. Earlier research findings relative to the effect of ginseng on endurance performance are equivocal. For example, one reviewer indicated that controlled studies of Asian ginseng found improvement in exercise performance with use of standardized extracts, long duration of supplementation, large numbers of subjects, and elderly subjects. However, most earlier studies reporting positive ergonomic effects have been associated with improper research methodology, including no control or placebo group, no double-blind protocol, no randomization of order of treatment, no statistical analysis, or the use of nonstandardized commercial ginseng preparations containing other potential ergonomic substances. Several recent studies have reported ergonomic effects of Panax ginseng. Others reported that Panax ginseng supplementation (1.35 g/day for 30 days) significantly increased cycle ergometer endurance time in untrained adults. Also using untrained adults, it was found that eight weeks supplementation with Panax ginseng extract (6 g/day) enhanced performance in treadmill running time which, based on serum levels of antioxidant enzymes, was attributed to decreased oxidative stress. However, this one-group study involved a control pre-test followed by a post-test after the eight-week supplementation period; no placebo was utilized. The vast majority of well-controlled studies have reported no significant effect of either Panax ginseng, eleutherooccus senticosus Maxim L, Ciwujia, or a standardized ginseng extract on cardiovascular, metabolic, or psychologic responses to either submaximal or maximal exercise performance, or on maximal or supramaximal performance capacity. Several recent extensive reviews of well-designed studies have concluded that there is an absence of compelling research evidence regarding the efficacy of ginseng use to improve physical performance in humans with one of the reviews focusing solely on eleutherococcus senticosus. Well-controlled studies and detailed reviews indicate that ginseng in its various forms does not enhance exercise or sport performance [06297].

In athletics today, there is no shortage of participants looking for an extra edge in competition. In addition, there is no shortage of nutritional supplements for athletes to use with hopes of reaping ergogenic benefits. Ginseng is and will continue to be one of these supplements consumed by athletes despite little or no scientific data to support its ergogenicity. Multiple different types of ginseng can be consumed; the most studied and most common types are Siberian, Chinese, and American. Although related, each has different active compounds, and likely, different effects on the body. There do not appear to be significant adverse effects when used for short periods, but further studies are needed to confirm this. Similarly, more studies are needed to address the ergogenic potential of
ginseng. At this time, ginsengs cannot be recommended to improve athletic performance, but there may be some utility for athletes by preventing viral upper respiratory infection and improving cognition. One review evaluated recently published literature on ginseng use in athletes [06333].

Ginseng has been one of the most popular herbs said to improve human exercise performance. Unclear and anecdotal information is known about the effect of ginseng on lactate threshold and aerobic performance in humans. The purpose of one study was to investigate the effect of ginseng supplementation on lactate threshold in physically active young men. Sixty men from the Thai Navy, aged 17-22 years old, were randomized into either the ginseng (n=30) or placebo (n=30) group. The ginseng group took 3 grams of 100 percent ginseng orally, while the placebo group took an equal amount of lactose powder each day, for 8 weeks. Blood lactic acid levels for determination of lactate threshold (LT) were measured during an incremental cycle ergometer work. LT exercise performance, and heart rate (HR) responses to exercise were determined at baseline and after 8 weeks of ginseng and placebo consumption. Substrate oxidation rates during steady state exercise were assessed upon study completion. Selected markers for liver and kidney functions, including serum aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, and creatinine were monitored for possible side effects of ginseng. LT before and after 8 weeks of supplementation in the ginseng group, and in the placebo group were not different. Both groups had a similar pattern of exercise heart rate, total exercise time, and peak power output. After 8 weeks, the magnitude of difference between ginseng and placebo groups on oxidation rates of fat or carbohydrate was not statistically significant. There were no abnormal changes of markers of liver and renal functions after ginseng administration. It was concluded that daily administration of 3 g of ginseng for an 8-week period did not improve LT nor did it affect physical performances. Therefore, ginseng supplementation did not exert an ergogenic property on aerobic fitness enhancement in well-fit individuals [07393].

“Chinese herbs” is a broad phrase encompassing species from eastern or central Asia purportedly possessing ergogenic, as well as other, properties. Supplements from these herbs are marketed globally and used by athletes around the world. In contrast to drugs, herbs are commonly grown outdoors, where they may experience considerable environmental variation from one crop to another. Harvesting, storage and extraction methods can also differ greatly between crops, or between manufacturers. All of these factors would impact on potential ergogenic effects. Considering that only a handful of studies exist for only a smattering of herbs, and that the studies themselves differ in experimental design and measures, on top of the variation discussed previously, it is not surprising that multiple groups studying the same species report different results. Ginseng is perhaps the most widely reported Chinese herbal supplement used by athletes. The common name encompasses both American and Asian species (genus Panax) as well as “Siberian ginseng” (genus Eleutherococcus, formerly Acanthopanax). Ginseng is claimed to improve cardiorespiratory function, increase aerobic and anaerobic performance, and improve mental acuity via its ginsenosides (triterpenoid saponins, a subclass of glycosides). However, reviews of original research on ginseng have found conflicting results. The most recent comprehensive reviews concluded that ginseng has not demonstrated efficacy as an ergogenic aid in athletes and, further, that the results of studies reporting benefits may be confounded by unsound methodologies such as inappropriate subject populations (e.g. non-athletes) or inappropriate/absent control groups. Despite numerous testimonials, the ergogenic properties of ginseng have not been substantiated scientifically [10252].

Ginseng is one of the most popular herbal supplements in the world. Although it is used for the treatment and prevention of many ailments, it is also used to increase work efficiency and is purported to increase energy and physical stamina. Athletes use ginseng for its alleged
performance-enhancing attributes. However, many studies examining the pharmacological effects of ginseng on physical performance have not employed sound scientific design and methodology. The purpose of one review was to provide an update on published empirical research focusing primarily on the efficacy of ginseng with respect to physical and athletic performance. Despite attempts in recent investigations to improve on the scientific rigor used in examining the ergogenic properties of ginseng, the authors conclude that many of the same methodological shortcomings observed in earlier studies persist. Enhanced physical performance after ginseng administration in well-designed investigations remains to be demonstrated [09366].

Ginseng is found in many products, with the most common forms being tablet and drinks, and is believed to work as a stimulant to enhance performance by increasing alertness in a similar manner to caffeine. Ginseng refers to Asian and American species of genera Panax and Eleutherococcus, although Panax ginseng root material is most commonly used in ergogenic aids (tablets, extracts and energy drinks). Ginsenosides (triterpinoid saponins; also known as panaxosides) are purported to be the primary bioactive compounds responsible for ginseng ergogenic properties, but other compounds such as caffeine and other methylxanthines are present in ginseng supplements. A recent review of ginseng in athletic contexts concluded that its ergogenic efficacy has not been scientifically validated and that many studies showing benefits may contain methodological flaws. Ginseng is an increasingly frequent ingredient of energy drinks (along with caffeine, taurine and guarana). A recent review of performance-enhancing properties of energy drinks suggested glucose and caffeine, not ginseng, are most likely responsible for observed effects. However, it is difficult to pinpoint the effects of ginseng specifically, as it is often found in conjunction with numerous other ingredients. There is no clear scientific support for ginseng as an ergogenic aid at this time. The case of ginseng may be similar to that of and other supplements where extraneous variability is confounding studies [11297].

Emerging evidence indicates exercise training could mediate mitochondrial quality control through the improvement of mitochondrial dynamics. Ginsenoside Rg3 (Rg3), one of the active ingredients in Panax ginseng, is well known in herbal medicine as a tonic and restorative agent. However, the molecular mechanism underlying the beneficial effects of Rg3 has been elusive. In one study, it was compared the effects of Rg3 administration with aerobic exercise on mitochondrial adaptation in cardiac muscle tissue of Sprague-Dawley (SD) rats. Three groups of SD rats were studied: (1) sedentary control, (2) Rg3-treated and (3) aerobic exercise trained. Both aerobic exercise training and Rg3 supplementation enhanced peroxisome proliferator-activated receptor coactivator 1 alpha (PGC-1alpha) and nuclear factor-E2-related factor 2 (Nrf2) protein levels in cardiac muscle. The activation of PGC-1alpha led to increased mRNA levels of mitochondrial transcription factor A (Tfam) and nuclear related factor 1(Nrf1), these changes were accompanied by increases in mitochondrial DNA copy number and complex protein levels, while activation of Nrf2 increased levels of phase II detoxifying enzymes, including nicotinamide adenine dinucleotide phosphate:quione oxidoreductase 1(NQO1), superoxide dismutase (MnSOD) and catalase. Aerobic exercise also enhanced mitochondrial autophagy pathway activity, including increased conversion of LC3-I to LC3-II and greater expression of beclin1 and autophagy-related protein 7 (ATG7), these effects of aerobic exercise are comparable to that of Rg3. These results demonstrate that Rg3 mimics improved cardiac adaptations to exercise by regulating mitochondria dynamic remodeling and enhancing the quantity and quality of mitochondria [13747].
**Ginkgo biloba**

The Chinese Ginkgo tree is the world's most ancient extant, originating two hundred million years ago, and is the source for Ginkgo biloba leaf extract. Ginkgo biloba is believed to exert its mode of action when its active ingredients, the flavonoids and terpenoids, work in concert. One of the tissue level effects is stimulated release of endothelium-derived relaxing factor, which may enhance muscle tissue blood flow through improved microcirculation. Such an effect could improve aerobic endurance by enhancing muscle tissue oxidation. Most Ginkgo biloba supplementation research has been conducted in the elderly, primarily for its vasoregulatory and cognition-enhancing effects. A meta-analysis and several subsequent studies have shown that Ginkgo biloba improves exercise performance, as evaluated by walking distance, in patients with peripheral arterial disease (PAD). However, although Ginkgo biloba supplementation may improve exercise endurance in patients with PAD, there is no evidence that similar effects occur in healthy young or older athletes [06297].

Ginkgo, traditionally available as ginkgo leaf extracts in teas, is now more commonly found as a supplement in tablet form, with evidence suggesting some utility hemorrheologically. Maidenhair tree (Ginkgo biloba, spelled “ginkgo” but often pronounced “gingko”) is native to eastern Asia. Traditionally, ginkgo leaves were extracted in teas to aid memory or improve circulation, but today supplements are more commonly tablets containing about 25 percent glycosides and about 6.5 percent lactones, the purported bioactive constituents. As an ergogenic aid, ginkgo has been considered for claudication (peripheral artery disease) therapy in older adults; however, studies have found that treatment with ginkgo + exercise therapy is equivocal to exercise therapy alone. Ginkgo extract has been considered for mountain sickness or to offset hemorrheological problems associated with exercise at altitude, with one study suggesting it may or may not mitigate altitude sickness contingent on product-specific differences, which may also explain heterogeneity in previous studies. Effects of ginkgo supplementation on blood pressure or salivary cortisol production differed by gender, nature of stress and time of day. The combined evidence suggests ginkgo may have some utility hemorrheologically, but effects are likely to be inconsistent due to the variation in manufacturing processes [11297].

**Garlic**

Garlic (Allium sativum) is a herb that is used as a food in many countries. From the early ages, it was believed to have different pharmaceutical and physiological properties. Raw garlic contains different compounds, the main one being alliin. It can be physically or chemically processed in order to obtain dry garlic powder, distilled oil, macerated oil or aged garlic extract. The latter is the most commonly used as a phytotherapeutic agent and has also been the most studied in human and animal models. It is claimed that garlic and garlic extracts show pharmaceutical properties such as nutritional, immunomodulatory, antioxidant, microvascular and rheological properties. For these reasons, it is assumed that garlic could be a candidate for an antifatigue agent, and thus it is advertised as an ergogenic agent when consumed alone or mixed with other compounds. According to existing scientific literature, it appears that good evidence exists to conclude that regular and high intakes (6-10 g fresh garlic daily) may improve the peripheral circulation. This effect is achieved through a better vascular relaxation, a change in membrane lipids from the red cells that could increase their flexibility and deformability and a negative effect on blood clotting and platelets adhesion. Regarding immunomodulatory and antioxidative capacity, a meta-analysis has shown anticarcinogenic (colorectal and stomach tumours) effects of regular garlic intakes, approx 18 g of fresh garlic or six cloves daily. The reported beneficial effects of garlic extracts on
oxidative stress and microvascular biology suggest that this food may be a potential candidate for an ergogenic and antifatigue compound. However, although some in vivo or animal studies showed promising results in terms of physical performance or delayed fatigue, this has not been confirmed so far in human athletes. There are some human clinical studies on garlic supplementation and fatigue, but the designs are dubious, and in some of them, garlic has been used in conjunction with other molecules, making the interpretation of the results complicated [11297].

More than three thousand publications in the past have confirmed the efficacy of garlic for the prevention and treatment of a variety of diseases, acknowledging and validating its traditional uses. Garlic is also used for the treatment of fatigue, although the mechanism involved remain unclear. The anti-fatigue function of garlic may be closely related to its many favorable biological and pharmacological effects. In animal studies, garlic has been shown to promote exercise endurance. Differences in the methods of processing garlic result in differences in the intensity of its anti-fatigue effect, and the most favorable form of processing has been shown to be extraction of raw garlic followed by its natural aging for a long period in a water-ethanol mixture. In human studies, it has been confirmed that garlic produces symptomatic improvement in persons with physical fatigue, systemic fatigue due to cold, or lassitude of indefinite cause, suggesting that garlic can resolve fatigue through a variety of actions. Recently, primarily in Japan, attempts have been made to measure the intensity of fatigue objectively and quantitatively using biomarkers. Currently available data strongly suggest that garlic may be a promising anti-fatigue agent, and that further studies to elucidate its application are warranted [07389].

Macroalgae

Macroalgae have for centuries been consumed whole among the East Asian populations of China, Korea, and Japan. Due to the environment in which they grow, macroalgae produce unique and interesting biologically active compounds. Protein can account for up to 47 percent of the dry weight of macroalgae depending on species and time of cultivation and harvest. Peptides derived from macroalgae are proven to have hypotensive effects in the human circulatory system. Hypertension is one of the major, yet controllable, risk factors in cardiovascular disease (CVD). CVD is the main cause of death in Europe, accounting for over 4.3 million deaths each year. In the United States it affects one in three individuals. Hypotensive peptides derived from marine and other sources have already been incorporated into functional foods such as beverages and soups. The purpose of one review was to highlight the potential of heart health peptides from macroalgae and to discuss the feasibility of expanding the variety of foods these peptides may be used in [11411].

Spirulina (microalgae)

Spirulina constitutes microalgae belonging to the cyanobacteria class, with nutritional supplements (tablets, flakes and powders) typically being produced from the cultivation of two species Arthrospira platensis and Arthrospira maxima. It is also used as a feed supplement in the aquaculture, aquarium and poultry industries. Spirulina was primarily recognised as being rich in proteins and essential fatty acids, but recently has attracted attention due to its content of phytochemicals with antioxidant and hypolipidemic properties. Claims for general health benefits from spirulina intake are centuries old. Claimed ergogenic benefits relate to antioxidant defence and favourable changes in substrate utilisation during exercise. Indeed, moderately trained runners who received spirulina supplementation for 4
weeks (6 g/day) showed increased fat utilisation, reductions in markers of antioxidant stress and increased endurance at high intensity exercise (95 % VO\textsubscript{2max}) after 2 hours of submaximal running, compared with a similar trial of supplementation with egg protein placebo. Similarly, enhancements in basal antioxidant status and time to fatigue during an incremental exercise test were reported when untrained students consumed 7.5 g/day of spirulina for 3 weeks. Although these results are of interest, limitations of the current literature include the failure to study well-trained individuals, failure to standardise or identify active ingredients, general issues related to monitoring antioxidant status and the lack of protocols related to sports performance. Spirulina is generally considered safe for human consumption; however, concerns related to spirulina supplements include contamination with heavy metals or toxins produced by blue-green algae. Spirulina may join the list of other antioxidant-rich food sources and extracts which require further investigation of potential benefits to exercise performance, along with mechanisms to explain them [12382].

**Phosphatidylserine**

One study investigated the effects of 750 mg of soybean-derived phosphatidylserine, administered daily for 7 d prior to a bout of eccentric exercise and for 2 d following exercise, on delayed onset of muscle soreness and markers of muscle damage, inflammation, and oxidative stress that followed prolonged downhill running. Following preliminary testing and a familiarization session, eight recreationally active males repeated an individualized downhill run at -17 percent for 51 min at 9 km/h on four occasions (trials 1-4). Trials 1 and 3 were presupplementation control trials. After trials 1 and 3 the subjects received, in a double-blind and crossover fashion, either phosphatidylserine or a glucose polymer placebo. Trials 2 and 3 were separated by a 4-week washout period. Venous blood, perceived soreness ratings, and feeling states were assessed prior to exercise, after exercise, and at 24 and 48 h after exercise during each trial. Downhill running led to elevations in perceived soreness creatine kinase activities, myoglobin concentrations, interleukin-6 (IL-6) concentrations, and lipid hydroperoxide concentrations. However, supplementation did not significantly attenuate these responses. These results suggest that supplementation with 750 mg/day phosphatidylserine for 10 d does not afford additional protection against delayed onset of muscle soreness and markers of muscle damage, inflammation, and oxidative stress that follow prolonged downhill running [06294].

Phosphatidylserine (PtdSer) is a ubiquitous phospholipid species that is normally located within the inner leaflet of the cell membrane. PtdSer has been implicated in a myriad of membrane-related functions. As a cofactor for a variety of enzymes, PtdSer is thought to be important in cell excitability and communication. PtdSer has also been shown to regulate a variety of neuroendocrine responses that include the release of acetylcholine, dopamine and noradrenaline. Additionally, PtdSer has been extensively demonstrated to influence tissue responses to inflammation. Finally, PtdSer has the potential to act as an effective antioxidant, especially in response to iron-mediated oxidation. The majority of the available research that has investigated the effects of PtdSer supplementation on humans has concentrated on memory and cognitive function; patients experiencing some degree of cognitive decline have traditionally been the main focus of investigation. Although investigators have administered PtdSer through intravenous and oral routes, oral supplementation has wider appeal. Indeed, PtdSer is commercially available as an oral supplement intended to improve cognitive function, with recommended doses usually ranging from 100 to 500 mg/day. The main sources that have been used to derive PtdSer for supplements are bovine-cortex (BC-PtdSer) and soy (S-PtdSer); however, due to the possibility of transferring infection through the consumption of prion contaminated brain, S-PtdSer is the preferred supplement for use in
humans. Although the pharmacokinetics of PtdSer have not been fully elucidated, it is likely that oral supplementation leads to small but quantifiable increases in the PtdSer content within the cell membrane. A small number of peer-reviewed full articles exist that investigate the effects of PtdSer supplementation in the exercising human. Early research indicated that oral supplementation with BC-PtdSer 800 mg/day moderated exercise-induced changes to the hypothalamo-pituitary-adrenal axis in untrained participants. Subsequently, this finding was extended to suggest that S-PtdSer 800 mg/day reduced the cortisol response to overtraining during weight training while improving feeling of well-being and decreasing perceived muscle soreness. However, equivocal findings from our laboratory might suggest that the dose required to undertake this neuroendocrine action may vary between participants. Interestingly, recent findings demonstrating that short-term supplementation with S-PtdSer 750 mg/day improved exercise capacity during high-intensity cycling and tended to increase performance during intermittent running might suggest an innovative application for this supplement. With the findings from the existing body of literature in mind, one article focused on the potential effects of PtdSer supplementation in humans during and following exercise [06295].

**Pycnogenol**

Pycnogenol (also referred to commonly as picnogel or pycnogel) is a combination of active bioflavonoids produced from the bark of the Pinus maritime pine tree. Pycnogenol supplementation has been reported to have a wide array of health benefits, including improved cognitive function, endothelial function, blood pressure regulation and venous insufficiency. Pycnogenol also may act as an anti-inflammatory agent. In most instances, reference is made to pycnogenol as a powerful antioxidant due to the proportionally high levels of procyanadins within the compound. Recommended doses of pycnogenol range widely and depend upon the treatment aim. For example, to combat chronic venous insufficiency, recommended doses range from 150 to 360 mg/day, whereas others have recommended approximately 75 to 90 mg/day to prevent oxidative tissue damage. In a majority of clinical trials, the duration of supplementation is generally 2 to 3 months. Side effects of pycnogenol supplementation are minimal. There is at least one commercial sports supplement based on pycnogenol which claims to enhance performance and fatigue resistance, but preliminary evidence of such benefits requires further substantiation. One problem of such supplements, however, is the lack of information on the dosage of claimed active ingredients [12462].

**Kava kava (kava)**

Kava kava, or kava, is the peeled and dried root of Piper methysticum G. Forster, a centuries-old South Pacific herb used as a ritual beverage for its relaxing or calming properties. Kava root contains kava lactones (kava pyrones). The neuropharmacologic effects of kava include analgesia, sedation, and skeletal muscle relaxation, but not central nervous system depression. The mechanism is not clear, but blockage of the GABA or norepinephrine neuroreceptors may be involved. Kava is theorized to reduce excess anxiety and/or hand tremor that may disrupt performance in many sports, such as archery and pistol shooting. Kava has been marketed for its antidepressant or anti-anxiety effects, a possible alternative to prohibited or potentially risky ergogenic drugs in such sports, such as alcohol and beta blockers. A recent meta-analysis has suggested that kava extract may be effective for reduction of anxiety symptoms [06297].
St. John's wort

St. John's wort consists of the dried parts of Hypericum perforatum. St. John's wort contains many phytochemicals, including flavonoids, phenolic acids, sterols, tannins, two naphthodianthrones (hypericin and pseudohypericin), and a phloroglucinol derivative (hyperforin). St. John's wort is used therapeutically as an antidepressant. Hyperforin is thought to be the primary active ingredient in antidepressant activity, but hypericin and pseudohypericin may also be important. All are thought to help maintain optimal brain neurotransmitter levels including serotonin, norepinephrine and dopamine. Comparable to kava, St. John's wort may be theorized to reduce anxiety and hand tremor in some athletes. Additionally, as serotonin is involved in appetite control, St. John's wort is theorized to help induce weight loss, which could confer a mechanical advantage to some athletes. Reviews and meta-analyses of research with St. John's wort conclude its effects on treatment of depression are inconsistent and confusing, some showing benefits comparable to standard antidepressant drugs while others noting minimal beneficial effects. Unfortunately, however, no research evaluating the potential ergogenic effect of St. John's wort supplementation on exercise or sport performance has been uncovered. Moreover, no data are available supporting St. John's wort as a means of promoting leanness in athletes [06297].

Quercetin

It was investigated whether 6 wk of antioxidant supplementation (AS) would enhance 30 km time trial (TT) cycling performance. Eleven elite male cyclists completed a randomized, double-blind, cross-over study to test the effects of twice daily AS containing essential vitamins plus quercetin (FRS), and AS minus quercetin (FRS-Q) versus a baseline TT (B). MANOVA analysis showed that time to complete the 30 km TT was improved by 3 percent on FRS compared to B, and by 2 percent over the last 5 km. Absolute and relative (\%HR_{max}) heart rates and percent VO_{2max} were not different between trials, but average and relative power (\% peak power) was higher on FRS. Rates of carbohydrate and fat oxidation were not different between trials. Thus, FRS supplementation significantly improved high-intensity cycling TT performance through enhancement of power output. Further study is needed to determine the potential mechanism(s) of the antioxidant efficacy [06296].

To investigate the effects of 6 weeks of quercetin supplementation on physical performance during military physical training it was used a randomized, double-blind, repeated-measures, placebo-controlled design, and 58 healthy, moderately trained men and women were randomly assigned to quercetin (Q) or placebo (P) groups. Peak oxygen uptake (VO_{2peak}) during maximal effort uphill treadmill running and four physical performance measures (Army Physical Fitness Test, Baumgartner Modified Pull-Up Test, Wingate Anaerobic Test and a 36.6-m sprint) were evaluated before and after 42-54 days of supplementation with 1 g/d of quercetin with vitamins and other substances in a soft chew or placebo chew. It was found that pretreatment-to-posttreatment changes in VO_{2peak} and physical performance were not significantly different in the two groups [10534].

The purpose of one study was to measure the influence of quercetin on plasma cytokines, leukocyte cytokine mRNA, and related variables in ultramarathoners competing in a 160-km run. Sixty-three runners were randomized to quercetin and placebo groups and under double-blinded methods ingested 1000 mg/day quercetin for 3 weeks before the run. Thirty-nine of the 63 subjects (n=18 for quercetin, n=21 for placebo) finished the race and provided blood samples the morning before the race and 15-30 min postrace. Significant prerace to
postrace runner increases were measured for nine proinflammatory and anti-inflammatory plasma cytokines, cortisol, serum C-reactive protein (CRP), and creatine kinase with no significant group differences. Interleukin-6 (IL-6) mRNA did not change after the run, with a significant decrease measured for leukocyte IL-8 mRNA and significant increases for IL-1Ra mRNA and IL-10 mRNA, with no significant differences between groups. In conclusion, quercetin ingestion (1 g/day) by ultramarathon athletes for 3 weeks before a competitive 160-km race significantly increased plasma quercetin levels but failed to attenuate muscle damage, inflammation, increases in plasma cytokine and hormone levels, and alterations in leukocyte cytokine mRNA expression [08431].

Quercetin, a natural polyphenolic flavonoid substance present in a variety of food plants, has been shown in vitro and in animal studies to have widespread health and performance benefits resulting from a combination of biological properties, including antioxidant and anti-inflammatory activity, as well as the ability to increase mitochondrial biogenesis. Little is known about these effects in humans, however, especially with respect to exercise performance. The authors determined whether quercetin ingestion would enhance maximal aerobic capacity and delay fatigue during prolonged exercise in healthy but untrained participants. Twelve volunteers were randomly assigned to 1 of 2 treatments: (a) 500 mg of quercetin twice daily dissolved in vitamin-enriched Tang or (b) a nondistinguishable placebo (Tang). Baseline VO$_{2\text{max}}$ and bike-ride times to fatigue were established. Treatments were administered for a period of 7 days using a randomized, double-blind, placebo-controlled, crossover study design. After treatment both VO$_{2\text{max}}$ and ride time to fatigue were determined. Seven days of quercetin feedings were associated with a modest but significant increase in VO$_{2\text{max}}$ (3.9 % vs placebo) along with a substantial (13.2 %) increase in ride time to fatigue. These data suggest that as little as 7 days of quercetin supplementation can increase endurance without exercise training in untrained participants. These benefits of quercetin may have important implications for enhancement of athletic and military performance. This apparent increase in fitness without exercise training may have implications beyond that of performance enhancement to health promotion and disease prevention [10256].

There is increased interest among such diverse groups as the military, athletes, and the aged for novel plant-derived dietary supplements to enhance performance and maintain/improve health. Quercetin, a flavonoid found in fruits and vegetables, has unique biological properties that are likely to improve mental/physical performance and reduce infection risk during intense exercise. These properties include antiinflammatory, antioxidant, and psychostimulant activity, as well as the ability to stimulate mitochondrial biogenesis, and they form the basis for potential benefits to overall health and disease resistance. However, most information regarding quercetin is based upon in vitro and animal studies. Therefore, there is a pressing need for well-designed clinical trials to evaluate this novel dietary supplement further. One article examined the recent scientific literature concerning the role of quercetin in mental and physical performance and health [09367].

Previous research indicates that ultramarathon exercise can result in blood oxidative stress. The purpose of one investigation was to examine the efficacy of oral supplementation with quercetin, a naturally occurring compound with known antioxidant properties, as a potential countermeasure against blood oxidative stress during an ultramarathon competition. In double-blind fashion, 63 participants received either oral quercetin (250 mg, 4x/day; 1,000 mg/day total) or quercetin-free supplements 3 weeks before and during the 160-km Western States Endurance Run. Results show that quercetin supplementation did not affect race performance. In response to the ultramarathon challenge, aqueous-phase antioxidant capacity (ferric-reducing ability of plasma) was similarly elevated in athletes in both quercetin and quercetin-free treatments and likely reflects significant increases in plasma urate levels.
Alternatively, trolox-equivalent antioxidant capacity was not altered by exercise or quercetin. Accordingly, neither F2-isoprostances nor protein carbonyls were influenced by either exercise or quercetin supplementation. In the absence of postrace blood oxidative damage, these findings suggest that oral quercetin supplementation does not alter blood plasma lipid or aqueous-phase antioxidant capacity or oxidative damage during an ultramarathon challenge [09368].

Previous evidence suggests that quercetin supplementation increases performance in humans. It was examined the effects of 3 weeks of quercetin supplementation on fuel utilization, gross efficiency (GE), and perceived effort during 3 h of cycling over 3 successive days. Forty cyclists were randomized into quercetin and placebo groups and tested for maximal oxygen consumption. For 3 weeks following maximal oxygen consumption testing, subjects supplemented either 1000 mg/day quercetin or placebo during normal training. Following supplementation, subjects cycled at 57 percent maximum power for 3 h, on 3 successive days, using their own bicycles fitted to CompuTrainer Pro Model trainers. Metabolic measurements were taken every 30 min for each 3-h ride. Muscle biopsies obtained from the vastus lateralis immediately pre-exercise and postexercise on days 1 and 3 were analyzed for muscle glycogen content. Power output remained constant for all 3 exercise trials, but significant decreases over time were measured for GE, cadence, respiratory exchange ratio, blood glucose, and muscle glycogen. Significant increases were measured for heart rate and volume of oxygen consumption over time. No quercetin treatment effect was observed for any of the outcome measures in this study. These data indicate that GE is reduced during an exhausting 3-h bout of exercise. However, quercetin did not significantly affect any outcomes in these already well-trained subjects [09369].

The purpose of one study was to measure the influence of quercetin on plasma cytokines, leukocyte cytokine mRNA, and related variables in ultramarathoners competing in the 160-km Western States Endurance Run (WSER). Sixty-three runners were randomized to quercetin and placebo groups and under double-blinded methods ingested 1000 mg/day quercetin for 3 weeks before the WSER. Thirty-nine of the 63 subjects (n=18 for quercetin, n=21 for placebo) finished the race and provided blood samples the morning before the race and 15-30 min postrace. Significant prerace to postrace WSER increases were measured for nine proinflammatory and anti-inflammatory plasma cytokines, cortisol, serum C-reactive protein (CRP) and creatine kinase (CK) with no significant group differences. Interleukin-6 (IL-6) mRNA did not change post-WSER, with a significant decrease measured for leukocyte IL-8 mRNA and significant increases for IL-1Ra mRNA and IL-10 mRNA, with no significant differences between groups. In conclusion, quercetin ingestion (1 g/day) by ultramarathon athletes for 3 weeks before a competitive 160-km race significantly increased plasma quercetin levels but failed to attenuate muscle damage, inflammation, increases in plasma cytokine and hormone levels, and alterations in leukocyte cytokine mRNA expression [07392].

Quercetin supplementation increases muscle oxidative capacity and endurance in mice, but its ergogenic effect in humans has not been established. One study investigated the effects of short-duration chronic quercetin supplementation on muscle oxidative capacity; metabolic, perceptual, and neuromuscular determinants of performance in prolonged exercise; and cycling performance in untrained men. Using a double-blind, pretest-posttest control group design, 30 recreationally active, but not endurance-trained, young men were randomly assigned to quercetin and placebo groups. A noninvasive measure of muscle oxidative capacity (phosphocreatine recovery rate using magnetic resonance spectroscopy), peak oxygen uptake ($V\text{O}_{2\text{peak}}$), metabolic and perceptual responses to submaximal exercise, work performed on a 10-min maximal-effort cycling test following the submaximal cycling, and voluntary and electrically evoked strength loss following cycling were measured before and
after 7-16 days of supplementation with 1 g/day of quercetin in a sports hydration beverage or a placebo beverage. Pretreatment-to-posttreatment changes in phosphocreatine recovery time constant, $V_{0\text{peak}}$, substrate utilization, and perception of effort during submaximal exercise, total work done during the 10-min maximal effort cycling trial, and voluntary and electrically evoked strength loss were not significantly different in the quercetin and placebo groups. Short duration, chronic dietary quercetin supplementation in untrained men does not improve muscle oxidative capacity; metabolic, neuromuscular and perceptual determinants of performance in prolonged exercise; or cycling performance. The null findings indicate that metabolic and physical performance consequences of quercetin supplementation observed in mice should not be generalized to humans [09370].

Maintenance of repeated-sprint performance is a goal during team-sport competition such as soccer. Quercetin has been shown to be an adenosine-receptor antagonist and may reduce oxidative stress via inhibition of the enzyme xanthine oxidase (XO). The purpose of the study was to determine the effect of quercetin consumption on performance of repeated sprints and, secondarily, the XO and inflammatory-marker response induced by repeated-sprint exercise. Fifteen recreationally active, young adult men completed 2 repeated-sprint tests (RST), 12 × 30-m maximal-effort sprints (S1-S12), each after 1 week supplementation with a placebo, a 6 percent carbohydrate commercial sports drink, or that drink with 500 mg of quercetin-3-glicoside, consumed twice a day (1,000 mg/d). Blood samples were collected before supplementation (B0), at baseline before each RST (B1), immediately after RST (B2), and 1 hr after RST (B3). Mean sprint time increased progressively and was significantly higher by S9 for both treatments (6 %); however, there were no significant differences between treatments. Percent fatigue decrement (%FD) for placebo (4 %) was significantly less than with quercetin (5 %). Changes in blood XO, IL-6, and uric acid from B1 to B2 were +47, +77, and +25 percent, respectively, with no difference by treatment. In conclusion, repeated-sprint performance was not improved by quercetin supplementation and was worse than with placebo when expressed as %FD. Quercetin did not attenuate indicators of XO activity or IL-6, a marker of the inflammatory response after sprint exercise [11296].

It has been demonstrated to be effective in increasing physical endurance in mice and humans. However, the mechanisms involved are not fully understood. In one study, male Kunming mice were fed a diet containing 0.1 percent quercetin for 14 days before swimming for 60 min. The overall serum metabolic profile was investigated by a $^1$H nuclear magnetic resonance-based metabolomic approach. Serum glucose, lactate, nonesterified fatty acids (NEFA), and nonprotein nitrogen (NPN), as well as hepatic and muscular glycogen were measured biochemically. The results of metabolomic analysis showed that swimming induced a significant change in serum metabolic profile. Relative increases in the levels of lactate, alanine, low-density lipoprotein-very low-density lipoprotein, and unsaturated fatty acids, and decreases in choline, phosphocholine, and glucose were observed after swimming. With quercetin supplementation, these changes were attenuated. The results of biochemical assays were consistent with the data obtained from metabolomic analysis, in that serum NEFA was increased while lactate and NPN decreased after exposed to quercetin in swimming mice. Similar change in NEFA was also found in liver and gastrocnemius muscle tissues. The current findings suggest that quercetin alters energy metabolism in swimming mice and increased lipolysis may contribute to the actions of quercetin on physical endurance [12469].

Epidemiological studies support multiple disease prevention benefits for individuals consuming foods rich in the flavonol quercetin. In vitro and animal studies indicate that quercetin is a strong antioxidant and anti-inflammatory agent, and exerts antipathogenic and immune regulatory influences. Quercetin supplementation studies in community-dwelling humans do not reflect these positive benefits, but research is continuing in order to
determine the proper outcome measures, dosing regimen and adjuvants that may amplify any perceived bioactive effects of quercetin in vivo. Quercetin supplementation studies in athletes have focused on potential influences on post-exercise inflammation, oxidative stress and immune dysfunction, illness rates following periods of physiological stress and exercise performance. Results thus far have been negative for quercetin's countermeasure effects on postexercise physiological stress indicators, such as immune perturbations. However, when quercetin supplementation is combined with other polyphenols and food components such as green tea extract, isoquercetin and fish oil, a substantial reduction in exercise-induced inflammation and oxidative stress occurs in athletes, with augmentation of innate immune function. Quercetin exerts strong antiviral activities when cultured with a wide variety of pathogens. In mice, quercetin supplementation for 7 days before inoculation with influenza virus and a 3-day period of heavy exertion partially reduced the exercise-induced increase in morbidity and mortality. A 12-week community trial showed a modest reduction in upper respiratory tract infections (URTI) among physically active subjects between the ages of 40 and 85 years consuming 1000 mg quercetin per day, but not among younger adults. Cyclists randomised to 1000 mg/day quercetin or placebo for five weeks experienced reduced URTI incidence during the two-week period following three days of exhaustive exercise. Quercetin supplementation over 7 days induces an increase in mitochondrial biogenesis and treadmill endurance performance (37%) and running distance in wheels in mice. The quercetin-related effects on performance in untrained humans are modest and far below those reported in mice. About 10 different exercise studies have been conducted and, despite conflicting results regarding the effect of quercetin supplementation on endurance exercise capacity, a meta-analysis indicated an ergogenic effect which the authors described as being between trivial and small (about 3%) but which was significant [12470].

It was aimed to test exercise-induced adaptations on skeletal muscle when quercetin is supplemented. Four groups of rats were tested: quercetin sedentary, quercetin exercised, placebo sedentary, and placebo exercised. Treadmill exercise training took place 5 days a week for 6 weeks. Quercetin groups were supplemented with quercetin, via gavage, on alternate days throughout the experimental period. Sirtuin 1 (SIRT1), peroxisome proliferator-activated receptor gamma coactivator-1alpha mRNA levels, mitochondrial DNA (mtDNA) content, and citrate synthase (CS) activity were measured on quadriceps muscle. Redox status was also quantified by measuring muscle antioxidant enzymatic activity and oxidative damage product, such as protein carbonyl content (PCC). Quercetin supplementation increased oxidative damage in both exercised and sedentary rats by inducing higher amounts of PCC. Quercetin supplementation caused higher catalase and superoxide dismutase activity in the non-exercised animals, but not when quercetin is supplemented during exercise. Quercetin supplementation increased SIRT1 expression, but when quercetin is supplemented during exercise, this effect is abolished. The combination of exercise and quercetin supplementation caused lower mtDNA content and CS activity when compared with exercise alone. Quercetin supplementation during exercise provides a disadvantage to exercise-induced muscle adaptations [13746].

Soldiers

The purpose of one study was to assess the short-term effects of quercetin supplementation on aerobically demanding soldier performance. In a double-blind crossover study, 16 male soldiers performed 3 days of aerobically demanding exercise under 3 conditions: Baseline (B), Placebo (P), and Quercetin (Q). Day 1 was a treadmill VO₂peak test. Days 2 and 3 were identical, consisting of 75 minutes of loaded treadmill marching (LM) and a subsequent cycling time trial (TT) to complete 200 kJ of work. After B condition, the soldiers consumed 2 energy bars, each containing 0 mg (placebo) or 500 mg of quercetin (1,000 mg/days) for 8.5 days. Beginning day 6 of supplementation, the soldiers performed the 3 exercise days. There
was a significant increase in plasma Q after Q supplementation. Repeated measures analyses of variance revealed no differences after P or Q supplementation as compared with B in VO₂peak or TT time. The respiratory exchange ratio during LM did not differ across treatments. Ratings of perceived exertion were not affected by Q supplementation during the VO₂peak test, LM or TT. Supplementation of 1,000 mg/day of quercetin for 8.5 days had no positive effect on aerobically demanding soldier performance. It is possible that a different dosing regimen, a combination of antioxidants or a different form of quercetin supplementation, may be needed to produce an increase in soldier performance.

Mice

Quercetin has been demonstrated to be effective in increasing physical endurance in mice and humans. However, the mechanisms involved are not fully understood. In one study, male Kunming mice were fed a diet containing 0.1 percent quercetin for 14 days before swimming for 60 min. The overall serum metabolic profile was investigated by a ¹H nuclear magnetic resonance-based metabolomic approach. Serum glucose, lactate, nonesterified fatty acids (NEFA), and nonprotein nitrogen (NPN), as well as hepatic and muscle glycogen were measured biochemically. The results of metabolomic analysis showed that swimming induced a significant change in serum metabolic profile. Relative increases in the levels of lactate, alanine, low-density lipoprotein-very low-density lipoprotein, and unsaturated fatty acids, and decreases in choline, phosphocholine, and glucose were observed after swimming. With quercetin supplementation, these changes were attenuated. The results of biochemical assays were consistent with the data obtained from metabolomic analysis, in that serum NEFA was increased while lactate and NPN decreased after exposure to quercetin in swimming mice. Similar change in NEFA was also found in liver and gastrocnemius muscle tissues. The findings suggest that quercetin alters energy metabolism in swimming mice and increased lipolysis may contribute to the actions of quercetin on physical endurance.

Capsaicin

Performance in many team sports is partially dependent on the ability to perform repeatedly at high intensity. Previous research demonstrates that capsaicin (CAP) has physiological and metabolic effects that could influence exercise performance and inflammation. The purpose of this study was to investigate the influence of CAP on performance of and the interleukin-6 (IL-6) response to repeated sprints. Nineteen healthy male experienced athletes, age 18-30 years, participated in a placebo (PCB)-controlled, crossover study. During 1 trial, they consumed 3 g/d cayenne (26 mg/d CAP) and the other a PCB for days. Directly after the supplementation period, they completed a repeated sprint test (RST) consisting of 30 maximal effort sprints on 35-second intervals with sprint times measured via an electronic dual-beam timing system. Fasted blood draws for IL-6 were taken at baseline before supplementation, 45 minute pre-RST, and immediately post-RST. Rate of perceived exertion (RPE), muscle soreness (MS), and gastrointestinal distress (GD) for 5 symptom subscales were measured 1-minute pretest, during, posttest, and 1-minute posttest. The MS was additionally measured for 3-day posttest. Relative to the PCB, CAP significantly increased the sum of ratings of GD symptoms by 6.3-fold. There was no difference between treatments in fastest or mean sprint time, fatigue, IL-6 response, RPE, or MS. In summary, CAP did not influence repeated sprint performance or the IL-6 response but caused substantial GD. The CAP is not recommended for athletes involved in repeated sprinting.
The Capsicum species (C. annuum; C. frutescens), native to tropical America, incorporates such peppers as the cayenne, red, and chili. The medicinal properties of the capsicum species are attributable to a compound known as capsaicin. The United States Pharmacopeia has classified capsaicin as a stimulant, and based on their previous research have related its physiological action to caffeine, i.e., ingestion may induce sympathetic activation of the central nervous system, increasing catecholamine secretion and enhancing lipid oxidation, sparing the use of glycogen. Some research supports this hypothesis. For example, when fed capsaicin (2 mg/kg), males at rest and exposed to immersion in cold water had a significant decrease in carbohydrate as an energy source. However, limited research is available regarding the effect of capsaicin on carbohydrate metabolism during exercise. In a well-designed study, it was evaluated the effect of a breakfast meal containing 10 g of dried, hot red pepper on energy substrate use in male runners during rest and exercise (cycling at about 60 percent VO\(_{2\text{max}}\)). For the red pepper trial, plasma epinephrine and norepinephrine concentrations were significantly elevated after 30 min, but not at 60 and 150 min of rest. The hot pepper meal significantly elevated the respiratory quotient (RQ) and blood lactate levels at rest and during exercise, but there was no effect on oxygen consumption or energy expenditure during rest or exercise. These results suggest that contrary to the theory of glycogen sparing, hot red pepper ingestion stimulates carbohydrate oxidation at rest and during exercise. The authors suggested that hot red pepper ingestion before exercise could decrease endurance performance in athletes due to associated muscle and/or liver glycogen depletion. Currently, no scientific evidence is available to support an ergogenic effect of capsaicin supplementation. Additional research is merited [06297].

**Yucca**

Yucca encompasses about 40-50 medicinally potent plant species that generally thrive in arid parts of southwestern USA and Mexico. Although medical research into yucca is very limited, the most researched species is Yucca schidigera (Mojave yucca), which is found in a variety of food supplements. Yucca has a reputed place in folk medicine for a variety of conditions with the most mentioned anti-inflammatory and antiarthritic effects. The yucca extract is widely used as an animal feed additive to increase growth rate, improve feed conversion efficiency and to ease joint pains in horses and dogs. Yucca has also been shown to have antioxidant, anticancer, antidiabetic, antimicrobial and hypcholesterolaemic properties. Steroidal saponins, resveratrol and yuccaol have been identified as active principles. Having identified this combination of substances, although there is no scientific evidence, there has been an interest in yucca from the sports medicine community. Yucca saponins are precursors to cortisone. Yuccaols and resveratrol, which are mainly found in the yucca bark, are known to have a variety of actions, including inhibitors of the nuclear transcription factor kappaB (NFκB) and thus anti-inflammatory, antioxidant and free radical scavengers. In addition, resveratrol has been shown to have an influence on muscle fibres, strength and possible ergogenic effects. Resveratrol, on its own, or in the form of a yucca extract, has several potential indications in sport and exercise medicine, which warrant further research efforts. However, there are currently no established oral doses or recommendations, and there is no evidence of an ergogenic benefit in sports performance [13749].

**Teribulus terrestris**

Tribulus terrestris, commonly known as puncture vine, is an herbal preparation that has been used medicinally as a diuretic as well as treatment for hypertension and
hypercholesterolemia, and has been used for centuries in Europe as treatment for impotence. The purported active ingredients are saponins and protodiosin. As ergogenic aids, Tribulus terrestris is used in the belief that they may elicit anabolic effects via increased testosterone production. Although limited, the available research does not support an ergogenic effect of Tribulus terrestris supplementation in humans. It was found that consumption of either 10 or 20 mg/kg body weight of Tribulus terrestris extract daily for four weeks had no effect on serum testosterone or androstenedione. Also, in a double-blind, placebo-controlled study, Tribulus terrestris supplementation exerted no effect on body weight, body composition, maximal strength or muscular endurance in resistance-trained males during training; plasma testosterone levels were not measured [06297].

Tribulus terrestris is a nutritional supplement highly debated regarding its physiological and actual effects on the organism. The main claimed effect is an increase of testosterone anabolic and androgenic action through the activation of endogenous testosterone production. Even if this biological pathway is not entirely proven, T. terrestris is regularly used by athletes. Recently, the analysis of two female urine samples by GC/C/IRMS (gas chromatography/combustion/isotope-ratio-mass-spectrometry) conclusively revealed the administration of exogenous testosterone or its precursors, even if the testosterone glucuronide/epitestosterone glucuronide (T/E) ratio and steroid marker concentrations were below the cut-off values defined by World Anti-Doping Agency. To argue against this adverse analytical finding, the athletes recognized having used T. terrestris in their diet. In order to test this hypothesis, two female volunteers ingested 500 mg of T. terrestris, three times a day and for two consecutive days. All spot urines were collected during 48h after the first intake. The $^{13}$C/$^{12}$C ratio of ketosteroids was determined by GC/C/IRMS, the T/E ratio and DHEA concentrations were measured by GC/MS and LH concentrations by radioimmunoassay. None of these parameters revealed a significant variation or increased above the WADA cut-off limits. Hence, the short-term treatment with T. terrestris showed no impact on the endogenous testosterone metabolism of the two subjects [08430].

Tribulus terrestris (caltrop, puncturevine) grows in southern Asia and is consumed primarily by anaerobic athletes for its purported strength-enhancing capacities. Steroidal saponins (glycosides) are the purported bioactive compounds, believed to upregulate testosterone production thereby increasing muscle mass and strength. A study of young male rugby players supplemented with T terrestris for 5 weeks (60 % saponins by content) showed no effect of supplementation on strength or fat-free mass. Dosing did not influence urinary testosterone/epitestosterone levels. This study is representative of the few others on the topic [10252].

Tribulus terrestris is an herbal nutritional supplement that is promoted to produce large gains in strength and lean muscle mass in 5-28 days. Although some manufacturers claim T. terrestris will not lead to a positive drug test, others have suggested that T. terrestris may increase the urinary testosterone/epitestosterone (T/E) ratio, which may place athletes at risk of a positive drug test. The purpose of one study was to determine the effect of T. terrestris on strength, fat free mass, and the urinary T/E ratio during 5 weeks of preseason training in elite rugby league players. Twenty-two Australian elite male rugby league players were match-paired and randomly assigned in a double-blind manner to either a T. terrestris (n=11) or placebo (n=11) group. All subjects performed structured heavy resistance training as part of the club’s preseason preparations. A T. terrestris extract (450 mg/day) or placebo capsules were consumed once daily for 5 weeks. Muscular strength, body composition, and the urinary T/E ratio were monitored prior to and after supplementation. After 5 weeks of training, strength and fat free mass increased significantly without any between-group differences. No between-group differences were noted in the urinary T/E ratio. It was concluded that T. terrestris did not produce the large gains in strength or lean muscle mass
that many manufacturers claim can be experienced within 5-28 days. Furthermore, T. terrestris did not alter the urinary T/E ratio and would not place an athlete at risk of testing positive based on the World Anti-Doping Agency's urinary T/E ratio limit of 4:1 [07391].

Two oligosaccharides and a stereoisomer of di-p-coumaroylquinic acid were isolated from the aerial parts of Tribulus terrestris along with five known compounds. The structures of the compounds were established as O-beta-D-fructofuranosyl-(2→6)-alpha-D-glucopyranosyl-(1→6)-beta-D-fructofuranosyl-(2→6)-beta-D-fructofuranosyl-(2→1)-alpha-D-glucopyranosyl-(6→2)-beta-D-fructofuranoside, O-alpha-D-glucopyranosyl-(1→4)-alpha-D-glucopyranosyl-(1→4)-alpha-D-glucopyranosyl-(1→2)-beta-D-fructofuranoside, 4,5-di-p-cis-coumaroylquinic acid by different spectroscopic methods including 1D NMR (¹H, ¹³C and DEPT) and 2D NMR (COSY, TOCSY, HMQC and HMBC) experiments as well as ESI-MS analysis. This is the first report for the complete NMR spectral data of the known 4,5-di-p-trans-coumaroylquinic acid. The antioxidant activity represented as DPPH free radical scavenging activity was investigated revealing that the di-p-coumaroylquinic acid derivatives possess potent antioxidant activity so considered the major constituents contributing to the antioxidant effect of the plant [13753].

**Arnica**

Arnica montana is a herbaceous plant, native to many regions or Europe. Its flowering heads have been used for medicinal purposes for millennia. Arnica extracts contain sesquiterpene lactones, volatile oils and flavonoids. They are said to have anti-inflammatory and antimicrobial activity. The former property has rendered arnica preparations popular remedies for sports injuries. There are two fundamentally different types of arnica preparations: herbal and homeopathic. Herbal arnica cream (administered exclusively topically because, given orally, arnica can be toxic) has not been extensively tested in controlled clinical trials: therefore it is not known whether it is effective or not. Nevertheless, such creams are extensively used and are for sale everywhere. However, there is no good evidence that herbal or homeopathic arnica preparations have a role in sports medicine [09362].

The aim of one study was to determine if topical Arnica is effective in reducing pain, indicators of inflammation and muscle damage, and in turn improve performance in well-trained males experiencing delayed onset muscle soreness (DOMS). Twenty well-trained males matched by maximal oxygen uptake completed a double-blind, randomised placebo-controlled trial. Topical Arnica was applied to the skin superficial to the quadriceps and gastrocnemius muscles immediately after a downhill running protocol designed to induce DOMS. Topical Arnica was reapplied every 4 waking hours for the duration of the study. Performance measures (peak torque, countermovement and squat jump), pain assessments (visual analogue scale (VAS) and muscle tenderness) and blood analysis (interleukin-1 beta, interleukin-6, tumour necrosis factor-alpha, C-reactive protein, myoglobin and creatine kinase) were assessed at seven time points over five days (pre-, post-, 4, 24, 48, 72 and 96 hours after the downhill run). Participants in the topical Arnica group reported less pain as assessed through muscle tenderness and VAS 72 hours post-exercise. The application of topical Arnica did not affect any performance assessments or markers of muscle damage or inflammation. Topical Arnica used immediately after intense eccentric exercise and for the following 96 hours did not have an effect on performance or blood markers. It did however demonstrate the possibility of providing pain relief three days post-eccentric exercise [13750].
Rhodiola rosea

Rhodiola rosea is a herb part of the Crassulacae family and is also known as Arctic root, rose root and golden root. It grows in the mountainous and Arctic regions of North America, Europe and Asia. It is purported that R. rosea possesses several ergogenic properties such as increasing physical and mental performance, enhancing cognitive and neural function and free radical mitigation. It has been described as an adaptogen because of its cardioprotective effects. Although the majority of research investigating the effects of R. rosea has been conducted in the animal model, there have been several studies done in humans. The dosages investigated in humans have ranged from 100 to 600 mg/day. Studies investigating its effects on exercise performance have been mixed. R. rosea supplementation in doses of 100 mg/day for 20 days and one acute dose of 200 mg/day were found to improve endurance exercise capacity by 6.5 percent and 5.0 percent, respectively. However, other studies have found no positive effects on VO$_{2peak}$, peak power, lactate threshold and ventilatory threshold. Studies investigating R. rosea supplementation on neural and cognitive performance have also produced mixed results. Doses of 100–555 mg/day have found positive effects on cognition but other studies have found no effect using doses 200 mg/day either acutely or for 5 weeks. R. rosea contains phenylpropanoids, phenolic compounds and flavonoids; some studies have found that supplementation can increase antioxidant levels decrease muscle-damage markers and mitigate free radicals. Based on the available literature, it remains unclear whether R. rosea supplementation in doses of 100–600 mg/day can enhance either mental and/or exercise performance. However, there is some evidence that R. rosea does possess antioxidant properties. Further tightly controlled studies in well-trained athletes need to be conducted in order to determine any performance-enhancing effects [12470].

Rhodiola rosea has been theorized to enhance endurance performance through a stimulating effect. In a preliminary study, it was found that an acute dose (200 milligrams) of Rhodiola rosea improved time to exhaustion by 3 percent on a cycle ergometer, but there was no significant effect following four weeks of supplementation with 200 milligrams daily. There was no effect on maximal strength or various measures of reaction time or movement time. Using combinations of such herbals have also been shown to have no ergogenic effect. It was evaluated the ergogenic effects of Rhodiola rosea-based supplement and reported no significant effects on oxygen dynamics, various physiological measures, or cycling time to exhaustion [06297].

Rhodiola rosea is an herb purported to possess adaptogenic and ergogenic properties and has recently been the subject of increased interest The purpose of this article was to review and summarize recent investigations of the potential performance-enhancing properties of Rhodiola rosea. Such studies have generated equivocal results. Several investigations conducted in Eastern Europe have indicated that Rhodiola rosea ingestion may produce such positive effects as improved cognitive function and reduced mental fatigue. Other research from this region has illustrated enhanced endurance exercise performance in both humans and rats. Studies conducted in Western Europe and in North America have indicated that Rhodiola rosea may possess substantial antioxidant properties but have produced mixed results when attempting to demonstrate an ergogenic effect during exercise in humans [06298].

Supplements from Rhodiola species (arctic or rose root; most commonly Rhodiola rosea) are purported to have many of the same ergogenic outcomes attributed to ginseng. Like ginseng, the supposed source of bioactivity is a subclass of glycosides (in this case, rosavins). Similar to ginseng again, the evidence substantiating ergogenic effects of Rhodiola supplementation is tenuous. A review concluded that this herb may possess some benefit for endurance (vs
strength) athletes, possibly through antioxidant or mitochondrial actions. However, but experimental differences between studies hamper direct comparisons, and several studies have not supported ergogenic claims [10252].

Rhodiola rosea, is an adaptogen plant which has been reported to promote fatty acids utilisation, to ameliorate antioxidant function, and to improve body resistance to physical strenuous efforts. The purpose of one study was to investigate the effects on physical performance as well as on the redox status of a chronic Rhodiola Rosea supplementation in a group of competitive athletes during endurance exercise. Following a chronic supplementation with Rhodiola Rosea for 4 weeks, 14 trained male athletes underwent a cardio-pulmonary exhaustion test and blood samples to evaluate their antioxidant status and other biochemical parameters. These data were compared with those coming from the same athletes after an intake of placebo. The evaluation of physical performance parameters showed that HR Max, Borg Scale level, VO$_{2\text{max}}$ and duration of the test were essentially unaffected by Rhodiola Rosea assumption. On the contrary, Rhodiola Rosea intake reduced, in a statistically significative manner, plasma free fatty acids levels. No effect on blood glucose was found. Blood antioxidant status and inflammatory parameters resulted unaffected by Rhodiola Rosea supplementation. Blood lactate and plasma creatine kinase levels were found significantly lower in Rhodiola Rosea treated subjects when compared to the placebo treated group. It was concluded that chronic Rhodiola Rosea supplementation is able to reduce both lactate levels and parameters of skeletal muscle damage after an exhaustive exercise session. Moreover this supplementation seems to ameliorate fatty acid consumption. Taken together those observation confirm that Rhodiola Rosea may increase the adaptogen ability to physical exercise [10255].

The aim of one study was to investigate the effect of Rhodiola rosea supplementation on the balance of oxidants and antioxidants in the serum and erythrocytes of competitive rowers. This double-blinded study included 22 members of the Polish Rowing Team who were participating in a preparatory camp. Participants were randomly assigned to the supplemented group (n=11), who received 100 mg of R. rosea extract twice daily for 4 wk, or the placebo group (n=11). At the beginning and end of the study, participants performed a 2,000-m maximum test on a rowing ergometer. Blood samples were taken from the antecubital vein before each exercise test, 1 min after completing the test, and after a 24-hr restitution period. The following redox parameters were assessed in erythrocytes: superoxide dismutase activity, glutathione peroxidase activity, and thiobarbituric-acid-reactive substances concentrations. In addition, creatine kinase activity and total antioxidant capacity were measured in plasma samples, lactate levels were determined in capillary blood samples, and uric acid concentrations were measured in serum. After supplementation, the total plasma antioxidant capacity was significantly higher in the supplemented group than in the placebo group, and superoxide dismutase activity in erythrocytes directly after and 24 hr after the ergometry was significantly lower in athletes receiving R. rosea extracts than in the placebo group. In conclusion, supplementation with R. rosea increased antioxidant levels in the plasma of professional rowers but had no effect on oxidative damage induced by exhaustive exercise [09371].

Rhodiola rosea (RR) is an adaptogenic herb suggested to improve exercise and cognitive performance and reduce stress responses. Further, some evidence reports that L-carnitine (LC) can alter metabolism during exercise and improve performance. This study examined the effects of acute ingestion of a commercially available RR beverage (250 mg, 3 % Rosavin) with and without LC (500 mg) on exercise performance, cognitive function and salivary cortisol. In a double-blind, randomised, crossover-design, 18 healthy, active men and women (age, 21 ± 6 years; maximal oxygen uptake, VO$_{2\text{max}}$ 43 ± 9 ml/min/kg) consumed 250 ml of a RR-LC, RR and placebo beverage 45 min before a cycle to fatigue at 77 ± 11
percent of VO$_{2\text{max}}$. Immediately before and after exercise, computerised cognitive tasks of Rapid Visual Information Processing (RVP), Visual Recognition Memory (VRM) and Stroop Colour-Word (Stroop) were completed. Expired gas and heart rate were recorded continuously during exercise. Capillary blood samples were taken for lactate analysis 2 min after cycling and saliva samples were collected before beverage ingestion, before and immediately after cognitive tests for cortisol analysis. Data were analysed using repeated measures ANOVAs. Time to fatigue, physiological and cognitive variables did not differ between treatments. Cortisol increased after tests, but there was no effect of treatment on this response. However, exercise elicited higher RVP sustained attention, a faster response time in Stroop, but VRM was reduced. These findings suggest that acute RR-LC ingestion does not alter cognitive function, exercise performance or salivary cortisol. However, chronic supplementation or greater dosage might affect these results. These results show that exhaustive exercise reduces reaction time and increases attention, but memory outcomes become decreased [11543].

As a society we are increasingly concerned about our physical appearance. For example, as much as 24 percent of people in developed countries admittedly exercise to improve their performance. Professional sportsmen and amateurs alike are in a constant search for new means that will enable them better sport results in shorter time. Among those means, a prominent place belongs to dietary supplements. However, the producers often advertise products whose use in sports is neither scientifically founded nor safe. This brings on an irrational use of herbal supplements which sometimes leads to unwanted side effects, but is more often of little use. Thus, the aim of one review was to systematically evaluate some of the herbal supplements that are used as adaptogenic and ergogenic aids in sport. The review will include available data on Rhodiola rosea, Withania somnifera, Schisandra chinensis, Tribulus terrestris, Vitis vinifera, Citrus aurantium, and others. Their effects, active ingredients as well as possible adverse effects will be discussed with special focus on clinical studies [13751].

**Lack of effect on marathon running**

Adaptogens modulate intracellular signaling and increase expression of heat shock protein 72 (HSP72). Rhodiola rosea (RR) is a medicinal plant with demonstrated adaptogenic properties. The purpose of this study was to measure the influence of RR supplementation on exercise-induced muscle damage, delayed onset of muscle soreness (DOMS), plasma cytokines, and extracellular HSP72 (eHSP72) in experienced runners completing a marathon. Experienced marathon runners were randomized to RR (n=24, 6 female, 18 male) or placebo (n=24, 7 female, 17 male) groups and under double-blinded conditions ingested 600 mg/day RR extract or placebo for 30 days prior to, the day of, and seven days post-marathon. Blood samples were collected, and vertical jump and DOMS assessed the day before, 15 min post- and 1.5h post-marathon. DOMS was also assessed for seven days post-marathon. Marathon race performance did not differ between RR and placebo groups. Vertical jump decreased post-marathon with no difference between groups. Post-marathon DOMS increased significantly but the pattern of change did not differ between groups. Myoglobin (Mb), creatine phosphokinase (CPK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), interleukin (IL)-6, IL-8, IL-10, monocyte chemotactic protein-1 (MCP-1), granulocyte-colony-stimulating factor (G-CSF), C-reactive protein (CRP), and eHSP72 all increased post-marathon, with no group differences over time. In conclusion, RR supplementation (600 mg/day) for 30 days before running a marathon did not attenuate the post-marathon decrease in muscle function, or increases in muscle damage, DOMS, eHSP72, or plasma cytokines in experienced runners [13752].
Cissus quadrangularis

Extracts and powders of Cissus quadrangularis have been used for many years to promote bone and tissues healing, as an analgesic, to treat infections, as an anabolic, and to promote weight loss and weight management. This review summarizes the studies in animals, humans and in vitro systems that have been conducted to determine the efficacy and safety of various Cissus preparations. Animal and in vitro studies provide support for the use of Cissus in promoting bone fracture healing and as an anti-osteoporotic. Several human studies support the use of Cissus extracts in weight management. No studies have been conducted demonstrating that Cissus exhibits anabolic and body building activities. Based on studies to date, Cissus extracts appear to be exceedingly safe and free of adverse effects at the doses commonly used. A wide variety of chemical constituents have been isolated and identified from Cissus extracts, including steroids, flavonoids, stilbenes, iridoids, triterpenes and gallic acid derivatives. However, in few cases have specific physiological effects been related to identifiable constituents. Better standardization of extracts and more well-controlled human studies are required [13754].

Cissus quadrangularis (CQ) is an ancient medicinal plant, found in warm regions of India, Sri Lanka, Malaysia, Java and West Africa. It has been used in traditional medicine to treat a number of ailments from healing bones to treating asthma. CQ has been reported to improve the healing time of bone fractures, potentially by increasing proliferation and differentiation of mesenchymal stem cells to osteoblasts. It has also been suggested to increase collagen synthesis through promoting alkaline phosphatase activity. These findings, along with supposed “anabolic steroidal substances” within CQ, have been used to promote its use within the body-building community for enhancing repair of damaged, connective tissue. However, there are no studies in athletic populations to support this [10253].

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Hydroxycut

Hydroxycut is a combination weight-loss supplement whose active ingredients include Cissus quadrangularis and caffeine. There are several subformulations containing various combinations of other botanical ingredients, including extracts of goji (Lycium barbarum), acerola (Malpighia glabra), wild mint (Mentha longifolia) and pomegranate (Punica granatum). As currently formulated, we could find no published studies assessing the product's safety and efficacy for weight loss in any population. The botanical ingredients lack evidence of efficacy for weight loss in high quality randomised trials in any population, and
the safety of individual ingredients in humans, and potential interactions, are largely unknown. Some of these botanical agents may have a significant polyphenol/antioxidant component, which may be beneficial during hard training and weight loss. A previous formulation of hydroxycut was withdrawn from the market after being associated with 23 cases of hepatotoxicity and one death. The suspected hepatotoxic ingredients included hydroxycitric acid (HCA) extracted from the garcinia cambogia fruit, chromium, and *Camellia sinensis* (green tea). These ingredients have been removed from the current formulation, although HCA continues to be available in other products, both alone and in combination with chromium and *Camellia sinensis*. Small, short-duration pilot studies have reported that 8 weeks of HCA supplementation resulted in statistically significant weight loss compared with placebo. In hydroxycut's current formulation, HCA has been replaced with *Cissus quadrangularis* (chromium and *Camellia sinensis* have also been removed). Small, short-duration pilot studies of *Cissus quadrangularis* have reported that 10 weeks of supplementation resulted in statistically significant weight loss compared with placebo. When taken as directed, the supplement provides 200 mg of caffeine per dose, with two doses suggested per day. In athletes, modest caffeine supplementation has been found to be an ergogenic aid that positively affects exercise capacity and performance; potential side-effects include irritability, tremor and an increase in heart rate. As is the case with many weight-loss supplements, hydroxycut contains potentially powerful pharmacoactive ingredients, but has never undergone high quality study to assess its safety and efficacy. To date the few published trials of *Cissus quadrangularis* (and HCA) effectiveness in non-athletes have methodological weaknesses including short duration and small size. For both HCA and *Cissus quadrangularis*, many published positive studies are funded by supplement manufacturers yet lack standard conflict of interest declarations. Because of the lack of good evidence of safety and efficacy, herbal weight-loss products are avoided by most performance nutrition practitioners in elite sport [11150].

**Echinacea**

The purpose of this investigation was to determine the effects of 4 weeks of oral Echinacea (ECH) supplementation on erythropoietin (EPO), red blood cell (RBC) count, running economy (RE), and VO$_{2\text{max}}$. Twenty-four men aged 25 years, height 179 cm, weight 88 kg, body fat 19 percent were grouped using a double-blind design and self-administered an 8,000 mg/day dosage of either ECH or placebo (PLA) in 5 × 400 mg × 4 times per day for 28 days. Blood samples were collected and analyzed for RBCs and EPO using automated flow cytometry and enzyme-linked immunosorbent assay. Maximal graded exercise tests (GXTs) were administered to measure VO$_{2\text{max}}$, RE, and heart-rate responses. Analysis of variance was used to determine statistically significant differences. The EPO increased significantly in ECH at 7 days, 14 days, and 21 days, and VO$_{2\text{max}}$ increased significantly after ECH. Running economy improved significantly in ECH as indicated by a decrease in submaximal VO$_{2\text{max}}$ during the first 2 stages of the GXT. These data suggest that ECH supplementation results in significant increases in EPO, VO$_{2\text{max}}$, and running economy [12473].

**Cordyceps sinensis**

Cordyceps sinensis is theorized to have favorable effects on the heart and circulation to improve oxidative capacity and endurance performance. Natural Cordyceps sinensis is rare, but a synthetic version is available; one version is CordyMax Cs-4. It was reported that 5 weeks of CordyMax Cs-4 supplementation had no effect on aerobic capacity or endurance exercise performance in endurance-trained male cyclists. It was evaluated the ergogenic
effects of a Cordyceps sinensis-based supplement and reported no significant effects on oxygen dynamics, various physiological measures, or cycling time to exhaustion [06297].

Labelled an ergogenic herb by some, Cordyceps sinensis is actually a fungus (not a plant) commonly called caterpillar fungus. Cordycepic acid and mannitol are the most frequently cited bioactive compounds; however, most studies of C sinensis in athletes have used a commercial product including other species (such as R rosea) or other isolated compounds. Thus, the ergogenic effects of C sinensis individually are difficult to ascertain. Its effects may lie in its ability to improve oxygen dynamics or upregulate ATP production. An investigation using short-term dosing showed no effect of a C sinensis supplement on muscle-associated parameters in young adult males. Similarly, earlier studies showed no effect of C sinensis on aerobic parameters [10252].

Cytoseira canariensis

Cytoseira canariensis has been marketed to increase muscle mass and decrease body fat by inhibiting myostatin, a growth and differentiation factor whose role is to inhibit (not promote) the growth of muscles. However, it was reported that 1,200 milligrams/day of C cytoseira canariensis supplementation during 12 weeks of resistance training had no effect on serum myostatin levels, not did it have any effect on muscle mass, muscle strength, or body fat [06297].

Smilax (sarsaparilla)

The plant genus Smilax includes several hundred species from the lily order that are distributed globally and commonly known as greenbriar or sarsaparilla (also spelled ‘zarzaporilla’ and often pronounced ‘sasparilla’). Belowground parts (rhizomes and roots) and bark from these plants are used in many traditional cultures to produce root beer, food or various herbal medicines. Smilax supplements are often produced from Smilax medica, Smilax officinalis and Smilax regelii belowground parts via extraction, and are rich in a category of plant sterols called saponins including sarsasapogenin (sarsapogenin), smilagenin (a derivative of sapogenin), sitosterol and stigmasterol. Bodybuilders consume Smilax supplements for their purported anabolic effects, mistakenly perceiving sarsaparilla sterols as prohormonal compounds that can be converted into testosterone by the human body and subsequently increase muscle mass, power, maximal performance and overall metabolism, though these possibilities have been disproved. Other benefits attributed to Smilax supplements include increasing endurance or energy, promoting recovery, lowering body fat and enhancing immune function. Documented side effects include stomach upset and kidney problems (including increased urination) and possible interactions with other supplements or drugs. Typical doses for Smilax supplements are 5-10 ml of extract or 1-4 g dried belowground parts daily. Sports nutrition authorities have found no evidence of ergogenic benefits from Smilax supplementation; therefore its use is discouraged [12459].

Yerba maté

Yerba maté tea (maté) is made from an infusion of the dried leaves of the Ilex paraguariensis tree, which is widely consumed in South America, but also found globally as both a herbal tea beverage and an ingredient in formulated foods and supplements. Numerous active phytochemicals have been identified in yerba maté tea: the two most prevalent compounds
are the polyphenols (chlorogenic acid) and xanthines (caffeine and obromine), followed by purine alkaloids (caffeic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid), flavonoids (quercetin, kaempferol and rutin), amino acids, minerals (phosphorus, iron and calcium) and vitamins (C, B1 and B2). However, unlike other teas, particularly white, green and oolong and, to a lesser extent black, yerba maté is a better alternative as it does not contain catechins, which have recently been linked with liver toxicity. Notably, the amount of caffeine in 150 mL of yerba maté tea is approximately 78 mg, which is similar to the amount found in a 250 mL cup of coffee. Hence, the primary association with sports performance is due to yerba maté being a natural source of caffeine and its associated ergogenic properties. Yerba maté has been shown to be hypocholesterolaemic, hepatoprotective, a central nervous system stimulant, diuretic, antioxidant, of benefit to the cardiovascular system, and associated with both the prevention and increased risk of some types of cancers. Also yerba maté, either on its own or in combination with other ingredients such as guarana and green tea, has been associated with weight loss. However, as with many herbal supplements, the specific evidence for an effect on athletic performance is limited and there is an increased risk of adverse effects [13749].

**Yohimbine**

Yohimbine, an alkaloid extracted from the bark of Corynanthe johimbe or Rauwolfia serpentina, has been used as a selective alpha-adrenergic receptor antagonist. The main aim of one study was to determine the effects of yohimbine supplementation on body composition and exercise performance in professional soccer players. The athletes (20 top-level male soccer players) were allocated to two randomly assigned trials. Subjects in the yohimbine group orally ingested tablets that contains yohimbine at a dose of 20 milligrams per day in two equal doses for 21 days. Subjects in the placebo group ingested an equal number of identical-looking pills that contained cellulose. There were no statistically significant changes in body mass and muscle mass within or between trials after the supplementation protocol. Percentage of body fat significantly decreased in the yohimbine group after the supplementation protocol. Furthermore, fat mass was significantly lower in the yohimbine versus placebo trial at post-supplementation assessment. There were no changes in exercise performance indicators (bench and leg press, vertical jump, dribble and power test results, shuttle run) within or between trials. No subject reported any side effects from yohimbine. The results of the current study indicate that supplementation with yohimbine combined with resistance training does not significantly alter the body mass, muscle mass, or performance indicators in professional soccer players. Nonetheless, yohimbine supplementation appears to be suitable as a fat loss strategy in elite athletes [06299].

Yohimbine is an alkaloid, that is, a naturally occurring secondary metabolite, derived from the bark of the West African tree, Pausinystalia yohimbe, and it can be bought over the counter as a herbal preparation. Traditionally, it has been used for erectile dysfunction and as an aphrodisiac. Yohimbine is an adrenergic alpha2 antagonist and, as yohimbine hydrochloride, is registered by the National Institutes of Health as a treatment for impotency where tolerance for sildenafil (Viagra) is poor. Evidence for this use in humans is not compelling but has been demonstrated in rats. Recent evidence suggests that yohimbine may benefit type 2 diabetes by increasing the blood flow to Islet cells, and it has been used for some time to improve saliva flow for those with xerostomia. Care should be taken over the provision of yohimbine, as it is known to cause agitation, anxiety, hypertension and tachycardia, with severity of these effects increasing with dose. In sport, yohimbine is perceived to reduce body fat and mobilise lipid, as well as to enhance endurance. Accordingly, it is often used in
bodybuilding and other aesthetic sports, and in sports where there is a significant aerobic component. However, despite these claims, research findings actually refute any ergogenic benefit for sport. In addition, as mentioned above, adverse effects have been established [13749].

**Bee pollen**

Bee pollen is a mixture collected by bees of pollen granules from the stamens of flowers and flower nectar. It is commercially available in granule, capsule or tablet preparations. These contain a wide and varying array of nutrients, including saccharides, amino acids, vitamins and minerals, as well as possible contaminants. Despite a long history in traditional medicine as a "superfood," there is little evidence to support the range of health claims for bee pollen. Interest in its ergogenic properties stems from anecdotal reports and testimonies from successful athletes. However, the few available studies from the 1970s and 1980s involving athletes and bee pollen supplementation (3-12-week protocols following manufacturers' recommended doses) have found minimal effects on: haemoglobin concentrations, strength and aerobic capacity, perceived exertion, time trial performance or repeated high-intensity exercise. One study did note fewer days lost to respiratory infections in swimmers supplementing with bee pollen. The limited empirical research suggests that bee pollen supplementation affords no additional benefit to athletic performance beyond that provided by a balanced diet [09135].

**Honey**

The purpose of this study was to examine the effects of natural honey supplementation on seminal plasma cytokines, oxidative stress biomarkers, and antioxidants during 8 weeks of intensive cycling training in male road cyclists. Thirty-nine healthy nonprofessional male road cyclists aged 18-28 years participated in this study. The participants were randomly assigned to exercise + supplement (E + S, n=20) and exercise (E, n=19) groups. All subjects participated in 8 weeks of intensive cycling training. Ninety minutes before each training session, subjects in the E + S group supplemented with 70 g of honey, whereas subjects in the E group received 70 g of an artificial sweetener. All subjects had an initial semen sampling at baseline (T(1)). The next 6 semen collections were collected immediately (T(2)) and 12 (T(3)) and 24 hours (T(4)) after the last training session in week 4, as well as immediately (T(5)) and 12 (T(6)) and 24 hours (T(7)) after the last training session in week 8, respectively. In the E group, 8 weeks of intensive cycling training significantly increased seminal interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-alpha, reactive oxygen species (ROS), and malondialdehyde (MDA) levels and significantly decreased the levels of seminal superoxide dismutase (SOD), catalase, and total antioxidant capacity (TAC). Significantly less elevation in seminal IL-1β, IL-6, IL-8, TNF-alpha, ROS, and MDA levels and significant increases in seminal SOD, catalase, and TAC concentrations were observed after the honey supplementation in the E + S group. It may be possible that honey supplementation following long-term intensive cycling training would be effective in attenuating the probable aggravating effects of intensive cycling training on spermatogenesis and fertility capacity in road cyclists [12466].

**Royal Jelly**

Royal jelly is secreted by worker bees to feed young larvae to produce a queen bee: it contains a source of amino acids, fatty acids, carbohydrate and B-vitamins, and has long
been used as a dietary supplement in complementary medicine. Little, however, is known about it in the context of exercise. It has been used sublingual doses of royal jelly, apparently combined with other components, on athletes undergoing rehabilitation in Irkutsk. It was not possible to deduce which components might have been responsible for the apparent improvements observed in the athletes' general well-being. Furthermore, royal jelly can cause allergic reactions [12459].

**Phlogenzym and wobenzym**

Phlogenzym and wobenzym are are traditionally paired and have been popular in the Eastern Bloc countries. The active ingredients found in phlogenzym are the hydrolase trypsin, the endopeptidase bromelain and the bioflavonoid rutin. Trypsin is a digestive enzyme produced by the pancreas and secreted into the small intestine, where it hydrolyses proteins. Bromelain is a proteolytic enzyme obtained from pineapples, and rutin is a bioflavonoid found in many plants, fruits and vegetables but the richest source is buckwheat. Similarly, wobenzym also contains trypsin, bromelain and rutin but also includes the proteolytic enzyme papain, the endopeptidase chymotrypsin and pancreatin which is an extract from the pancreas of animals that contains pancreatic enzymes. Phlogenzym and wobenzym are commonly known as hydrolytic enzymes or systemic enzymes and have been purported to possess anti-inflammatory, fibrinolytic and analgesic properties as well as having positive effects on oedema. Studies investigating the efficacy of phlogenzym and wobenzym in the athletic population are lacking but several studies have investigated their effects on recuperation following injury, disease and health. In a double-blind prospective randomised study, phlogenzym was compared with diclofenac in the treatment of activated osteoarthritis of the knee in 63 patients. Phlogenzym supplementation for 3 weeks in doses of six tablets per day (540 mg bromelain, 288 mg trypsin, 600 mg rutin) was found to be more effective than diclofenac in reducing pain over the 3-week period and phlogenzym was superior to diclofenac in reducing pain 3 weeks after supplementation had stopped. In a similar study, phlogenzym supplementation was found to be just as effective and well tolerated as diclofenac in the management of osteoarthritis over 3 weeks of treatment. Studies investigating phlogenzym supplementation for the treatment of lateral ankle ligament injury is mixed. Phlogenzym supplementation in conjunction with dietary counselling and acupuncture was more effective in treating rotator cuff tendinitis compared with an exercise group. However, these findings should be interpreted with caution as the group that received phlogenzym also received diet counselling and acupuncture; therefore, the significant improvement could have been attributed to other factors as there was no treatment group that solely received phlogenzym. Although studies in the athletic population are lacking and findings have been mixed, the research investigating the effects of phlogenzym and wobenzym suggests that they could be used as an effective anti-inflammatory and analgesic. However, future research should also investigate the potential mechanisms of action [12467].

**Cytochrome C**

Cytochrome C, a small haem protein, is found in the mitochondria where it is involved in the electron-transport chain. Endurance training and antioxidant supplementation have been shown to increase muscle cytochrome C concentrations. It has been hypothesised that supplementation with cytochrome C may also increase muscle levels, enhancing exercise performance by increasing maximum oxygen-carrying capacity, reducing blood lactate accumulation, and raising anaerobic threshold. However, the use of cytochrome C as an ergogenic aid to enhance aerobic exercise capacity cannot be supported [10347].
Glandulars

Glandulars are extracts from animal glands, which are normally dried, ground-up and sold in powder or tablet form. The most common glandular supplements include: thyroid; adrenal; thymus; testis and ovary; followed by glandulars from the pituitary, kidney, liver, pancreas, spleen, lung, heart, brain, uterus and prostate. Glandulars are claimed to enhance the function of the equivalent gland in the human body. The theory is that glandular tissues contain intrinsic cell-specific, but not species-specific, factors that are distinct from vitamins and minerals. Glandulars are popular with bodybuilders who believe that their ingestion will produce anabolic effects by boosting the body's production of hormones. However, glandular extracts are degraded during the digestive process and are inactive when absorbed. Therefore, it is not surprising that there is no scientific evidence that glandular tissue concentrates enhance organ and gland activities, or work ergogenically, other than through their vitamin, mineral and protein content [11297].

Lactobacillus casei Shirota

The purpose of this study was to examine the effects of a probiotic supplement during 4 mo of winter training in men and women engaged in endurance-based physical activities on incidence of upper respiratory tract infections (URTIs) and immune markers. Eighty-four highly active individuals were randomized to probiotic (n=42) or placebo (n=42) groups and, under double-blind procedures, received probiotic (PRO: Lactobacillus casei Shirota, LcS) or placebo (PLA) daily for 16 weeks. Resting blood and saliva samples were collected at baseline and after 8 and 16 wk. Weekly training and illness logs were kept. Fifty-eight subjects completed the study (n=32 PRO, n=26 PLA). The proportion of subjects on PLA who experienced 1 or more weeks with URTI symptoms was 36 percent higher than those on PRO. The number of URTI episodes was significantly higher in the PLA group than in the PRO group. Severity and duration of symptoms were not significantly different between treatments. Saliva IgA concentration was higher on PRO than PLA, significant treatment effect; this difference was not evident at baseline but was significant after 8 and 16 weeks of supplementation. Regular ingestion of LcS appears to be beneficial in reducing the frequency of URTI in an athletic cohort, which may be related to better maintenance of saliva IgA levels during a winter period of training and competition [11298].

Colostrum

The aim of one pilot investigation was to examine the influence of bovine colostrum protein concentrate (CPC) supplementation on salivary hormones, salivary IgA and heart rate variability over consecutive days of competitive cycling. Ten highly-trained male road cyclists were randomly assigned to a control (n=6, 10 g whey protein concentrate/day) or bovine CPC group (n=4, 10 g bovine CPC/day). Cyclists provided a baseline saliva sample before commencing eight weeks of supplementation, and competing in a five day cycle race. Cyclists provided saliva samples and measured heart rate variability (HRV) each day of the race. Saliva samples were analysed for cortisol, testosterone and IgA concentrations. Bovine CPC supplementation was associated with increased morning cortisol concentration on the first day of racing when compared to the control group and significantly prevented a decrease in testosterone concentration over the race period. Across the race period parasympathetic indices of HRV were elevated in the bovine CPC group and reduced in the control group, while there were no significant differences in salivary IgA between groups. It
was concluded that bovine CPC supplementation maintained salivary testosterone concentration and modulated autonomic activity over consecutive days of competitive cycling. One pilot study provides justification to explore the effects of bovine CPC on recovery in endurance athletes further \[13748\].

One study examined the effects of bovine colostrum on exercise-induced modulation of antioxidant parameters in the skeletal muscles in mice. Adult male BALB/c mice were randomly divided into four groups (control, colostrum alone, exercise and exercise with colostrum) and each group had three subgroups (day 0, 21 and 42). Colostrum groups of mice were given a daily oral supplement of 50 mg/kg body weight of bovine colostrum and the exercise group of mice were made to exercise on the treadmill for 30 minutes per day. Total antioxidants, lipid hydroperoxides, xanthine oxidase and super oxide dismutase level were assayed from the homogenate of hind limb skeletal muscles. Exercise-induced a significant oxidative stress in skeletal muscles as evidenced by the elevated lipid hydroperoxides and xanthine oxidase levels. There was a significant decrease in skeletal muscle total antioxidants and superoxide dismutase levels. Daily colostrum supplement significantly reduced the lipid hydroperoxides and xanthine oxidase enzyme level and increased the total antioxidant levels in the leg muscle. Thus, the findings of the study showed that daily bovine colostrum supplementation was beneficial to the skeletal muscle to reduce the oxidant-induced damage during muscular exercise [12465].

Colostrum is the milk produced by mammals 24-72 h after giving birth. It is rich in immune, growth and antimicrobial factors that support neonate development. The primary source of colostrum for supplementation by athletes is bovine, which is similar in composition to human colostrum. However, the concentration of immune and growth factors is up to 100 times greater in bovine colostrum. Supplements may be in liquid, capsule, tablet or powder form. The superiority of one form over another is yet to be established. Supplement quality is affected by the timing and methods of processing: non-heat-treated, early-collection colostrum is superior. Colostrum contains growth factors (125 ml/day, containing 8.4 µg/day of IGF-I). It has been found increased serum IGF-I after 8 days' supplementation during resistance training, but no increase in maximal strength. Importantly, the IGF-I content of bovine colostrum supplements may vary considerably (1.7–120 µg/day). Although bovine colostrum supplementation is associated with increases in lean body mass, limb cross-sectional area and circulating essential amino acids, these have not translated into significant improvements in maximal strength. Improved immunocompetence has been suggested by several studies in which bovine supplementation was associated with increased salivary IgA and, more importantly, a reduction in symptoms of upper respiratory tract illness after 8 weeks of supplementation. Given its varied constituents, bovine colostrum supplementation may have modest effects on numerous pathways combining to provide potential ergogenic effects. To date, however, there is limited literature to support any consistent benefits. The safety of long-term dosing (>12 weeks) is yet to be established. Those allergic to cow's milk proteins should avoid bovine colostrum supplements [10251].

The aim of one study was to investigate the influence of low-dose bovine colostrum protein concentrate (CPC) supplementation on selected immune variables in cyclists. Twenty-nine highly trained male road cyclists completed an initial 40-km time trial (TT(40)) and were then randomly assigned to either a supplement (n=14, 10 g bovine CPC/day) or placebo group (n=15, 10 g whey protein concentrate/day). After 5 weeks of supplementation, the cyclists completed a second TT(40). They then completed 5 consecutive days of high-intensity training (HIT) that included a TT(40), followed by a final TT(40) in the following week. Venous blood and saliva samples were collected immediately before and after each TT(40), and upper respiratory illness symptoms were recorded over the experimental period. Compared with the placebo group, bovine CPC supplementation significantly increased preexercise

2007
serum soluble TNF receptor 1 during the HIT period. Supplementation also suppressed the postexercise decrease in cytotoxic/suppressor T cells during the HIT period and during the following week. Bovine CPC supplementation prevented a postexercise decrease in serum IgG(2) concentration at the end of the HIT period. There was a trend toward reduced incidence of upper respiratory illness symptoms in the bovine CPC group. In summary, low-dose bovine CPC supplementation modulates immune parameters during normal training and after an acute period of intense exercise, which may have contributed to the trend toward reduced upper respiratory illness in the bovine CPC group [07390].

The aim of one experiment was to investigate the influence of low dose bovine colostrum supplementation on exercise performance in cyclists over a 10 week period that included 5 days of high intensity training (HIT). Over 7 days of preliminary testing, 29 highly trained male road cyclists completed a VO\textsubscript{2max} test (in which their ventilatory threshold was estimated), a time to fatigue test at 110 percent of ventilatory threshold, and a 40 km time trial (TT40). Cyclists were then assigned to either a supplement (n = 14, 10 g/day bovine colostrum protein concentrate, CPC) or a placebo group (n= 15, 10 g/day whey protein) and resumed their normal training. Following 5 weeks of supplementation, the cyclists returned to the laboratory to complete a second series of performance testing (week 7). They then underwent five consecutive days of HIT (week 8) followed by a further series of performance tests (week 9). The influence of bovine CPC on TT40 performance during normal training was unclear. However, at the end of the HIT period, bovine CPC supplementation, compared to the placebo, elicited a 1.9 ± 2.2 percent improvement from baseline in TT40 performance and a 2.3 ± 6.0 percent increase in time trial intensity (% VO\textsubscript{2max}), and maintained TT40 heart rate. In addition, bovine CPC supplementation prevented a decrease in ventilatory threshold following the HIT period. It was concluded that low dose bovine CPC supplementation elicited improvements in TT40 performance during an HIT period and maintained ventilatory threshold following five consecutive days of HIT [06300].

**Effect on salivary IgA**

Secretory IgA in saliva (s-IgA) is a potential mucosal immune correlate of upper respiratory tract infection (URTI) status. Nutritional supplements may improve mucosal immunity, and could be beneficial to athletes who are at increased risk of URTI. In this study, 35 distance runners (15 female, 20 male, age 35 to 58 years) consumed a supplement of either bovine colostrum or placebo for 12 weeks. Saliva samples were taken prior to training at baseline, monthly during supplementation, and 2 weeks post supplementation. Median levels of s-IgA increased by 79 percent in the colostrum group after 12 week intervention, and the time-dependent change from baseline value was significant. This significance was still apparent after adjusting for training volume and self-reporting of upper respiratory symptoms. The study has demonstrated increased s-IgA levels among a cohort of athletes following colostrum supplementation. While this result is statistically significant, its physiological interpretation must be viewed with caution due to the small numbers in this study and the large variability in s-IgA levels [06301].

**Fermented papaya**

Fermented papaya preparation (FPP) (a product of yeast fermentation of Carica papaya Linn) is a food supplement. Studies in chronic and degenerative disease conditions (such as thalassemia, cirrhosis, diabetes and aging) and performance sports show that FPP favorably modulates immunological, hematological, inflammatory, vascular and oxidative stress damage parameters. Neuroprotective potential evaluated in an Alzheimer’s disease cell
model showed that the toxicity of the beta-amyloid can be significantly modulated by FPP. Oxidative stress trigger apoptotic pathways such as the c-jun N-terminal kinase (JNK) and p38-mitogen activated protein kinase (MAPK) are preferentially activated by pro-inflammatory cytokines and oxidative stress resulting in cell differentiation and apoptosis. FPP modulated the H$_2$O$_2$-induced ERK, Akt and p38 activation with the reduction of p38 phosphorylation induced by H$_2$O$_2$. FPP reduces the extent of the H$_2$O$_2$-induced DNA damage, an outcome corroborated by similar effects obtained in the benzo[a]pyrene treated cells. No genotoxic effect was observed in experiments with FPP exposed to HepG2 cells nor was FPP toxic to the PC12 cells. Oxidative stress-induced cell damage and inflammation are implicated in a variety of cancers, diabetes, arthritis, cardiovascular dysfunctions, neurodegenerative disorders (such as stroke, Alzheimer's disease, and Parkinson's disease), exercise physiology (including performance sports) and aging. These conditions could potentially benefit from functional nutraceutical/food supplements (as illustrated here with fermented papaya preparation) exhibiting anti-inflammatory, antioxidant, immunostimulatory (at the level of the mucus membrane) and induction of antioxidant enzymes [10535].

**Ultrasonography-guided interventions**

Increasing histological and radiological understanding of the processes involved in soft-tissue injury is leading to novel targeted treatments. A number of reviews have recommended that these treatments should be performed with image guidance. One review described current ultrasound-guided interventions and injections, together with the level of evidence for these. Discussion of guided interventions will include; percutaneous lavage (barbotage), brisement, dry needling, electrocoagulation, and of guided injections; corticosteroids, autologous substances (blood and platelet rich plasma), sclerosants, and prolotherapy (hyperosmolar dextrose). Representative imaging illustrating some of these techniques is included for correlation with the methods described. As these procedures are often performed in sportspeople, it is essential that the radiologist is aware of prohibited substances and methods outlined in an annual publication from the World Anti-Doping Association (WADA) [11300].
GENE DOPING

Overviews

Hugh Montgomery's discovery of the first of more than 239 fitness genes together with rapid advances in human gene therapy have created a prospect of using genes, genetic elements, and cells that have the capacity to enhance athletic performance (to paraphrase the World Anti-Doping Agency's definition of gene doping). A brief overview covered the main areas of interface between genetics and sport, attempts to provide a context against which gene doping may be viewed, and predicted a futuristic legitimate use of genomic (and possibly epigenetic) information in sport [10257].

Gene doping is the misuse of gene therapy to enhance athletic performance. It has recently been recognised as a potential threat and subsequently been prohibited by the World Anti-Doping Agency. Despite concerns with safety and efficacy of gene therapy, the technology is progressing steadily. Many of the genes/proteins which are involved in determining key components of athletic performance have been identified. Naturally occurring mutations in humans as well as gene-transfer experiments in adult animals have shown that altered expression of these genes does indeed affect physical performance. For athletes, however, the gains in performance must be weighed against the health risks associated with the gene-transfer process, whereas the detection of such practices will provide new challenges for the anti-doping authorities [08432].

Some spectacular results from genetic manipulation of laboratory rodents and increasing developments in human gene therapy raise the spectre of genetic modification or "gene doping" in sports. Candidate targets include the induction of muscle hypertrophy through overexpression of specific splice variants of insulin-like growth factor-1 or blockade of the action of myostatin, increasing oxygen delivery by raising the hematocrit through the use of erythropoietin, induction of angiogenesis with vascular endothelial growth factors or related molecules and changes in muscle phenotype through expression of peroxisome-proliferator-activated receptor- delta and associated molecules. Some of these potential genetic enhancements, particularly where the genetic modification and its action are confined to the muscles, may be undetectable using current tests. This had lead to exaggerated predictions that gene doping in athletics will be common within the next few years. However, a review of the methods of gene transfer and the current state of the art in development of genetic treatments for human disease show that the prospects for gene doping remain essentially theoretical at present. Despite this conclusion, it will be important to continue to monitor improvements in the technology and to develop methods of detection, particularly those based on identifying patterns of changes in response to doping as opposed to the detection of specific agents [08433].

Sports authorities fear that a new form of doping called gene doping, based on the misuse of gene therapy, represents an emerging important problem and so far no methods are available for detecting it. The World Anti-Doping Agency (WADA) has included since 2003 for the first time gene doping methods in the "Prohibited List of Substances and Methods", thus detection of this new form of doping is challenging for analytical chemists. In this work, we apply affinity-based biosensors (ABBs), in particular DNA piezoelectric sensing, for detection of target DNA sequences selected as transgenosis markers. In this work, two sequences widely used in transgenosis experiments have been identified as markers: the enhanced green fluorescence protein (EGFP) gene and the promoter of cytomegalovirus (CMV). The biosensors are characterized in their analytical performances using synthetic
oligonucleotides and amplified DNA obtained from purified plasmid used as a template. Finally they have been applied to transgenic human cell cultures (human embryonic kidney HEK-EGFP), transformed with the same plasmid and carrying the target markers. This represents the closest human real matrix available for our transgenes [09375].

Performance enhancing polymorphisms (PEPs) are examples of natural genetic variation that affect the outcome of athletic challenges. Elite athletes, and what separates them from the average competitor, have been the subjects of discussion and debate for decades. While training, diet, and mental fitness are all clearly important contributors to achieving athletic success, the fact that individuals reaching the pinnacle of their chosen sports often share both physical and physiological attributes suggests a role for genetics. That multiple members of a family often participate in highly competitive events, such as the Olympics, further supports this argument. In one review, it was discussed what is known regarding the genes and gene families, including the mitochondrial genome, that are believed to play a role in human athletic performance. Where possible, it was described the physiological impact of the critical gene variants and consider predictions about other potentially important genes [09376].

Our ever-increasing understanding of the genetic control of cardiovascular and musculoskeletal function together with recent technical improvements in genetic manipulation generates mounting concern over the possibility of such technology being abused by athletes in their quest for improved performance. Genetic manipulation in the context of athletic performance is commonly referred to as gene doping. A review of the literature was performed to identify the genes and methodologies most likely to be used for gene doping and the technologies that might be used to identify such doping. A large number of candidate performance-enhancing genes have been identified from animal studies, many of them using transgenic mice. Only a limited number have been shown to be effective following gene transfer into adults. Those that seem most likely to be abused are genes that exert their effects locally and leave little, if any, trace in blood or urine. It was concluded that there is currently no evidence that gene doping has yet been undertaken in competitive athletes but the anti-doping authorities will need to remain vigilant in reviewing this rapidly emerging technology. The detection of gene doping involves some different challenges from other agents but a number of promising approaches are currently being explored [09377].

The disclosure of repeated cases of athletes who abuse performance-enhancing drugs is an ordinary phenomenon in the athletic community. In addition, the completion of sequencing of the human genome and the unprecedented advances in molecular biology have enabled the progression from traditional drug enhancement to hypothetical (at present) gene transfer. Scientific, sports, and antidoping authorities fear that gene doping will be the next major challenge. The misuse of gene therapy to improve athletic ability, which is commonly referred to as “gene doping,” represents a practice as dangerous and unethical as any type of conventional doping. However, gene doping appears to be more attractive than traditional forms of doping because it is still undetectable and thus much less presentable [09378].

Gene doping is the newest threat to the spirit of fair play in sports. Its concept stemmed out from legitimate gene therapy trials, but anti-doping authorities fear that they now may be facing a form of doping that is virtually undetectable and extremely appealing to athletes. One paper presented studies that generated mouse models with outstanding physical performance, by manipulating genes such as insulin-like growth factor 1 (IGF-1) or phosphoenolpyruvate carboxykinase (PEPCK), which are likely to be targeted for gene doping [09379].

Recent studies in somatic gene therapy indicate that long-term presence of transgenic DNA
(tDNA) following various gene transfer protocols can be found in DNA isolated from whole blood using conventional PCR protocols. Application of these protocols for the direct detection of gene doping would require almost complete knowledge about the sequence of the genetic information that has been transferred. Now it was developed and described the novel single-copy primer-internal intron-spanning PCR (spiPCR) procedure that overcomes this difficulty. Apart from the interesting perspectives that this spiPCR procedure offers in the fight against gene doping, this technology could also be of interest in biodistribution and biosafety studies for gene therapeutic applications [09380].

Gene doping is the term given to the potential misuse of gene therapy for the purposes of enhancing athletic performance. Insulin-like growth factor-I (IGF-I), the prime target of growth hormone action, is one candidate gene for improving performance. In recent years, a number of transgenic and somatic gene transfer studies on animals have shown that up-regulation of IGF-I stimulates muscle growth and improves function. This increase in muscle IGF-I is not reflected in measurable increases in circulating IGF-I. Whilst the responses obtained in the animal studies would appear to give clear benefits for performance, the transfer of such techniques to humans still presents many technical challenges. Further challenges will also be faced by the anti-doping authorities in detecting the endogenously produced products of enhanced gene expression [09381].

"Gene doping" is the term used to describe the potential abuse of gene therapy as a performance-enhancing agent. Gene doping would apply the techniques used in gene therapy to provide altered expression of genes that would promote physical superiority. For example, insulin-like growth factor 1 (IGF-1) is a primary target for growth hormone; overexpression of IGF-1 can lead to increased muscle mass and power. Although gene doping is still largely theoretical, its implications for sports, health, ethics, and medical genetics are significant [10258].

As clinical gene therapy has progressed toward realizing its potential, concern over misuse of the technology to enhance performance in athletes is growing. Although gene doping is banned by the World Anti-Doping Agency, its detection remains a major challenge. In one study, it was developed a methodology for direct detection of the transferred genetic material and evaluated its feasibility for gene doping detection in blood samples from athletes. Using erythropoietin (EPO) as a model gene and a simple in vitro system, it was developed real-time PCR assays that target sequences within the transgene corresponding to exon/exon junctions. As these junctions are absent in the endogenous gene due to their interruption by introns, the approach allows detection of trace amounts of a transgene in a large background of the endogenous gene. Two developed assays and one commercial gene expression assay for EPO were validated. On the basis of ability of these assays to selectively amplify transgenic DNA and analysis of literature on testing of gene transfer in preclinical and clinical gene therapy, it was concluded that the developed approach would potentially be suitable to detect gene transfer through gene transfer by analysis of small volumes of blood using regular out-of-competition testing [10259].

Genetic research is used to identify the relative contributions made by inherent abilities (nature) versus environmental effects (nurture) in human performance. The same approach allows a better understanding of how injuries or illnesses can result from sport or physical activity. Having identified the genes involved in athletic performance, there are the intriguing possibilities of using this information for talent search, developing individualized training programs and prevention of sports-related injuries. There are many interacting genes involved in athletic performance. This class of genes is often described as "complex" and the mode of inheritance is called "multifactorial". Discovery of these genes is difficult using the conventional case control (association) studies. Recent genomic-based developments
allowing high throughput SNP analysis are very promising. Potentially more exciting is the availability in the near future of cheaper and faster whole-genome sequencing technologies. Genetic research in exercise science has produced a lot of data including the ability to draw a human exercise gene map. However, progress at the genetic level has been slow because gene-based association studies are not powerful enough to detect multiple small but cumulative gene effects. In future, the more efficient genomic-based research approaches will accelerate the finding of “sports genes”. Data generated will be enormous, making it essential to have a direct link between the laboratory researcher and bioinformatics expertise. It was finally underlined that genetics research has moved to the genomics era, i.e. the simultaneous testing of multiple genes is now possible [10260].

Gene doping abuses the legitimate approach of gene therapy. While gene therapy aims to correct genetic disorders by introducing a foreign gene to replace an existing faulty one or by manipulating existing gene(s) to achieve a therapeutic benefit, gene doping employs the same concepts to bestow performance advantages on athletes over their competitors. Recent developments in genetic engineering have contributed significantly to the progress of gene therapy research and currently numerous clinical trials are underway. Some athletes and their staff are probably watching this progress closely. Any gene that plays a role in muscle development, oxygen delivery to tissues, neuromuscular coordination, or even pain control is considered a candidate for gene dopers. Researchers today are racing the clock because assuring the continued integrity of sports competition depends on their ability to develop effective detection strategies in preparation for the 2012 Olympics, which may mark the appearance of genetically modified athletes [10261].

The concept of gene doping grew out of the important development in the early 1970s of a novel approach in medicine that promised to treat human disease by attacking underlying genetic defects. Thus was born the idea of gene therapy. In early, phase I safety studies, gene therapy has produced effective treatments for a number of diseases, such as pediatric immune deficiency, a genetic form of blindness, and neurodegeneration, with more sure to come in the very near future. While the efficacy of treatments has not yet been confirmed in more extensive phase III studies, the success so far teaches us is that it is clearly possible to introduce new genetic functions into human beings in forms efficient and stable enough to modify traits that produce serious disease and thus to ameliorate life-threatening illness and ease suffering. The same methods can undoubtedly be used to enhance normal human traits, including traits that affect athletic ability. One might readily envision genetic modification of healthy young athletes to augment functions useful for athletic performance, such as muscle growth and contraction, endurance, blood production, pain perception, and oxygen delivery to exercising muscle. But how close we are to gene doping in sports is a matter of debate [10262].

Many forms of human enhancement are becoming more feasible, sought-after, and even justifiable in the quest for healthier, happier, and longer lives. Around the world, people have been exposed to the notion of human enhancement through sport, as some athletes seek a boost to success, stardom, and financial reward. In the past, doping and cheating in sport have been enabled by advances in pharmacology and physiology. The successful development of gene therapy has provided the concepts, tools, opportunity, and, for some, justification for genetic modification of functions that affect normal human traits, including athletic performance. As science progresses and sport and antidoping authorities express increasing concerns, the time is right to look at how advances in genetics are affecting sport in ways unexpected just a decade ago [10263].

Some early experimental studies illustrate the potential of gene therapy for treating diseases. Although most gene therapy approaches involve gain-of-function expression of exogenous...
transgenes, other methods for genetic modification have also emerged. A definitive approach to genetic modification for therapy would involve an emerging technology of site-specific sequence correction of disease-causing mutations, as through the use of zinc finger-associated recombinational methods. Although highly effective in some models, these gene therapy techniques are imperfect and still highly risky, as demonstrated by severe adverse events such as treatment-induced leukemia, or even deaths. Nevertheless, it is inevitable that, as the science and techniques mature, these same methods and concepts will be applied to broader nontherapeutic uses, including gene-based “enhancement” of human traits linked to sport [10263].

Genetic methods have been used, for instance, to demonstrate enhanced muscle function from the insulinlike growth factor (IGF-1) or follistatin transgenes and stably increased, regulatable, erythropoietin-enhanced blood production in primates. One of the most widely discussed transcriptional modulation approaches has involved small molecule modulators of peroxisomal proliferator-activated receptor delta (PPAR-delta), which regulates expression of genes involved in lipid metabolism, energy utilization, and insulin action and that increases the production of slow twitch oxidative energy-effi cient muscle fi bers. These effects have important implications for therapy of diabetes, obesity, and muscle disease. Furthermore, mice overexpressing a PPAR-delta transgene or treated with a PPAR-delta agonist show enhanced endurance performance [10263].

Traditional approaches to detection of doping in sport have been based on chemical or molecular detection of the doping agent or of markers reflecting the physiological or metabolic effect(s) of the agent (e.g. chemical assays for steroids and stimulants, molecular identifi cation of foreign erythropoietin, and detection of abnormally high erythrocyte production following exogenous erythropoietin exposure). Although this is the most direct approach, new assays are constantly needed to respond to chemical modifications that make some drugs more difficult to detect, and therefore more prone to doping abuse. A potentially more powerful detection method has emerged, based on the concept that chemical, biological, or genetic doping agents are likely to produce broad metabolic, genetic, and proteomic changes. These changes are now detectable by techniques such as microarray- or sequence-based transcriptional profiling and proteomic and metabolomic analyses that can defi ne molecular “signatures” of exposure to specifi c doping agents, or families of drugs, or methods. Such signatures may be used to identify perturbed physiological systems, even in the absence of knowledge of, or assays for, specifi c doping agents. This approach is similar to that commonly used in searches for molecular signatures of oncogenesis, developmental disorders, and so on. The World Anti-Doping Agency has sponsored international research teams with early results providing growing credence to the utility of molecular signatures in doping detection. For instance, exposure of murine myoblasts to IGF-1 has been shown to induce transcriptional and proteomic changes that may eventually constitute a “signature” specifi c for exogenous IGF-1 exposure. Of course, the application of these kinds of global assays would require rigorous validation of a connection with specifi c doping agents or methods [10263].

Currently there is no clear evidence that gene doping has already found its way to the sports ground; however it is defi nitely only a question of time when fi rst athletes will start a try. The World Anti-Doping Agency is taking the probability of such malpractice seriously, and, in 2003, the term gene doping was enumerated in the official list of banned substances and methods. As gene doping is based on the intuitive concept of administering genetic information, thus enabling the athlete’s cells to produce its own doping substances – which in most cases would be indistinguishable from endogenous proteins – it is commonly believed that gene doping detection strategies should aim at metabolic, transcriptomic or proteomic changes as a consequence of the delivery and/or expression of the transgene. However, it
remains an open issue if incontrovertible doping signatures can be established that are independent of any confounding variables such as age, gender, physical or ethnic background. It must be keep in mind that elite athletes are “exceptional people” with “exceptional skills” and “exceptional physical abilities” that will also be mirrored in “exceptional signature patterns”, for example, because of distinct genetic polymorphisms, methylation patterns or histone modifications [10418].

Somatic gene therapy carries immense potential as a therapeutic modality for the treatment of inherited or acquired diseases by supplying functional copies in substitution of mutated genes, and by improving the body's natural ability to cope with diseases and infections. Although apparently simple in concept, the practical realities of translating gene therapy strategies into clinical practice have proven to be tremendously challenging. However, previous experiences have shown that even drugs that are still in the experimental phases of research may rapidly find their way into the athletic world. Several gene therapy trials aimed at the treatment of anemia, muscular dystrophy and peripheral vascular diseases have been initiated or are currently on the way. Moreover, impressive results from preclinical animal trials might entice some athletes to try these drugs, especially as no detection method is yet at hand. Dramatically improved muscle mass and muscle performance has been shown by rAAV-mediated intramuscular transfer of insulin-like growth factor 1 and follistatin in mice. Growth hormone serum levels in rodents could be significantly increased by salivary gland and intraperitoneal administration of rAAV vector systems. Intramuscular transfer of rAAV-VEGF has been shown to improve muscle survival and regeneration following muscle damage in mice. Using a tetracycline-inducible rAAV-based vector system, sustained functional neovascularization could be achieved after intramuscular gene transfer of VEGF in a murine ischemia model. By exploiting a similar Tet-On system, precise regulation of blood hemoglobin levels could be demonstrated after intramuscular electro-mediated gene transfer of erythropoietin in mice [10418].

Among the various application strategies and vector constructs that have been established in gene therapy trials, attempts to transfer gene therapy into doping practice would most likely primarily focus on intramuscular gene transfer using AAV-derived vectors as gene delivery systems. As current non-viral vectors have proven to be highly inefficient in humans for in vivo applications, recombinant AAV would be most suitable to deliver the transgene of choice in a gene doping scenario. This is because of its low immunogenicity, stable support of gene expression in slowly dividing or postmitotic cells, and the possibility of improving tissue specificity by pseudotyping the transgene cassette into alternative AAV vector capsids. Production of these vectors is by now a routine task in many molecular biology labs, and custom-made viral vectors are offered by commercial suppliers as ready-to-use stocks. Considering application strategies, in vivo intramuscular gene transfer would be the most practicable and promising approach, as skeletal muscle is easily accessible for vector administration, is sufficiently vascularized and enables prolonged expression of the transgene because of its postmitotic state [10418].

Several detection strategies for gene doping are currently under investigation. As expression of the transgene cassette is uncoupled from intrinsic regulatory pathways, most detection strategies aim at deciphering biomarkers and expression profiles (transcriptomic, proteomic and metabolomic approaches) that are indicative of “unnatural” gene expression. It remains an open issue whether such indirect methods can be sufficiently validated to exclude all natural causes for aberrant signature patterns. Because in sports jurisdiction a positive doping test alone establishes comfortable satisfaction of guilt, athletes charged with doping offenses are functionally presumed guilty until proven innocent. But how can an athlete provide evidence for an exceptional genetic or epigenetic predisposition when current knowledge of the complex orchestration of genomic, epigenomic, transcriptomic and
proteomic regulatory networks is spotty at best? Undoubtedly, a direct detection method that unambiguously identifies the doping agent should always be preferable. The test laboratory must be designed and operated in a way that prevents contamination of reactions with amplified products from previous assays and cross-contamination between samples, both of which can lead to false-positive results. Unidirectional workflow, separation of workspace and appropriate control procedures are fundamentally crucial. As a matter of course, these prerequisites cannot be completely fulfilled in a small molecular biology lab that is routinely dealing with a bulk of cDNA preparations. Substitution of positive control cDNAs by recombinant controls that are distinguishable from transgenic DNA will further reduce the risk of cross-contamination, thus making our procedure amenable to high-throughput screening in accredited laboratories [10418].

The misuse of somatic gene therapy for the purpose of enhancing athletic performance is perceived as a coming threat to the world of sports and categorized as “gene doping”. As clinical gene therapy has progressed toward realizing its potential, concern over misuse of the technology to enhance performance in athletes is growing. Although “gene doping” is banned by the World Anti-Doping Agency, its detection remains a major challenge. In one study, it was developed a methodology for direct detection of the transferred genetic material and evaluated its feasibility for gene doping detection in blood samples from athletes. Using erythropoietin (EPO) as a model gene and a simple in vitro system, it was developed real-time PCR assays that target sequences within the transgene complementary DNA corresponding to exon/exon junctions. As these junctions are absent in the endogenous gene due to their interruption by introns, the approach allows detection of trace amounts of a transgene in a large background of the endogenous gene. Two developed assays and one commercial gene expression assay for EPO were validated. On the basis of ability of these assays to selectively amplify transgenic DNA and analysis of literature on testing of gene transfer in preclinical and clinical gene therapy, it is concluded that the developed approach would potentially be suitable to detect gene doping through gene transfer by analysis of small volumes of blood using regular out-of-competition testing [10419].

When the concept of gene doping first emerged a decade or so ago, some critics considered it improbable and far from imminent. One of my most respected colleagues, who had a prominent role in the gene therapy oversight process, called the potential for using genetic modification methods for gene doping “a lot of gale-force hand waving.” In contrast, others saw it to be the obvious next and inevitable step in doping and cheating technology and believed it offered potential advantages over drug-based doping – that it might be more effective and more difficult to detect. Many feared that gene doping would enter the world of competitive sports very quickly; in fact, the sports media have predicted that every Olympic Games in the last decade would probably be the first genetically doped games. Indeed, several instances have come to light that can only be interpreted as serious attempts at gene doping. An athletic coach in Germany was found to be making diligent efforts to obtain a gene vector called Repxygen that contains and expresses the erythropoietin gene and was developed to increase blood production in patients with serious diseases such as cancer and chronic kidney disease. The product of the gene, erythropoietin, is in fact one of the most widely used drugs in the world for treatment of these disorders and, of course, is known to be heavily abused in some endurance sports, such as cycling. In addition, shortly before the Beijing Games in 2008, a German investigative television team broadcast a program identifying a Chinese scientist who was reported to be offering genetic manipulation for athletes [10262].

There is no documented case of genetic techniques having been used to enhance athletic ability in people. We do know that genetic manipulations have produced mice and, in some cases, primates with enhanced traits crucial for athletic performance, including increased
muscle function, prolonged endurance, and elevated blood production. If gene therapy is becoming increasingly feasible and available and if the pressure for gene doping is so great, why has it not yet been documented in athletes? One reason may be that the procedures for safe, successful, and legitimate genetic manipulation for medical purposes are extremely complicated, lying outside the capability of most rogue operations. Even though the production of gene doping materials is achievable using standard graduate school or even undergraduate molecular biology technology, the truly difficult aspect of gene therapy is its execution: bringing about the safe and effective performance of complicated human clinical manipulations by methods consistent with international ethical standards of human clinical work. Even for legitimate gene therapy, it has taken several decades of experimental refinement and testing to learn how to express added genetic information safely in human patients. Gene therapy remains a highly experimental and potentially risky technique, and even some of the successful therapies have caused serious side effects, including leukemia and even death [10262].

We know from vast experience that, like all technology, the use of apparently sophisticated genetic doping methods will not await demonstration of safety, much less efficacy, before being applied in sports. For that reason, the World Anti-Doping Agency has included genetic doping in its list of banned methods since 2004 and has instituted major research projects to identify potential methods for gene doping and for detection. And yet, those intent on using illicit methods are likely to pay little attention to WADA lists or to comply with the multiple layers of local and national oversight and regulation required for gene therapy – review and approval of local institutional review boards, human subjects committees, and federal oversight and regulatory bodies. The financial and other rewards are too great and the sources of funding too deep in sports for those intent on gene doping to be concerned about such troublesome niceties [10262].

Sport is a deeply human activity dependent on an honest and transparent rule-based “contract” between participants. Those who love it deserve protection from those who would weaken or destroy its rules and introduce unethical, ineffective, and probably harmful materials and tools. Those who practice genetic manipulation, evading requirements for ethical and scientific review and applying genetic tools without full disclosure and informed consent, should certainly be considered guilty of scientific or medical malpractice and professional misconduct [10262].

The challenges posed to sports organizations concerned with gene doping are compounded by the ubiquity of the Internet, relatively unconstrained by geographical boundaries, which, when fueled with private commercial interests, creates a powerful marketing tool for promotion and distribution of performance-enhancing agents. An industry has emerged to cater to the desire of athletes and their coaches to find a competitive edge. Athletes are an especially vulnerable population in the marketing of performance enhancement. Reputable athletes or coaches with little knowledge of genetics are at a disadvantage in assessing “scientific” claims that appear in advertisements. Marketing is particularly worrisome when the science is still a work in progress, when a person’s health can be adversely affected, and when consumer knowledge about genetics is low. Although advertisements promoting products that promise to enhance athletic performance have pervaded the Internet for many years, recently it has become home for advertisements that promote products to “alter muscle genes...by activating your genetic machinery”, or that state “your genetic limitations are a thing of the past!” Although commercial web sites may be “biased, and unreliable by rigorous scientific standards, they are a principal source of information for many athletes and should be monitored when looking for evidence of developing trends in doping”. Some athletes and coaches will be tempted, prematurely and unwisely, to take advantage of results
packaged by some as performance enhancement "breakthroughs," even if they are untested in humans and the only "breakthrough" is faster or stronger mice [10263].

Our ever-increasing understanding of the genetic control of cardiovascular and musculoskeletal function together with recent technical improvements in genetic manipulation generates mounting concern over the possibility of such technology being abused by athletes in their quest for improved performance. A review of the literature was now performed to identify the genes and methodologies most likely to be used for gene doping and the technologies that might be used to identify such doping. A large number of candidate performance-enhancing genes have been identified from animal studies, many of them using transgenic mice. Only a limited number have been shown to be effective following gene transfer into adults. Those that seem most likely to be abused are genes that exert their effects locally and leave little, if any, trace in blood or urine. There is currently no evidence that gene doping has yet been undertaken in competitive athletes but the anti-doping authorities will need to remain vigilant in reviewing this rapidly emerging technology. The detection of gene doping involves some different challenges from other agents and a number of promising approaches are currently being explored [10264].

The misuse of somatic gene therapy for the purpose of enhancing athletic performance is perceived as a coming threat to the world of sports and categorized as "gene doping". One article describes a direct detection approach for gene doping that gives a clear yes-or-no answer based on the presence or absence of transgenic DNA in peripheral blood samples. By exploiting a priming strategy to specifically amplify intronless DNA sequences, we developed PCR protocols allowing the detection of very small amounts of transgenic DNA in genomic DNA samples to screen for six prime candidate genes. The detection strategy was verified in a mouse model, giving positive signals from minute amounts (20 mikroL) of blood samples for up to 56 days following intramuscular adeno-associated virus-mediated gene transfer, one of the most likely candidate vector systems to be misused for gene doping. To make our detection strategy amenable for routine testing, we implemented a robust sample preparation and processing protocol that allows cost-efficient analysis of small human blood volumes (200 microL) with high specificity and reproducibility. The practicability and reliability of the detection strategy was validated by a screening approach including 327 blood samples taken from professional and recreational athletes under field conditions [11301].

With the advances in gene therapy fears of an abuse in sports arise. The WADA's definition of the term strictly differentiates between gene doping and gene therapy. There are in vivo and ex vivo practices to manipulate the different phases of gene expression in the organism, with viral vectors being looked upon as the most efficient ones. IGF-1, PPAR-delta, MSTN and EPO play the most important roles in today's scientific research. Their potential was proven in various animal studies, showing a significant improvement of performances. Potential risks for human users include severe immune reactions, mutagenesis, and raised risk for cancer. Big efforts are being put into the development of ways of detection, however until now there are neither practicable methods of control nor any reported cases of manipulated humans. Still, a usage of gene doping that has already taken place cannot be ruled out and is highly likely [11302].

Legitimate uses of gene transfer technology can benefit from sensitive detection methods to determine vector biodistribution in pre-clinical studies and in human clinical trials, and similar methods can detect illegitimate gene transfer to provide sports-governing bodies with the ability to maintain fairness. Real-time PCR assays were developed to detect a performance-enhancing transgene (erythropoietin, EPO) and backbone sequences in the presence of endogenous cellular sequences. In addition to developing real-time PCR assays, the steps involved in DNA extraction, storage and transport were investigated. By real-time PCR, the
vector transgene is distinguishable from the genomic DNA sequence because of the absence of introns, and the vector backbone can be identified by heterologous gene expression control elements. After performance of the assays was optimized, cynomolgus macaques received a single dose by intramuscular (IM) injection of plasmid DNA, a recombinant adeno-associated viral vector serotype 1 (rAAV1) or a rAAV8 vector expressing cynomolgus macaque EPO. Macaques received a high plasmid dose intended to achieve a significant, but not life-threatening, increase in hematocrit. rAAV vectors were used at low doses to achieve a small increase in hematocrit and to determine the limit of sensitivity for detecting rAAV sequences by single-step PCR. DNA extracted from white blood cells (WBCs) was tested to determine whether WBCs can be collaterally transfected by plasmid or transduced by rAAV vectors in this context, and can be used as a surrogate marker for gene doping. It was demonstrated that IM injection of a conventional plasmid and rAAV vectors results in the presence of DNA that can be detected at high levels in blood before rapid elimination, and that rAAV genomes can persist for several months in WBCs [11303].

To achieve success in sports, many athletes consume doping substances, such as anabolic androgenic steroids and growth hormones, and ignore the negative influence of these drugs on their health. Apart from the unethical aspect of doping in sports, it is essential to consider the tremendous risk it represents to their physical condition. The abuse of pharmaceuticals which improve athletic performance may alter the expression of specific genes involved in muscle and bone metabolism by epigenetic mechanisms, such as DNA methylation and histone modifications. Moreover, excessive and relentless training to increase the muscle mass, may also have an influence on the health of the athletes. This stress releases neurotransmitters and growth factors, and may affect the expression of endogenous genes by DNA methylation, too. One paper focuses on the relationship between epigenetic mechanisms and sports, highlights the potential consequences of abuse of doping drugs on gene expression, and describes methods to molecularly detect epigenetic changes of gene markers reflecting the physiological or metabolic effects of doping agents [11304].

The quest for athletic excellence holds no limit for some athletes, and the advances in recombinant DNA technology have handed these athletes the ultimate doping weapons: recombinant proteins and gene doping. Some detection methods are now available for several recombinant proteins that are commercially available as pharmaceuticals and being abused by dopers. However, researchers are struggling to come up with efficient detection methods in preparation for the imminent threat of gene doping [07395].

After only a short history of three decades from concept to practice, gene therapy has recently been shown to have potential to treat serious human diseases. Despite this success, gene therapy remains in the realm of experimental medicine, and much additional preclinical and clinical study will be necessary for proving the efficacy and safety of this approach in the treatment of diseases in humans. However, a potential complicating factor is that advances in gene transfer technology could be misused to enhance athletic performance in sports, in a practice termed "gene doping". Moreover, gene doping could be a precursor to a broader controversial agenda of human "genetic enhancement" with the potential for a significant long-term impact on society. One review addressed the possible ways in which knowledge and experience gained in gene therapy in animals and humans may be abused for enhancing sporting prowess. It was provided an overview of recent progress in gene therapy, with potential application to gene doping and with the major focus on candidate performance-enhancement genes. It was also discussed the current status of preclinical studies and of clinical trials that use these genes for therapeutic purposes. Current knowledge about the association between the natural "genetic make-up" of humans and their physical characteristics and performance potential is also presented. It was addressed issues associated with the safety of gene transfer technologies in humans, especially when used...
outside a strictly controlled clinical setting, and the obstacles to translating gene transfer strategies from animal studies to humans. We also address the need for development and implementation of measures to prevent abuse of gene transfer technologies, and to pursue research on strategies for its detection in order to discourage this malpractice among athletes [07396].

Implications of novel genetic interventions fascinated not only researchers, physicians, and gene therapists, but also coaches, athletes, and trainers looking for athletic performance enhancement of biologic parameters, such as strength, power, and oxygen delivery, to create a critical edge in sporting competition. The creation of a superman or superwoman athlete could be planned by well-placed genetic physiologic tweaks. Without a single known human incident of gene doping, WADA bestowed the technique of gene doping a dishonored place on the list of prohibited substances. For an entire biomedical technique to be banned, before even acquiring regulatory approval by any government or before acceptance by any branch of organized medicine, seems to be unprecedented. Defined as “the transfer of genetic material to human cells for the treatment or prevention of a disease or disorder,” gene therapy uses genetic materials, such as DNA, RNA, or genetically altered cells. In the simplest form, gene therapy introduces a “therapeutic gene” (transgene) into an organism by way of a vector, often an inactive virus. Within the organism itself, the new “transgene” synthesizes the defective/missing protein or biologic substance to correct dysfunctional tissues and organs. Other gene therapy strategies involve manipulation of genes, turning them on or off as the desired physiologic response dictates. Initial gene therapy trials included protocols to treat an X-linked immunodeficiency disease and hemophilia variant. A trial of human vascular endothelial growth factor produced positive results in patients who had angina. More than 1000 gene therapy trials have been ongoing in various states of clinical study. No gene therapy protocols have been approved for medical practice by the US Food and Drug Administration, the regulatory agency charged with overseeing the development and clinical use of the medical procedure [07046].

Drug doping uses therapeutic advances in exercise physiology and clinical pharmacology to provide unfair advantages to athletes who covertly use anabolic drugs, thus dramatically enhancing competitive performance. Similar to drug doping, gene doping manipulates scientific advances originally developed for the treatment of disease. Rather than drug interventions, the gene-doping athletes appropriate advances in gene therapies. Gene doping, in concept, uses scientific developments that manipulate DNA in the most basic regulation of biologic processes, to dramatically improve aspects of athletic performance, such as speed, power, or endurance Molecular biology, particularly the “discovery” of DNA by Watson and Crick in 1953, revolutionized biology and medicine. The rate of genetic discovery in molecular biology rapidly accelerated throughout the last half of the twentieth century and into the twenty-first century. The new millennium was to be the dawning of practical, effective treatments for genetic diseases, such as muscular dystrophy, X-linked hemophilia, and other single-gene disorders. Further, with molecular biology advances that included insertions of new genes into organisms, cloning of organisms, and use of human stem cells, more than single-gene diseases could be treated. Any number of serious or fatal medical conditions might be altered with the introduction of genes that would produce “in vivo pharmacies” delivering biochemicals, including proteins and hormones, to injured or impaired tissue [07046].

Gene therapy also could improve a general disadvantageous condition, such as aging-related muscle atrophy, by introducing a transgene to produce the depleted factors involved in muscle repair and regeneration. Therapeutic genes could be targeted directly into cells, tissues, and organs limiting effects to a localized site, thus reducing the systemic side effects produced by a typical drug administration. With the advent of gene therapy, a more direct
way to deliver proteins and hormones to an athlete’s tissues and organs became reality. The sophistication and the power of these biologic alterations piqued the ingenuity of the drug-cheating coaches and athletes. A substance that can alter the basic genetic expression of DNA – such that muscles grow larger, contract more forcefully, and recover more quickly than non-doped muscles – and cannot be detected by anti-doping laboratories would be ideal to gain a competitive advantage while not running afoul of the regulatory officials. In the broad sense, genetic therapies include several categories of biotechniques [07046]

- use of recombinant DNA techniques to produce new peptides or drugs (rEPO)
- pharmacogenetics, or the use of knowledge of the specific genome of an athlete to tailor pharmacologic interventions
- somatic cell modification, which produces genetically modified cells (e.g. modified RBCs to increase blood-carrying capacity)
- germ-line modification, where the gametes or early embryos undergo gene modification to express more athletically expedient traits
- genetic preselection, where a gene scan would inform parents about the distribution of desired genes in a potential offspring
- genetic selection, where individuals are selected for particular traits (widely practiced in animal husbandry)

The common inheritance of approximately 20,000 genes defines each of us as human. However, substantial variation exists between individual human genomes, including ‘replication’ of gene sequences (copy number variation, tandem repeats), or changes in individual base pairs (mutations if <1% frequency and single nucleotide polymorphisms if >1% frequency). A vast array of human phenotypes (e.g. muscle strength, skeletal structure, tendon elasticity, and heart and lung size) influences sports performance, each itself the result of a complex interaction between a myriad of anatomical, biochemical and physiological systems. This article discusses the role for genetic influences in influencing sporting performance and injury, offering specific exemplars where these are known. Many of these preferable genotypes are uncommon, and their combination even rarer. In theory, the chances of an individual having a perfect sporting genotype are much lower than 1 in 20 million – as the number of associated polymorphisms increase, the odds decrease correspondingly. Many recently discovered polymorphisms that may affect sports performance have been described in animal or other human based models, and have been included in this review if they may apply to athletic populations. Muscle performance is heavily influenced by basal muscle mass and its dynamic response to training. Genetic factors account for approximately 50-80 percent of inter-individual variation in lean body mass, with impacts detected on both “training-naive” muscle mass and its growth response. Several cytokines such as interleukin-6 and -15, ciliary neurotrophic factor and insulin-like growth factor (IGF) have myoanabolic effects. Genotype-associated differences in endocrine function, necessary for normal skeletal muscle growth and function, may also be of significance, with complex interactions existing between thyroxine, growth hormone and the downstream regulators of the anabolic pathways (such as IGF-1 and IGF-2). Almost 200 polymorphisms are known to exist in the vitamin D receptor (VDR) gene. VDR genotype is associated with differences in strength in premenopausal women. VDR expression decreases with age and VDR genotype is associated with fat-free mass and strength in elderly men and women. Muscle fibre type determination is complex. Whilst initial composition is likely to be strongly influenced by genetic factors, training has significant effects on fibre shifts. Polymorphisms of the peroxisome proliferator-activated receptor alpha (PPARalpha) gene and R577x polymorphism of the ACTN3 gene are both associated with specific fibre compositions. Alterations in cardiac size have been associated with both increased performance and excess cardiovascular mortality. PPARalpha is a ligand-activated
transcription factor that regulates genes involved in fatty acid uptake and oxidation, lipid metabolism and inflammation. Psychology plays an important role in training, competition, tolerance of pain and motivation. However, the role of genetic variation in determining psychological state and responses remains poorly understood; only recently have specific genes been implicated in motivational behaviour and maintenance of exercise. Thyroid hormone receptors exist within the brain and influence both neurogenesis and behaviour. With the current state of knowledge, the field of genetic influences on sports performance remains in its infancy, despite over a decade of research [11544].

Gene therapy was first successfully applied in 1990 in the treatment of a four-year-old child suffering from a severe combined auto-immuno deficiency (SCAD). A number of further successes were followed by a heavy setback in 1999 when a young man died as a consequence of a gene therapy. Presently gene therapy is once more on its way to becoming a normal therapeutic measure where conventional therapies fail. The increasing practice of gene therapy of course also implies the risk of misuse in sport – gene doping. The World Anti-Doping Agency, founded in 1999, organized its first scientific workshop on gene doping in 2002 in New York. Further conferences in Stockholm (2005) and St Petersburg (2008) followed and various national and international meetings have been dedicated to the specific issue of misusing knowledge arising from biotechnological advances in medicinal research. In 2010, The Federal Institute of Sports Science of Germany organized its second conference on gene doping to assess the potential danger of misuse of modern methods of molecular biology. It featured three special areas of genetics with a view to elucidating both the possibilities of misuse and its detection: genome, transcriptome and epigenetics. Polymorphism at the myostatin gene locus and EPOreceptor gene locus are of particular interest when aiming at muscle growth and elevated oxygen capacity, respectively. Many other gene loci are related to performance in sport but are not as unilocular as gene loci previously enumerated. Results of family studies and other comparative investigations show various participating genes but significance is rarely given and ‘lucky punches’ of significance of relatively small samples lose their ground when the investigated population grows larger. Polymorphism of the gene locus UGT2B has been shown to influence the metabolism and elimination of testosterone and epitestosterone and their concentration in urine. Consequently, the impact of this phenomenon on modern sports drug testing, a field that considers the steroid profile as one of the most important sources of information in doping controls, was elucidated. Further, research concerning the role of mitochondrial DNA (regarding the energy cycle and mitochondrial myopathies related to defects of mtDNA) was presented in the context of gene therapy; its misuse potential in sport, however, has been implausible so far [11557].

The genome is not an isolated double strand of DNA in the nucleus but is encapsulated by histones, a group of protecting proteins surrounding the DNA forming strings of spheres. In a figurative sense, these shields are cut at distinct places as the first step of transcribing a part of DNA, for example, by so-called zincfinger proteins. Evidence or indication of misuse in sports has not (yet) been provided; methylation and demethylation of DNA, as another epigenetic regulatory mechanism of gene expression, may offer a tool of manipulation. The transcriptome is a mirror of genetic activity and is essential for transporting genetic information. The mRNA generated in the nucleus consists of exons and space-filling introns; the latter are removed immediately by enzymes in the cytoplasm so that the active mRNA is composed of exons only. This offers one option to detect the misuse of gene therapy because only RNA can be copied to cDNA in the cytoplasm for inserting this cDNA with a gene ferry (e.g. a virus). The resulting gene then consists of exons only and can be identified as xenobiotic. Introns will also be digested to different forms of RNA which act as regulators of further translational processes. Although a lot of knowledge about these effects is at hand, they are not sufficiently calculable yet. Manipulations on tissues also result in free circulating
DNA (cDNA) which might be useful for sports drug testing purposes. cDNA today is a powerful diagnostic tool for various diseases, for example, different forms of cancer. Samples for the detection of doping usually consist of urine and particularly blood. The latter is often preferred as genetic manipulation can be tissue-specific and not detectable in urine. Detection in blood might be possible but limited in respect to its specificity. One of the most likely target tissues of manipulation is the muscle. In order to determine whether muscle tissue has been the subject of gene doping, muscle biopsies are presumably the only way to obtain an adequate sample of cells for assaying. Although a micro biopsy may be a possibility to obtain a sufficient number of cells without injuring the muscle, which would imply a functional reduction, such specimens are currently not in the scope of anti-doping authorities. In summary, gene doping still seems to be an emerging problem in high-level sports as gene therapy develops to become a controllable tool in medicine and in the manipulation of livestock [11557].

Over the course of the past decade, technical progress has enabled scientists to investigate genome-wide RNA expression using microarray platforms. This transcriptomic approach represents a promising tool for the discovery of basic gene expression patterns and for identification of cellular signalling pathways under various conditions. Since doping substances have been shown to influence mRNA expression, it has been suggested that these changes can be detected by screening the blood transcriptome. In one review, it was critically discuss the potential but also the pitfalls of this application as a tool in doping research. Transcriptomic approaches were considered to potentially provide researchers with a unique gene expression signature or with a specific biomarker for various physiological and pathophysiological conditions. Since transcriptomic approaches are considerably prone to biological and technical confounding factors that act on study subjects or samples, very strict guidelines for the use of transcriptomics in human study subjects have been developed. Typical field conditions associated with doping controls limit the feasibility of following these strict guidelines as there are too many variables counteracting a standardized procedure. After almost a decade of research using transcriptomic tools, it still remains a matter of future technological progress to identify the ultimate biomarker using technologies and/or methodologies that are sufficiently robust against typical biological and technical bias and that are valid in a court of law [11545].

RNA interference represents a comparably new route of regulating and manipulating specific gene expression. Promising results were obtained in experimental therapies aim at the treatment of different kinds of diseases including cancer, diabetes mellitus or Duchenne muscular dystrophy. While studies on down-regulation efficiency are often performed by analyzing the regulated protein, the direct detection of small, interfering RNA molecules and antisense oligonucleotides is of great interest for the investigation of the metabolism and degradation and also for the detection of a putative misuse of these molecules in sports. Myostatin down-regulation was shown to result in increased performance and muscle growth and the regulation of several other proteins could be relevant for performance enhancement. One mini-review summarizes current approaches for the mass spectrometric analysis of siRNA and antisense oligonucleotides from biological matrices and the available data on biodistribution, metabolism, and half-life of relevant substances are discussed [11546].

The issue of gene doping has chaperoned scientific accomplishments in gene therapy for at least a decade, and despite considerable reservations as to what kind of benefit cheating athletes could possibly expect as well as detrimental health and legal consequences. Two major scenarios of gene doping are described with one being the abuse of “classical” gene therapy, i.e. introduction of synthetic DNA sequences via viral vehicles into the organism, and the other being based on RNA interference strategies. The latter has recently been considered the more promising approach in therapeutic settings, which however also implies
that there is a higher risk of its abuse in sports. The most common approach to directly determine synthetic exogenous DNA relies on the amplification by polymerase chain reaction (PCR), exploiting the presence of exon-exon junctions in exogenous DNA sequences. Using such strategies, the determination of incorporated exogenous DNA was traceable in white blood cells up to 57 weeks after intramuscular injection. In order to improve the method’s sensitivity and exclude false negative results, an internal threshold control (ITC) was suggested that should compensate for sample preparation and analysis issues. The approach was applied to a non-human primate EPO gene doping model providing proof-of-concept data, which should be corroborated with further analyses demonstrating that the principle can be applied to other DNA targets as well. In a different study, an attempt was conducted to determine the intramuscularly administered plasmid (cytomegalovirus-focal adhesion kinase) in rats using PCR. While tissue sampling of the transfected muscle allowed for the detection of the exogenous DNA sequence for up to seven days, essentially all serum samples returned negative test results, demonstrating the challenging aspect of sports drug testing since tissue sampling will not be an option in doping controls. As the most common route of gene transfer is through viral vehicles, a complementary indirect approach was presented, aiming at the detection of backbone sequences of the employed vector, seconded by the analysis of a so-called construct-specific marker. The latter comprises parts of the promoter and the transgene, representing a non-natural target for PCR amplification and analysis, supporting the differentiation of a coincidentally present virus in the host from a modified recombinant vector backbone. The methodology was applied to transduced laboratory mice and proof-of-concept was obtained for blood, urine, tears, and various tissues; however, detection windows were comparably small (1-6 days) [12475].

With one recently recommended gene therapy in Europe and a number of other gene therapy treatments now proving effective in clinical trials it is feasible that the same technologies will soon be adopted in the world of sport by unscrupulous athletes and their trainers in so called gene doping. In this article an overview of the successful gene therapy clinical trials is provided and the potential targets for gene doping are highlighted. Depending on whether a doping gene product is secreted from the engineered cells or is retained locally to, or inside engineered cells will, to some extent, determine the likelihood of detection. It is clear that effective gene delivery technologies now exist and it is important that detection and prevention plans are in place [12475].

It has been established that excessive training, by inducing stress and releasing neurotransmitters and cytokines, may naturally affect the expression of endogenous genes via DNA methylation. However, the abuse of certain physical performance-enhancing substances, such as AAS or GH, may also change the expression of genes via epigenetic mechanisms such as DNA methylation and histone modifications, this technique bearing the name of “gene doping”. Gene or cell doping is defined by WADA as “the non-therapeutic use of cells, genes, genetic elements, or of the modulation of gene expression, having the capacity to enhance athletic performance’.’ The main targets of gene doping are the myostatin gene, EPO and IGF-1. For example, removal or decrease of the expression of the myostatin gene is capable of elevating hypertrophy and muscle power. The discovery of “physical performance genes”, which have led to novel techniques for gene transfer for the purposes of gene doping, is a particularly highly detrimental feature of modern day doping because of the extreme difficulty in detecting it. Direct as well as indirect testing methods have been implemented by WADA to assist in the challenging task of detecting gene doping. Direct methods search for recombinant proteins or gene insertion vectors, while indirect methods, by nature more subjective, are based on the clinical examination of the athlete in an attempt to register bodily changes or structural differences between endogenous and recombinant proteins. This raises the urgent need for sophisticated molecular biology techniques, such as the use of lab-on-a-chip techniques and nanoparticles, to enable the
distinction between the “normal” and “modified” genome, while complex methodology is required to trace gene doping. A real-time PCR assay targeting sequences within the transgene complementary DNA corresponding to exon/exon junctions – which, due to their interruption by introns, are absent in the endogenous gene – may allow detection of trace amounts of a transgene against a broad background of the endogenous gene. This is an effective method to detect exogenous DNA. The incorporation of an internal threshold control (ITC) serves to avoid confounding false positive or false negative results, while simultaneously obtained fluorescence emission signals determine the cycle thresholds for amplification of the target and ITC sequences. This method, according to the authors, may enhance the detection capability of gene doping. Gene doping is a highly complex issue as there is a very fine line between legitimate medical intervention and nonlegitimate exploitation of an individual’s genetic make-up for competitive advantage [12011].

Gene therapy generally involves the delivery of genetic material encoding the expression of proteins that are either endogenous or biological for treatment of disease. Gene therapy has great potential to treat genetic diseases and holds much promise as a mode of delivering biological molecules in more widespread conditions. After initial hype and several lows the first gene therapy product has now been recommended for approval in Europe and there have been a number of recent clinical trials that have demonstrated the effectiveness of this treatment modality. The possibility of gene doping, defined as the transfer of nucleic acid sequences and/or the use of normal or genetically modified cells to enhance sport performance, is also a real concern in sports medicine. The abuse of knowledge and techniques gained in the area of gene therapy is a form of doping, and is prohibited for competitive athletes. As yet there is no conclusive evidence that that gene doping has been practiced in sport. However, given that gene therapy techniques improve continuously, the likelihood of abuse will increase. All gene therapy approaches use a vector to deliver the genetic material to cells and also utilize the transcriptional machinery of the cell for gene expression. The delivery vector can be as simple as naked plasmid DNA or as complex as replication deficient recombinant viruses that have innate ability to deliver genes. The first gene therapy successes were achieved in X-linked severe combined immunodeficiency (SCID) patients through ex vivo engineering their bone marrow stem cells (BMSC) with retrovirus encoding a correct copy of the common cytokine-receptor gamma chain prior to re-implantation. Although only a small percentage of cells are corrected these have a selective advantage and are able to populate the patient with a functioning immune system. This approach of engineering BMSC has been similarly adopted in the successful treatment of other genetic conditions including adenosine deaminase (ADA) SCID and X-linked chronic granulomatous disease [13755].

A literature search was conducted to identify the most relevant proteins based on their current gene doping potential using articles from Pubmed, Scopus and Embase published between 2006 and 2011. The final list of selected proteins were erythropoietin, insulin-like growth factor, growth hormone, myostatin, vascular endothelial growth factor, fibroblast growth factor, endorphin and enkephalin, α actinin 3, peroxisome proliferator-activated receptor-delta (PPAR-delta) and cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C). We discuss these proteins with respect to their potential benefits, existing gene therapy experience in humans, potential risks, and chances of detection in current and future anti-doping controls. It was identified PPARdelta and PEPCK-C as having high potential for abuse. But we expect that for efficiency reasons, there will be a preference for inserting gene target combinations rather than single gene doping products. This will also further complicate detection [13755].

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sports medicine. The abuse of knowledge and techniques gained in the area of gene therapy is a form of doping, and is prohibited for competitive athletes. As yet there is no conclusive evidence that that gene doping has been practiced in sport. However, given that gene therapy techniques improve continuously, the likelihood of abuse will increase. A literature search was conducted to identify the most relevant proteins based on their current gene doping potential using articles from Pubmed, Scopus and Embase published between 2006 and 2011 [13011].

Given that gene therapy techniques have improved considerably, the likelihood of gene doping has increased. Today, most gene therapy studies examine hereditary diseases and cancer. Gendicine (recombinant Human Ad-p53 Injection) and Glybera (alipogene tiparvovec) are the first approved gene therapy products for human use in the USA and the EU, respectively. Gendicine is designed to place a p53 gene in cancer cells to inhibit cell growth and the Glybera gene therapy has been approved for treatment of life-threatening pancreatitis attacks in patients with lipoprotein lipase deficiency [13011].

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Gene therapist Ted Friedmann and multiple Olympic gold medallist Johann-Olav Koss were the first to describe the possibility of misusing the techniques and experiences of gene therapy in the athletic arena. In 2006, before the Turin Winter Olympic games, the president of the World Anti-Doping Agency (WADA), Dick Pound, called gene doping “the new threat that is now a reality.” Although Pound did not expect gene doping to pose a problem in Turin, he indicated that it could be a problem at the Summer Games, 2 years hence in Beijing. In fact, the problem did not materialise in China, in 2008, nor at the London 2012 Olympics, as far as the then available detection measures could determine. Yet again, we have to operate on the assumption that there may be athletes out there willing to test gene doping at the 2016 Rio de Janeiro Olympics. After all, an Olympic gold medal means considerable social and economic benefit. Historical doping control statistics show that somewhere between 1.1 and 2 percent of all athletes test positively for doping. The real number of doping users is expected to be higher, despite the fact that the governing bodies of sport place immense pressure on athletes by a strict liability rule that makes them responsible for everything in their bodies. Although the detection of doping is constantly improving, it generally trails actual practice [13011].

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The issue of gene doping has chaperoned scientific accomplishments in gene therapy for at least a decade, and despite considerable reservations as to what kind of benefit cheating athletes could possibly expect as well as detrimental health and legal consequences, there is an urgent need to pursue anti-doping efforts concerning the manipulation of the sportsmen's genetic material. Two major scenarios of gene doping are described with one being the abuse of “classical” gene therapy, i.e. introduction of synthetic DNA sequences via viral vehicles into the organism, and the other being based on RNA interference strategies. The latter has recently been considered the more promising approach in therapeutic settings, which however also implies that there is a higher risk of its abuse in sports [13012].

The most common approach to directly determine synthetic exogenous DNA relies on the amplification by polymerase chain reaction (PCR), exploiting the presence of exon-exon junctions in exogenous DNA sequences. Using such strategies, the determination of incorporated exogenous DNA was traceable in white blood cells up to 57 weeks after intramuscular injection. In order to improve the method's sensitivity and exclude false-negative results, an internal threshold control (ITC) was suggested that should compensate for sample preparation and analysis issues. The approach was applied to a non-human primate EPO gene doping model providing proof-of-concept data, which should be corroborated with further analyses demonstrating that the principle can be applied to other DNA targets as well. In a different study, an attempt was conducted to determine the intramuscularly administered plasmid (cytomegalovirus-focal adhesion kinase) in rats using PCR. While tissue sampling of the transfected muscle allowed for the detection of the exogenous DNA sequence for up to seven days, essentially all serum samples returned negative test results, demonstrating the challenging aspect of sports drug testing since tissue sampling will not be an option in doping controls [13012].

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The potential of gene doping was once more demonstrated in a multisite adeno-associated virus-IGF-1 gene transfer experiment with mice. Besides significant endurance performance enhancement (as assessed by exhaustive swimming tests), substantial alterations in the muscle proteome were recognized, affecting both energy expenditure pathways as well as structural and contractile proteins [13012].

During the last 2 decades, progress in deciphering the human gene map as well as the discovery of specific defective genes encoding particular proteins in some serious human diseases have resulted in attempts to treat sick patients with gene therapy. There has been considerable focus on human recombinant proteins which were gene-engineered and produced in vitro (insulin, growth hormone, insulin-like growth factor-1, erythropoietin). Unfortunately, these substances and methods also became improper tools for unscrupulous athletes. Biomedical research has focused on the possible direct insertion of gene material into the body, in order to replace some defective genes in vivo and/or to promote long-lasting
endogenous synthesis of deficient proteins. Theoretically, diabetes, anaemia, muscular
dystrophies, immune deficiency, cardiovascular diseases and numerous other illnesses could
benefit from such innovative biomedical research, though much work remains to be done.
Considering recent findings linking specific genotypes and physical performance, it is
tempting to submit the young athletic population to genetic screening or, alternatively, to
artificial gene expression modulation. Much research is already being conducted in order to
achieve a safe transfer of genetic material to humans. This is of critical importance since
uncontrolled production of the specifically coded protein, with serious secondary adverse
effects (polycythaemia, acute cardiovascular problems, cancer, etc.), could occur. Other
unpredictable reactions (immunogenicity of vectors or DNA-vector complex, autoimmune
anaemia, production of wild genetic material) also remain possible at the individual level.
Some new substances (myostatin blockers or anti-myostatin antibodies), although not gene
material, might represent a useful and well-tolerated treatment to prevent progression of
muscular dystrophies. Similarly, other molecules, in the roles of gene or metabolic activators
[5-aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside (AICAR), GW1516], might
concomitantly improve endurance exercise capacity in ischaemic conditions but also in
normal conditions. Undoubtedly, some athletes will attempt to take advantage of these new
molecules to increase strength or endurance. Antidoping laboratories are improving detection
methods. These are based both on direct identification of new substances or their
metabolites and on indirect evaluation of changes in gene, protein or metabolite patterns
(genomics, proteomics or metabolomics) [13756].

The science behind gene doping

Definitions and history

When gene therapy is used to increase the performance of a healthy person, it is considered
gene doping by WADA. Gene doping presents the same advantages over regular doping as
gene therapy does for regular medicine, but detection of gene doping is more difficult. Gene
doping has been prohibited by the International Olympic Committee (IOC) since 2003. In
2004, WADA took responsibility for publishing the Olympic doping list, and they added gene
doping. The following new methods with the potential to enhance sport performance are
prohibited [13011]:

1. The transfer of nucleic acids or nucleic acid sequences
2. The use of normal or genetically modified cells

As for gene therapy, every known gene can be used for gene doping. Currently, only about
500 genes in the human genome are used in existing drugs, thus a significant number of the
remaining genes could bring new options for doping. At least 100 genes are already linked to
athletic performance and the number is increasing every year. Although not all of these
genes can be considered to be potential gene doping candidates, the increasing number of
genes used in medications raises expectations for the potential advantages of gene doping.
The fear is that athletes might not wait for gene therapy to be fully developed and tested
before misusing it [13011].

The explosion of genetic knowledge in the latter part of the twentieth century has given rise
to new ways of thinking about human biology in health and disease. These advances have
begun to deliver on their promise of new and more effective approaches to the prevention
and treatment of human disease. This genetic revolution started long ago in the middle of the
nineteenth century with the work of Gregor Mendel and the concepts of inheritance that
emanated from his studies of the properties of peas and other plants in the monastery in

2028
Brno in the Austro-Hungarian Empire. Mendel discovered that the physical properties of these living systems are inherited in predictable and reproducible ways and that there are biological factors in the plants that carry these traits from one generation to the next. Mendel’s work was forgotten by the scientific community until the beginning of the twentieth century when his studies were “rediscovered” independently by several scientists and, for the first time, applied to human disease by the English clinician Sir Archibald Garrod during the first decade of the twentieth century. In his clinical work, Garrod was treating patients suffering from a number of familial conditions. The rules that Mendel had worked out in plants were found to be equally important to human beings in determining human disease. Garrod coined the term “inborn errors of metabolism” to describe the underlying processes that led to the appearance of these and other inherited human conditions. The structure or composition of the factors discovered by Mendel, and first applied to human disease, was not known for many years. The discovery of the true chemical nature of the gene was made in 1944 through the work of Oswald Avery and his group at the Rockefeller Institute in New York. Avery and his team found that they could permanently and heritably change the infectivity properties of a strain of the bacterium pneumococcus by introducing deoxyribonucleic acid (DNA) from a different strain. With the discovery of the precise three-dimensional structure of DNA by James Watson and Francis Crick in Cambridge in 1953 and the elucidation of the genetic code by several groups of molecular biologists came the first real functional understanding of DNA. The “golden age” of molecular biology from 1953 through the 1970s and through the subsequent decades of one startling advance after another, have now culminated in the total sequence characterization of the human genome. Through all of these advances we know that:

- the genetic information of living systems, from microbes to humans, is contained in the sequence of four simple basic purine and pyrimidine chemical-building blocks
- many naturally occurring differences in that sequence account for normal variations among individuals of a species
- some differences in the sequence of genes, naturally occurring or induced by environmental influences, cause severe disruption of normal metabolic, cellular functions to cause disease.

**Gene transfer approach to therapy**

In the late 1960s and early 1970s, during the explosion of knowledge of molecular genetics, techniques emerged that began to promise precisely the new kind of approach to therapy. The genetic components of human disease were recognized, detailed mechanisms of gene expression were obtained, animal models of more and more human diseases were available for study in the laboratory, and early techniques for transferring foreign genes into human and other mammalian cells appeared. Inevitably, the connection of these advances in basic genetics with the need for definitive new methods of disease therapy was made, and the concept of “gene therapy” was born. From this conception more than three decades ago, it was clear that there were many difficult scientific, ethical, and policy obstacles to overcome before such techniques could be applied to humans for treatment of disease. Because of several ill-conceived and inappropriate applications of highly immature gene transfer technologies to human patients, an extensive set of oversight and regulation mechanisms have been put into place over the years. Gene transfer vectors have been constructed from many viruses (e.g. herpes viruses, pox viruses, flu viruses, even HIV). In such cases, the genes of the viruses themselves are removed or otherwise inactivated, leaving the virus with the only function of acting as a moving van, or in more surreptitious terms, a Trojan horse, to sneak foreign genes into the cells in a way that will be completely harmless to the cell.
**Therapeutic gene transfer vectors introduced into human beings**

Two principal methods have been developed to introduce gene transfer vectors into research subjects and patients. These methods are now called the “ex vivo” and “in vivo” methods and differ by whether the gene transfer vector is introduced directly into the tissues of the patient or research subject (“in vivo”) or alternatively into cells of the patients in the laboratory followed by the reintroduction of the genetically corrected cells into the patient (“ex vivo”). For instance, cells can be obtained from the bone marrow of a patient suffering from a disease that prevents normal production of blood cells. These cells can then be modified genetically by exposure to a gene transfer vector in the laboratory and the corrected cells can then be transfused back into the patient. Under these conditions, the corrected cells make their way into the defective bone marrow again, set up residence there, and replace the abnormal cells. In another ex vivo approach, skin cells can be obtained from a patient through a very small skin biopsy, grown in culture dishes in the laboratory, genetically modified as discussed earlier, and then put back into the patient in a number of tissues, including the brain where they can produce a product to correct a genetic deficiency in that organ. The use of a patient’s own cells for this ex vivo approach to transfer has the great advantage of avoiding the immune rejection of cells derived from other donors, whether they be closely related donors, unrelated donors, or possibly even nonhuman cells. In the latter cases, cells could be first put into microcapsules that prevent contact of the “foreign” cells with the host immune system that would cause an immune rejection response by the host and elimination of the therapeutic cells. In the case of the in vivo approach to gene transfer, a vector or even a “naked” piece of DNA can be introduced by injection directly into a tissue to transfer a foreign gene into the cells of that tissue [06305].

**Muscle cells**

One of the early surprises of the in vivo approach of gene transfer was that injection of DNA directly into skeletal muscle was a relatively effective way of causing a genetic modification of muscle cells and of providing prolonged expression of a foreign gene to alter muscle function. Most of these early studies involved genes that are needed specifically in the muscle cells themselves and that are involved in diseases such as muscular dystrophy. But muscle is also an excellent platform from which one can deliver many kinds of gene products into the blood stream for delivery to the entire body, and many foreign genes other than those producing muscle-specific functions have also been introduced into and expressed by muscle, including genes encoding growth factors, erythropoietin, and others. To achieve more efficient levels of gene transfer than those possible with naked DNA alone, virus vectors have also been injected directly into many tissues and organs in vivo, including many kinds of cancer, skeletal and even heart muscles, the brain, joints, the skin, the liver, the lungs and other parts of the airway, and many other sites [06305].

**Reversing the effects of transplanted genes**

We know now that our tools are still too immature to prevent serious harm to some people taking part in gene transfer experiments. It seems clear that the more effective we become with today’s tools and methods, the greater are the chances of serious and harmful side effects. Therefore, it is prudent to incorporate some procedures to remove the foreign gene or even to kill the cells carrying the transferred gene. In some cases, it might be possible to remove the cells surgically, especially if the gene transfer is performed into skin, an accessible muscle, or other tissue that can be accessed readily and can be removed with impunity. In those cases in which the foreign gene is placed into the body in the form of a small capsule from which the therapeutic gene product can be secreted into a local tissue or
into the blood stream, the capsule can be removed. One such method of selective cell removal involves the incorporation into the gene transfer vector of a “suicide” gene, i.e. a second gene in addition to the therapeutic gene that selectively acts only on cells that have been genetically modified and produces a toxin that kills them while not affecting the neighboring unmodified cells. Such an approach has been used in a number of human gene transfer studies aimed at the elimination of certain kinds of cancer cells, but until now the results of these studies have generally been imprecise. Nevertheless, these kinds of selective killing, or removal methods, in the event that the genetically modified cells grow out of control, or otherwise produce harm, hold promise [06305].

**Medical uses of gene therapy**

Gene therapy can be used to treat a variety of illnesses. It may be applied to weaken or kill cancer cells by triggering apoptosis, to enable target cells to produce a protein that otherwise has to be administered or to upregulate the production of a specific protein. Though a couple of gene therapy products have been marketed outside China to date, at least 1843 gene therapy trials have been conducted worldwide with thousands of patients suffering from cancer diseases, cardiovascular and neurological diseases and a range of other diseases. Early clinical trials in Europe and the USA had limited results and even fatalities were reported from gene therapy; however, examples of successful gene therapy include treatment of SCID-X1 and Leber’s congenital amaurosis. The proof of concept of various transfer strategies in gene therapy shows that we are at least at the beginning of a gene therapy revolution for patients with monogenic diseases [13011].

**Animal use of gene doping**

Since gene doping can increase performance, it is likely to be used in animal competitions as well. If money can be earned by betting or trading with superior animals, gene doping would be lucrative. If the achievements of horses, dogs, camels or pigeons could be improved, then it is quite possible that gene doping will be tried on these animals before human applications [13011].

**Genes versus environmental influence**

Physical traits, such as our hair color and height, as well as our personalities, intelligence, talents, and emotional properties, are the result of the potential provided by our genes and the nurturing and shaping of that potential by the environment in which we develop and grow. Furthermore, the presumed mix of the effects of “nature” and “nurture” – genetics and our environment – applies not only to what makes us normal but also to the “abnormal” traits that cause so many human diseases and suffering. In many cases, such as sickle-cell anemia and cystic fibrosis, the genetic factors are predominant. These diseases result from defects – “mutations” – in single genes, and chances are high that people who have inherited abnormal genes will develop the clinical disease, no matter how the environment is manipulated. “Nature” is enough to cause the disease. For other diseases, such as, various forms of diabetes, most heart diseases and cancer, psychiatric diseases, and so on, there may be a number of genetic factors that have to interact with each other and that also are expressed in many different ways depending on the environmental influences. “Nature”
interacts with “nurture” to bring about the clinical disease. In such cases, making changes in “nurture” may have a very significant effect on how that disease trait is expressed – hence, the reasonably effective treatments for many diseases with drugs, surgery, vaccines, and so on. It is not only disease characteristics that are affected by the influence of genes and environment but athletic performance that is affected by many of these, mixed “nature” and “nurture” factors. Of course, to be successful in sports, one must have the physical talents and capabilities – the appropriate body build, keen eyesight, and rapid hand-eye coordination to satisfy the physical demands of the sports. But it is just as important for the underlying athletic talent and physical capability to emerge in an environment that fosters participation and training – an encouraging family with sufficient resources to support purchase of equipment and the expenses of trainers and a school environment that provides athletic opportunities. It is hard to imagine becoming a successful athlete without having both the physical characteristics as well as the milieu for those traits to develop [06006].

The parts of this formula for success in sports can be, and often are, manipulated to enhance the likelihood for successful athletic performance. Only by ensuring the best possible training facilities and opportunities for athletes and providing the best medical support and care for them can we ensure that a sport maintains its important role in one’s society in fostering a healthy sense of striving for and achieving lofty goals, building a rewarding sense of physical accomplishment in the young, and providing our society with the inspiration and entertainment of sports achievement. Enlightened programs exist in many parts of the world to encourage participation in sports, provide sports facilities in schools and communities for recreational participation in sports, as well as for training selected athletes in sports academies and other sports facilities for more professional and elite levels of sports. Unfortunately, the society can manipulate other aspects of the sports environment in sly, duplicitous, surreptitious, dangerous, and venal ways, as indicated by the pervasive problem of drug abuse, doping, in sports. This is not a new phenomenon in sports, since it is recognized that athletes for centuries, as far back as ancient Greece and probably even earlier, have pursued victory by using any available means, including specialized ointments, lotions and salves, diets, and so on, openly or secretly, to achieve athletic success. In the past, many of these kinds of tools and tricks were more or less ineffective and of little use, other than for psychological support of the athlete or intimidation of competitors. However, the problems associated with doping in sports today are different from the mere psychological effects in the past because the potency of drugs in common use today give them the potential not only for having powerful and real effects on athletic performance effect but also for equally powerful but harmful physiological effects. Today’s drugs are not yesterday’s placebos. Physical traits, such as muscle size and strength, blood circulatory properties, the efficiency of our energy utilization, and others, seem to be the most common targets of genetic manipulation just as these have been vulnerable to manipulation by “traditional” drug-based doping [06006].

**Gene therapy versus gene doping**

One of the most difficult steps in gene therapy is delivering the gene into host cells. Three major techniques used for delivering genes are injecting naked DNA, viruses or modified cells 130011].

Gene doping is defined by the World Anti-Doping Agency (WADA) as “the non-therapeutic use of genes, genetic elements and/or cells that have the capacity to enhance athletic performance”. Gene therapy approaches deliver genetic material to the body's own cells which then produce the encoded protein. In this way the expressed protein can potentially be
indistinguishable from the endogenous version of the same protein encoded from chromosomal genes. This similarity is important for treatment of disease to ensure that the expressed protein is not targeted by the immune system so that long term production of the gene therapy protein is achieved. This ability to produce biologically active molecules that are potentially identical to endogenous proteins is also appealing for unscrupulous athletes as a way to deliver undetectable performance enhancing molecules [13011].

When gene therapy is used to increase the performance of a healthy person, it is considered gene doping by WADA. Gene doping presents the same advantages over regular doping as gene therapy does for regular medicine, but detection of gene doping is more difficult. Since gene doping is a powerful tool to boost performances, it may have a significant impact on the professional sports world. Gene doping has been prohibited by the International Olympic Committee (IOC) since 2003. In 2004, WADA took responsibility for publishing the Olympic doping list, and they added gene doping. The following new methods with the potential to enhance sport performance, are prohibited:

- the transfer of nucleic acids or nucleic acid sequences
- the use of normal or genetically modified cells

As for gene therapy, every known gene can be used for gene doping. Currently, only about 500 genes in the human genome are used in existing drugs, thus a significant number of the remaining genes could bring new options for doping. At least 100 genes are already linked to athletic performance and the number is increasing every year. Although not all of these genes can be considered to be potential gene doping candidates, the increasing number of genes used in medications raises expectations for the potential advantages of gene doping. The great benefits expected from gene doping make it likely that actual misuse is close at hand. As illustrated by the BALCO-affair, among other incidents, athletes are known to take more risks than average people. BALCO was an American-based company that officially advised numerous world-class athletes on nutrition, but secretly instigated cooperation between chemists, trainers and athletes to purposely evade doping controls with new and undetectable doping substances [13011].

As the London 2012 Olympics are approached, sport finds itself facing a highly specific threat to its ethos of fair play. Defined by the World Anti-Doping Agency (WADA) as "the non-therapeutic use of genes, genetic elements and/or cells that have the capacity to enhance athletic performance" gene doping presents a range of challenges to those involved in sport and exercise medicine and an urgent need for an understanding of its potential impact to healthcare. Gene doping has a molecular basis similar to gene therapy, whereby selected genes are intentionally introduced into targeted human somatic cells to facilitate the production of the "normal" physiological variant of a protein. Therapy is therefore not intended to be curative nor prevent transmission of the genetic mutation to any future children (since any modification to the human germline is universally illegal). Although several variations in the technology exist, it is essentially dependent upon a therapeutic gene coupled with a regulatory element to ensure its appropriate expression; and a vector’ (viral or synthetic) to enable its delivery to cells. Early researchers came to see two basic principles emerging that could be applied when considering the probable efficacy of gene therapy in various conditions; with the highest probable efficacy occurring if:

1. The disease process is solely due to a lack of normal protein production by its associated target cells
2. The associated target somatic cells are readily accessible

Furthermore, in order for a suitable therapeutic gene for a condition to be developed, the
genetic basis of the disorder must be well understood in order to not only ensure the desired therapeutic outcome but also that the therapy itself does not cause unforeseen harm to the recipient. However, despite early optimism, by 2011 only a handful of conditions had been successfully treated by gene therapy. Moreover, profound concerns have been voiced surrounding the safety of gene therapy following reports of severe adverse effects having occurred in trial subjects; including the development of leukaemias in a few children treated for SCID-X1 deficiency, and the unexplained death of a participant in a gene therapy trial involving rheumatoid arthritis patients. Accordingly, it is widely held that with advancing methods, the use of gene therapy will become safer, more efficient and, as such, increasingly widespread [11547]

**Epigenetics**

To achieve success in sports, many athletes consume doping substances, such as anabolic androgenic steroids and growth hormones, and ignore the negative influence of these drugs on their health. Apart from the unethical aspect of doping in sports, it is essential to consider the tremendous risk it represents to their physical condition. The abuse of pharmaceuticals which improve athletic performance may alter the expression of specific genes involved in muscle and bone metabolism by epigenetic mechanisms, such as DNA methylation and histone modifications. Moreover, excessive and relentless training to increase the muscle mass, may also have an influence on the health of the athletes. This stress releases neurotransmitters and growth factors, and may affect the expression of endogenous genes by DNA methylation, too. One paper focused on the relationship between epigenetic mechanisms and sports, highlights the potential consequences of abuse of doping drugs on gene expression, and describes methods to molecularly detect epigenetic changes of gene markers reflecting the physiological or metabolic effects of doping agents. Rapid growth and development of muscles by high physical activity is based on an integrated mechanism linking exercise with a variety of anabolic responses. The physical adaptation to environmental conditions, such as strongly developed muscles and tendons and a more resilient blood circulation, might influence epigenetic mechanisms. The so-called eustress in sports releases neurotransmitter and growth factors which may cause a change in gene expression by epigenetic mechanisms. The effects of environmental exposures can even be transmitted for several generations, suggesting transgenerational inheritance of induced epigenetic variation. The illegal intake of anabolic substances can affect the body-own gene activity in the form of an activation or repression of gene expression. The most frequently used doping agents are growth hormones and anabolic steroids that are readily detectable in urine using gas chromatography and mass spectrometry. It is estimated that every fourth sportsman takes combinations of anabolic steroids and IGF-1 (insulin-like growth factor 1). Although it is little known about the long-term effects on gene expression, these substances could influence the epigenetic pattern of the genome. Epigenetics covers three molecular mechanisms: DNA methylation, histone modifications, and remodulation of the chromatin. These processes control the transcription of genes responsible for the variability of the phenotypes. DNA methylation affects directly or indirectly the gene expression. In general, DNA methylation inhibits the access of factors to their target sequences on promoters and introns (e.g. enhances, silencer), and thus, leading to the inactivation of transcriptional expression. Moreover, the degree of chromatin condensation has a regulatory role on gene expression. In this regard, switching on and off distinct sets of genes to achieve lineage-specific activation or repression could, hence, improve the physical activity. It may be speculated, that drug-induced long-lasting changes of gene expression can increase the risk of cancer. Therefore, increasing attention should be paid to the role of DNA methylation and histone modifications in doping. To date, a main challenge of World Anti-Doping Agency
(WADA) and International Olympic Committee (IOC) is to prevent serious health risks and reduce unfair competitive advantage among athletes. Hence, specific, sensitive, and validated methods are needed or should be adapted to the sport setting, in order to document the modulated gene expression profile underlying the epigenetic influences of doping. Physical alterations caused by sports, for example, increased muscle fibres by training or drug abuse, may induce a changed epigenetic profile leading to a changed gene expression. Doping control laboratories have begun to develop methods for detection of peptide hormones using electrophoretic, chromatographic, and mass spectrometric approaches. However, sensitive and specific techniques which reveal the molecular and epigenetic outcome of doping agents are lacking. Thus, the effect of these substances on the genomic methylation pattern remains to be addressed. Therefore, efforts to translate currently available information on the molecular mechanisms of doping agents should be made [114075].

To achieve success in sports, many athletes consume doping substances, such as anabolic androgenic steroids and growth hormones, and ignore the negative influence of these drugs on their health. Apart from the unethical aspect of doping in sports, it is essential to consider the tremendous risk it represents to their physical condition. The abuse of pharmaceuticals which improve athletic performance may alter the expression of specific genes involved in muscle and bone metabolism by epigenetic mechanisms, such as DNA methylation and histone modifications. Moreover, excessive and relentless training to increase the muscle mass, may also have an influence on the health of the athletes. This stress releases neurotransmitters and growth factors, and may affect the expression of endogenous genes by DNA methylation, too. One paper focused on the relationship between epigenetic mechanisms and sports, highlights the potential consequences of abuse of doping drugs on gene expression, and describes methods to molecularly detect epigenetic changes of gene markers reflecting the physiological or metabolic effects of doping agents [11548].

**Evolving definitions**

The genotype of the athlete influences his or her performance in sport as well as the daily training. By genetic screening and by gene therapy it may be possible to assist the sportsmen in nonacceptable manner to the success. As possible health risks, immunological reactions caused by the transfer of inactivated viruses, autoimmunological reactions, negative influence on the growth of the myocardium or leukaemia are described. In the meantime, beside sports-juridical sanctions (lex sportiva), numerous countries have sanctions for doping in sport in their state norms (lex extra sportiva). The sanctions can refer to the use of prohibited substances or prohibited methods, like gene doping. One paper first describes the development of the definitions of gene doping by the World Anti-Doping Agency (WADA). Knowledge of genetic influences provides an opportunity for medical diagnostic and therapeutic attempts. Beside risks and therapeutic aspects, however, the possibilities of abuse for gene doping purposes in sports also exist. Genetic screening or gene therapy may have an advantage for athletes who use these methods. In juridical comments, it is pointed out that gene doping so far plays no role in sports, but that the legislator must consider a development in this area. Preventing abuse requires legal regulations. These regulations can include sanctions. This paper deals with the gene doping prohibition of the World Anti-Doping Agency (WADA) as confirmed and accepted by the monitoring group according to Articles 10 and 11 of the European Anti-Doping Convention by the Council of Europe, the prohibition of (gene) doping in sports of the German Medicinal Products Act (Arzneimittelgesetz) and the German Doping Agents Amounts Ordinance (Dopingmittel-Mengen-Verordnung) of the German Federal Ministry of Health. The
comprehensibility of the doping ban on the norm addressee was tested with a questionnaire. In connection with legal regulations of the German constitution, gene doping is discussed and problems which may arise by a state doping prohibition are pointed out. Criteria for including substances or methods on WADA’s prohibited list are according to Article 4.3.1 of the WADA Code 2009 that a substance or a method like gene doping fulfils any two of three criteria:

- Medical or other scientific evidence, pharmacological effect or experience that the substance or method, alone or in combination with other substances or methods, has the potential to enhance or enhances sport performance
- Medical or other scientific evidence, pharmacological effect or experience that the use of the substance or method represents an actual or potential health risk to the athlete
- WADA’s determination that the use of the substance or method violates the spirit of sport described in the introduction to the code

In addition, a substance or method also shall be included in the prohibited list according to Article 4.3.2 of the WADA Code 2009, if there are effects and/or experience of a potential to mask, for instance, prohibited substances. Since 2003, gene doping is specifically mentioned in WADA’s prohibited lists. Several changes of the gene doping definition have taken place since the first definition in 2003 [11514].

- **2003**: Gene or cell doping is defined as the non-therapeutic use of genes, genetic elements and/or cells that have the capacity to enhance athletic performance
- **2004**: Gene or cell doping is defined as the non-therapeutic use of genes, genetic elements and/or cells that have the capacity to enhance athletic performance
- **2005**: The non-therapeutic use of cells, genes, genetic elements, or of the modulation of gene expression, having the capacity to enhance athletic performance, is prohibited
- **2006**: The non-therapeutic use of cells, genes, genetic elements, or of the modulation of gene expression, having the capacity to enhance athletic performance, is prohibited
- **2007**: The non-therapeutic use of cells, genes, genetic elements, or of the modulation of gene expression, having the capacity to enhance athletic performance, is prohibited
- **2008**: The non-therapeutic use of cells, genes, genetic elements, or of the modulation of gene expression, having the capacity to enhance athletic performance, is prohibited
- **2009**: The transfer of cells or genetic elements or the use of cells, genetic elements or pharmacological agents to modulating expression of endogenous genes having the capacity to enhance athletic performance, is prohibited. Peroxisome Proliferator Activated Receptor (PPAR) agonists (e.g. GW 1516) and PPAR-AMP-activated protein kinase (AMPK) axis agonists (e.g. AICAR) are prohibited
- **2010**: The following, with the potential to enhance athletic performance, are prohibited:
  - The transfer of cells or genetic elements (e.g. DNA, RNA)
  - The use of pharmacological or biological agents that alter gene expression. Peroxisome Proliferator Activated Receptor (PPAR) agonists (e.g. GW 1516) and PPAR-AMP-activated protein kinase (AMPK) axis agonists (e.g. AICAR) are prohibited
- **2011**: The following, with the potential to enhance sport performance, are prohibited:
  - The transfer of nucleic acids or nucleic acid sequences
  - The use of normal or genetically modified cells;
The use of agents that directly or indirectly affect functions known to influence performance by altering gene expression. For example, Peroxisome Proliferator Activated Receptor (PPAR) agonists (e.g. GW 1516) and PPAR-AMP-activated protein kinase (AMPK) axis agonists (e.g. AICAR) are prohibited.

**RNA interference**

RNA interference represents a comparably new route of regulating and manipulating specific gene expression. Promising results were obtained in experimental therapies aim at the treatment of different kinds of diseases including cancer, diabetes mellitus or Duchenne muscular dystrophy. While studies on down-regulation efficiency are often performed by analyzing the regulated protein, the direct detection of small, interfering RNA molecules and antisense oligonucleotides is of great interest for the investigation of the metabolism and degradation and also for the detection of a putative misuse of these molecules in sports. Myostatin down-regulation was shown to result in increased performance and muscle growth and the regulation of several other proteins could be relevant for performance enhancement. One mini-review summarized current approaches for the mass spectrometric analysis of siRNA and antisense oligonucleotides from biological matrices and the available data on biodistribution, metabolism, and half-life of relevant substances are discussed [11549].

**Genetic enhancement**

Gene doping also could use the technique of genetic enhancement/engineering. In practice in agricultural settings, genetic enhancement places advantageous genes into organisms, not to cure disease, but to confer advantages to the organism that would improve the organism's survival or the organism's “product”: hardiness, better insect resistance, or greater yield. Genetic enhancement exploits the same techniques as gene therapy; however, it can be applied outside of medicine. Using basic principles of gene therapy (and genetic enhancement), gene doping injects genes directly into the athlete's body by one of two methods: in vitro delivery and ex vitro delivery. In gene therapy, the clinician introduces a gene that covers for a deficient gene or modulates the activity of an existent gene to correct a disease state. The goals of gene doping include the injection of novel genes or the modulation of existing genes too; however, the gene doper introduces the gene products for the enhancement of physiologic parameters expedient to the athlete's competitive tasks, rather than the treatment of a medical illness [07046].

**In vivo gene doping**

The delivery of the new gene into the athlete can be through biologic, physical, or chemical methods. Viruses can be modified to biologically insert the artificial gene into cells in a specific organ or target tissue or into cells throughout the competitor's body. Virus lines modified to transfer genes to mammalian cells include retroviruses, adenoviruses, and lentiviruses. Physical methods to deliver genes into cells use microsyringes, or gene guns. Biochemical injection vehicles use plasmids or liposomes [07046].

**Ex vivo gene doping**

The technique of exogenous gene doping involves gene transfer to cells in culture first, then implantation of the tissue into the recipient. Once implanted into the athlete's cells, the new
genes express hormones or biochemicals that again enhance tasks of the athlete in competition [07046].

**Possibilities for gene doping**

Human gene therapy involves the insertion of DNA (or RNA) into somatic cells to produce a therapeutic effect. Gene therapy was first envisaged as an approach to treating genetic disorders. In this scenario, missing or mutant genes could be replaced or repaired. Today, gene therapy has broader applications, with trials covering many clinical problems including genetic diseases, cancer, infections such as HIV, and degenerative diseases. The transfer of genetic material into cells can be undertaken in many ways, most commonly using a viral vector. For this, viruses are genetically engineered to remove infectious potential while retaining the capacity to carry a therapeutic gene(s) into selected target cells. The inserted sequences can encode a missing or mutant product as might occur in the case of cancer, or alternatively could be used to inhibit a foreign protein as would be found in HIV infection. Viral vectors have been derived from a number of different viruses. Some, such as the adenovirus, are associated with relatively mild human infections, whereas others are associated with more serious disease, for example HIV. Certain viral properties are particularly useful for gene therapy, such as the capacity to permanently integrate introduced genetic sequences into the host cell genome [06306].

Apart from viruses, there are numerous physicochemical methods for introducing DNA (or RNA) into somatic cells. The most relevant in the context of sport involves direct injection of DNA that has been formulated with a chemical carrier for more efficient uptake by cells. None of the physicochemical approaches has been successful in human trials, as the levels of gene transfer achieved are insufficient for therapeutic benefit. The results in gene therapy have generally been disappointing despite over 1000 clinical trials since 1990. Only two diseases have been successfully treated by gene therapy. Both are forms of severe combined immunodeficiency, SCID-X1 and ADA-deficiency. Unfortunately, success has come at a cost, with three of 18 infants with SCID-X1 treated developing leukaemia. This has now been shown to have been caused by insertional mutagenesis, which had previously been considered a remote theoretical risk associated with the integrating gene transfer technology used. At present, there are three limitations to gene therapy:

- gene transfer technologies are not efficient enough for most applications
- therapeutically useful integrating gene transfer technologies carry unresolved risks
- there remains an inadequate understanding of the biology of therapeutically relevant target cell populations.

Sports men and women and sporting administrators faced with the prospect of drug cheating and blood doping now need to consider gene doping. Although therapeutic benefit from gene therapy is difficult to achieve, gene doping is paradoxically more feasible because a very large output from the introduced gene may not be required, and the desired effect need only be short term. Regular injections at the time of sporting events may suffice. Gene doping is further simplified as it would not be necessary to have the transferred gene regulated so that its output corresponds to specific cellular requirements as might be the case for treating disease [06306].

Depending on the desired effect and the type of sport and athlete, gene doping might enhance performance. Athletes who compete in endurance sports, like marathons and long-distance swimming, may look to gene therapy to boost their oxygen supply or delay the
sense of fatigue. Sprinters and weight lifters, who mainly need power, may consider gene therapy to increase muscle mass or improve their injury recovery time. Boxers would appear to be most interested in improved pain tolerance from gene doping [13011].

The variables that control oxygen delivery include erythrocyte number in the blood, degree of tissue vascularization and rate of blood flow. Oxygen is delivered to the body through breathing and so improving lung function is a potential target for gene doping. Gene delivery to the lungs has primarily been developed for the treatment of cystic fibrosis, a genetic condition caused by a mutation in the gene for the protein cystic fibrosis transmembrane conductance regulator (CFTR) which regulates the movement of chloride and sodium ions across epithelial membranes, such as the alveolar epithelia located in the lungs. Gene therapy for the condition requires delivery of the correct CFTR gene to the lungs of patients for expression in the epithelium. Expressing genes that improve oxygen delivery could be achieved in lower airways if gaseous exchange could be improved. Alternatively targeting pulmonary muscle could facilitate improved lung function [13011].

**Gene-based doping for muscle function**

An obvious first approach to gene-based athletic enhancement might involve “improvement” of muscle function. Most competitive sport requires optimum muscle function – maximal and controlled force of contraction, optimal delivery of nutrients and optimized energy utilization in exercising muscle, and efficient removal of metabolic wastes. These properties of muscle and of blood circulation can be modified in many ways; certainly through the classic route of intensive training. Scientists are coming to learn a great deal about the genes that regulate muscle function in health and in disease and even the ways in which muscle function is affected by normal kinds of training. It has been proven that the injection into skeletal muscle of a muscle growth factor, insulin-like growth factor-1 (IGF-1), or of the gene that encodes this growth factor causes skeletal muscle to become hypertrophic. This causes the muscle to contract with greater force, recover from work more efficiently, and repair from injury more quickly. These studies were undertaken as part of efforts to develop therapies for muscular dystrophy and other degenerative diseases, and these have been extended to the degenerative muscle changes in normal aging and even to normal rats to determine the effects of this and other growth factors on muscle in normal, young animals. Such a gene therapy approach would be useful but certainly not ideal for correction of the muscular dystrophies since many muscles not accessible to direct injection, such as the diaphragm and heart muscle, would not be easily corrected by this approach. However, many investigators are developing methods for delivering genes and gene transfer virus vectors through the circulatory system to large tissues and even to entire limbs, and it seems that such methods would be feasible in humans in carefully monitored settings [06307].

Another approach to muscle function is through the potential manipulation of a different gene that has a powerful “braking” effect on muscle growth. All genes in the body are subject to complex regulatory processes, some of which, like IGF-1, promote cell and tissue growth, while others act as brakes to dampen the positive stimulatory factors in order to achieve a balanced state of growth – neither too much nor too little. Myostatin is one such braking gene in muscle. It acts to counter the growth-stimulatory properties of IGF-1 and similar muscle growth factors. In an effort to improve meat production, cattle breeders managed more than 100 years ago to create two breeds of cattle, the Belgian Blue and the Piedmontese, which were markedly more muscular than normal cattle. Both breeds of cattle were found to have defects in the expression of the myostatin gene, resulting in a reduced inhibition of muscle growth and thereby an increased amount of muscle growth. Mouse geneticists then engineered a breed of mice in which the myostatin gene was inactivated and the resulting
mice also had bigger, stronger muscles, similar to those of the IGF-1 “Schwarzenegger” mice of Sweeney and colleagues [06307].

**Gene-based doping for oxygen delivery to exercising tissues**

Erythropoietin is a normal hormone of humans and other mammals, and it is vital in the process of normal blood production. Erythropoietin is turned on under conditions in which an animal is exposed to lowered amount of oxygen in the environment, and it acts to increase the production of the oxygen-carrying red blood cells in the bone marrow. It is one of the world’s most important and widely prescribed therapeutic drugs, producing enormous benefits to patients in whom blood production is suppressed by diseases such as many kinds of cancer and chronic or end-stage kidney disease. It is a life-saving medicine. Unfortunately, athletes taking part in endurance sports, such as bicycling, realized that an EPO-induced rise in the level of their red blood cells also resulted in a marked improvement in their performance. The result was an uncontrolled and uninformed epidemic of EPO doping in the 1990s during which the allure of possible benefits of EPO doping was not balanced by a corresponding awareness or concern with the hazards of excessive and poorly regulated red blood cell production. In order to improve the treatment of humans with life-threatening disease, clinical scientists have been examining gene-based methods for delivering the EPO gene so that one might have a stable source of EPO production in the body without the need of repeated injections of the EPO hormone itself. In studies reported in 2003 by scientists at Stanford University in California, the normal mouse EPO gene was introduced into a virus vector in a way that allowed this gene to be expressed only in the presence of a cortisone-like steroid. In the presence of the steroid, EPO could be produced by the vector, but in the absence of the drug, no EPO could be produced. This is the sort of arrangement that one would want for genes expressed in off–on ways. The vector was used to infect human skin cells that were being grown in the laboratory, and the genetically modified cells were then transplanted back to the mice. In the absence of the inducing steroid, mice containing grafts of the genetically modified cells had perfectly normal levels of EPO and normal levels of red blood cells in their circulation. However, when the glucocorticosteroid was applied to the grafted cells in the form of a simple topical cream, the genetically modified cells responded with an increased production of EPO that was, in turn, taken up by the blood circulation to the grafts and transported to the mouse bone marrow, where production of red blood cells was turned on. The level of red blood cells rose dramatically and could be maintained in the presence of the inducing glucocorticosteroid. In its absence, the level of red blood cells eventually fell slowly back to normal as the newly produced cells went through their usual lifetime of several months [06307].

All muscle and tissue functions require carefully regulated production and utilization of metabolic energy. Muscle is a complex tissue containing a number of different kinds of muscle cells that burn energy at different rates and therefore affect muscle function and athletic performance. The slow-twitch muscle fibers are particularly fatigue-resistant, probably because of their high content of mitochondria, the power-producing elements in all cells that enable them to convert fat to energy more efficiently in contrast to the fast-twitch muscle fibers that contain fewer mitochondria and that rely completely on energy production from glucose. It was reported that mice expressing an excessive amount of a foreign gene, peroxisome proliferator-activated receptor (PPAR delta), developed an increased number of the slow-twitch fibers. The genetically altered mice showed lower levels of intramuscular triglycerides, which are associated with insulin resistance and diabetes in obese humans. Furthermore, the mice demonstrated a reduced amount of body fat and surprisingly also became more efficient energy utilizers during endurance exercising. They were known as “marathon” mice. Because the mechanism of this effect is related to the burning of calories from fat, the genetic model is becoming important for the control and treatment of obesity,
but the effects of this and probably other related genes on athletic performance are not lost on the athletic community [06307].

The variables that control oxygen delivery include erythrocyte number in the blood, degree of tissue vascularization and rate of blood flow. Oxygen is delivered to the body through breathing and so improving lung function is a potential target for gene doping. Gene delivery to the lungs has primarily been developed for the treatment of cystic fibrosis, a genetic condition caused by a mutation in the gene for the protein cystic fibrosis transmembrane conductance regulator (CFTR) which regulates the movement of chloride and sodium ions across epithelial membranes, such as the alveolar epithelia located in the lungs. Gene therapy for the condition requires delivery of the correct CFTR gene to the lungs of patients for expression in the epithelium. Expressing genes that improve oxygen delivery could be achieved in lower airways if gaseous exchange could be improved. Alternatively targeting pulmonary muscle could facilitate improved lung function [13011].

Further possibilities of detecting gene doping

It can be used methods where light emit signals in proportion to the extent to which the gene is expressed. In just several days, one can determine how the entire collection of human genes responds to exposure to any agent – a drug, an environmental toxin, etc. This is precisely the approach that is also being taken so effectively to determine the differences, for instance, between normal and cancer cells. In addition, it has become possible through an approach called “proteomic analysis” to examine simultaneously all of the thousands of proteins that are expressed by genes and that are found in blood, urine, other body fluids and in all cells. If one imagines how these and other immensely powerful new detection methods can be useful in sport doping, envision the great difficulty posed by new, “designer steroids” that are created by rogue chemists precisely to avoid detection because they are "invisible" to existing chemical tests. It is safe to presume that most or even all anabolic steroids will all use common pathways to achieve their effect, and those effects will be reflected in changes in gene expression and in the distribution of proteins (the "proteome") of affected tissues. If this approach proves effective, it would go a long way toward the goal of developing relatively noninvasive testing and monitoring procedures for drug testing and monitoring in sport doping. It is of great interest to the gene therapy community to keep track of genes that are introduced into subjects and patients, to determine how well, how long and at what levels foreign genes are expressed [06308].

Doping targets

Approximately 200 “fitness genes” are now known. Certain target genes have begun to emerge as “front runners” in the development of doping agents, particularly within fields in which a competitive advantage has been demonstrated through the use of synthetic substances and the physiological basis of this advantage is well understood [11547].

Glucose metabolism

Genetic delivery would also offer new doping approaches such as the local expression of proteins within a target organ. The liver plays an essential role in both glucose storage (glycogenesis) and in the generation of glucose (gluconeogenesis) from non-carbohydrate carbon substrates including lactate which is converted into pyruvate by lactate dehydrogenase through the Cori cycle. Through local gene delivery to the liver it may be possible to enhance either storage or liberation of glucose in the liver. There have been no
gene therapy studies that have aimed to alter glucose metabolism directly in healthy subjects but clearly treatment of diabetic patients is a potential application of gene therapy. One study in diabetic obese mice has shown that continuous expression of glucagon-like peptide (GLP)-1 from an adenoviral vector administered by intravenous injection resulted in long term remission of diabetes by improving insulin sensitivity through restoration of insulin signalling and reduction of hepatic gluconeogenesis [13011].

Red blood cell activity/delivery

Since its introduction as a therapeutic agent in the 1980s, synthetic erythropoietin (EPO) has been a drug of relevance across a number of sports, particularly those, such as cycling, in which the aerobic threshold of an athlete is known to be a major source of performance limitation. In 1997, it was reported the results of their trial in which the administration of a genetic-based EPO agent to mice and primate subjects with EPO-responsive anaemias caused a substantial increase in the subjects' haematocrit levels from 49 to 81 percent and 40 to 70 percent, respectively. Moreover, the rise was then sustained for over one year in the mice and 12 weeks in the primates; and, upon assessment of its safety profile (among the primate subjects), no adverse effects were found to have occurred in any of the monkeys to whom it was administered. One of the most significant recent advances in genetic EPO agents has been the development of “Repoxygen” by Oxford Biomedica in 2002. Comprised of a viral-vector with a human EPO gene, intra-muscular injection of the product has been trialled in the treatment of anaemia associated with renal diseases and chemotherapy. In addition, the EPO gene is under the control of a hypoxia response element and is, therefore, self-regulated, only being induced (when required) under hypoxic conditions. However, while promising results from pre-clinical trials on mice subjects were widely publicized, Oxford Biomedica ultimately chose not to develop the product any further, as it was thought that it would not be financially viable in a market where exogenous EPO agents are already widely available. Of associated interest has been the potential for the use of vascular endothelial growth factor (VEGF) and/or other angiogenic factors in order to increase tissue blood supply, thus improving tissue oxygenation and nutrition as well as the efficient removal of local waste products. It has also been raised the possibility that the use of transcription factors such as hypoxia-inducible factor 1 alpha (HIF1 alpha) may facilitate therapeutic progress. Known to activate the production of angiogenic factors and, crucially, itself be activated by muscle hypoxia and endurance training, HIF1 alpha has been shown to increase the levels of VEGF and EPO mRNA after exercise [11547].

Erythropoietin

EPO is a hormone with 165 amino acids produced mainly in the renal cortex and its production is quickly induced by hypoxia. After being released, EPO binds to the EPO receptor stimulating erythropoiesis (the production of new erythrocytes), which increases the number of haemoglobin carrying erythrocytes in the blood. Haemoglobin binds to oxygen with high affinity; although this affinity is reduced by heat or high carbon dioxide concentrations; conditions found in active muscle tissue. Thus, EPO increases the oxygen supply for muscle tissue and muscles can work longer before they build up lactic acid. The result is that maximal oxygen uptake in muscles is increased, which increases endurance. Recombinant human erythropoietin (rHuEPO) was introduced in 1988 as the protein drug epoetin-alpha in Europe (and in 1989 in the USA). It is used to treat anaemia caused by kidney disease, cancer or HIV, or for blood loss following surgery or trauma. Instead of giving a patient–donor blood to increase erythrocytes, the patient is injected with EPO to stimulate erythropoiesis. Although studies have shown the stimulation of steroidogenesis in Leydig cells by EPO leads to infertility over time, EPO has also been found to have neuroprotective properties. The first documented illicit use of rHuEPO was in the 1989 Tour de France, and
more cases have since been documented. Since EPO stimulates erythropoiesis, it increases the viscosity of the blood, thus raising the risk of microcirculation blockage, heart failure and strokes, which makes overdosing and overexpression a risk. EPO has been prohibited by the IOC since 1990 and is currently on the WADA prohibited list [13011].

Gene therapy with EPO was first tested in macaques in the late 1990s and was shown to double the number of red blood cells in 10 weeks, which increased aerobic capacity and performance. Unfortunately, EPO also made the macaques’ blood rather viscous, although the macaques did not go into cardiac arrest and survived. The macaques also had autoimmune reactions against EPO causing anaemia. In a follow-up study, regulated gene expression allowed safe production of EPO for at least 6 years. Furthermore, ex vivo gene therapy has been performed in mice causing expression of functional EPO. It has already been shown that EPO-screening of urine samples, as currently used in WADA doping controls, can identify EPO genetic therapy. Since muscle tissue produces EPO with posttranslational modifications that differ from EPO produced by the kidneys, illegal use could be detected using isoelectric focusing (a technique using differences in pH-dependent electric charges). On the basis of the promising animal studies, Repoxygen is a viral vector containing the EPO-gene and a hypoxia-response element used to treat anaemia. However, due to safety problems in in vivo testing – erythrocytosis, thrombosis and ischaemia and immune reactions – Repoxygen has not been clinically tested to date. Despite the rather problematic safety profile of Repoxygen, a 2006 incident raised fear of abuse when a German track coach was accused of supplying Repoxygen to his athletes. However, the only evidence was email correspondence with a Dutch general practitioner about the issue. Although in its infancy, given the availability of an EPO gene-therapy product, it is the most likely protein to be used for gene doping. However, the availability of a broad range of conventional EPO-products and the likelihood that the current urine EPO-detection test would identify this type of gene doping rather easily speak against the use of EPO gene doping in the sport's arena [13011].

Erythropoietin (EPO) is a glycoprotein produced predominantly by the kidney, which acts on erythroid progenitor cells in the bone marrow to regulate red blood cell production by the process of erythropoiesis which increases haemoglobin and haematocrit and therefore increases oxygen delivery. This type of systemic delivery of a protein can potentially be achieved by gene therapy. Gene delivery of EPO has been demonstrated in several experimental studies and using a variety of vectors including intramuscular injection of EPO encoding AAV in non-human primates [13011].

Because gene therapy can also be used to have local effects at the site of gene delivery it is also possible to produce proteins locally whose effects are restricted to the injection site for example the induction of the growth of new blood vessels by the process of angiogenesis. Increased blood supply will enhance oxygen delivery which could be significant in many clinical settings such as peripheral vascular disease (PVD), coronary artery disease (CAD) and wound healing. There has been progress in the development of gene therapy for the induction of angiogenesis for the treatment of CAD and PVD. Several clinical trials have examined expression of angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) from vectors including plasmid DNA and adenovirus. Whist there are reports of safety with these approaches there are no clear cut demonstrations of therapeutic effect in well controlled clinical trials [13011].

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binds to the EPO receptor stimulating erythropoiesis (the production of new erythrocytes), which increases the number of haemoglobin carrying erythrocytes in the blood. Haemoglobin binds to oxygen with high affinity; although this affinity is reduced by heat or high carbon dioxide concentrations – conditions found in active muscle tissue. Thus, EPO increases the oxygen supply for muscle tissue and muscles can work longer before they build up lactic acid. The result is that maximal oxygen uptake in muscles is increased, which increases endurance. Recombinant human erythropoietin (rHuEPO) was introduced in 1988 as the protein drug epoetin-a in Europe (and in 1989 in the USA). It is used to treat anaemia caused by kidney disease, cancer or HIV, or for blood loss following surgery or trauma. Instead of giving a patient–donor blood to increase erythrocytes, the patient is injected with EPO to stimulate erythropoiesis. Although studies have shown the stimulation of steroidogenesis in Leydig cells by EPO leads to infertility over time, EPO has also been found to have neuroprotective properties [13011].

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Since muscle tissue produces EPO with posttranslational modifications that differ from EPO produced by the kidneys, illegal use could be detected using isoelectric focusing (a technique using differences in pH-dependent electric charges) [13011].

On the basis of the promising animal studies, Biomedica, a British company in Oxford, developed Repoxygen® to be used in the treatment of cancer, diabetic neuropathy and Parkinson’s disease. Repoxygen is a viral vector containing the EPO-gene and a hypoxia response element used to treat anaemia. However, due to safety problems in in vivo testing – erythrocytosis, thrombosis and ischaemia and immune reactions – Repoxygen has not been clinically tested to date. Despite the rather problematic safety profile of Repoxygen, a 2006 incident raised fear of abuse when a German track coach was accused of supplying Repoxygen to his athletes. However, the only evidence was email correspondence with a Dutch general practitioner about the issue. In conclusion, the potential benefits and experience with EPO gene doping are quite reasonable relative to other gene-doping candidates discussed below. Although in its infancy, given the availability of an EPO gene-therapy product, it is the most likely protein to be used for gene doping. However, the availability of a broad range of conventional EPO-products and the likelihood that the current urine EPO-detection test would identify this type of gene doping rather easily speak against the use of EPO gene doping in the sport’s arena [13011].

**Skeletal muscle size, strength and endurance**

Anabolic factors known to improve muscle mass such as human growth hormone (hGHD) and insulin-like growth factor-1 (IGF-1) have already been cloned. IGF-1 is known to be important in the regulation of skeletal muscle mass through stimulating hypertrophy and enabling repair
after injury. In a study, mice that had been injected as embryos with a transgene encoding an isoform of IGF-1 demonstrated a 15 percent increase in muscle bulk even in the absence of any exercise programme. Moreover, a separate study involving the injection of an IGF-1 isoform (via an adenovirus vector) to rat subjects, has indicated that such effects may be further amplified when combined with subsequent resistance training. In the study, while all injected rats demonstrated an increase in muscle mass, the effect was considerably amplified when combined with resistance training; with a 32 percent increase in muscle mass noted among exercised rats vs. the 15 percent increase in mass in non-exercised subjects. It is also important to highlight that, in contrast to the aforementioned EPO study involving anaemic mice, subjects in both of the IGF-1 trials were essentially normal/healthy animals. As such, it seems hard to ignore the potential connotations of such trials in relation to the possible use of IGF-1 isoforms as performance-enhancing agents. Perhaps of more concern is the potential development of mechano-growth factor (MGF) gene therapy. MGF is an isoform of IGF-1 that is released locally from skeletal muscle in response to mechanical factors, such as exercise, rendering it virtually undetectable in blood or urine samples. Originally studied with regards to its possible therapeutic application in conditions relating to skeletal muscle atrophy such as Duchenne muscular dystrophy, it has been suggested that MGF may be particularly beneficial as a doping agent in light of its involvement in skeletal muscle repair following injury. It has also been demonstrated that an adenovirus-mediated shRNA expression system could be used to down regulate myostatin expression via inhibition of the enzyme “muscle atrophy F-box” (MAFbx); resulting in an increase in the muscle mass of injected mice subjects [11547].

**Insulin-like growth factor**

Although increased endurance offers major benefits for athletes like long-distance runners, EPO offers limited benefits for athletes for whom power is essential (e.g., weight lifters). For this class of athletes, IGF may be more useful since it enhances muscle growth and performance. Medical researchers currently focus on developing methods to stimulate the endogenous production of IGF to prevent muscle loss due to a range of conditions such as degenerative muscle conditions, cancer, HIV or ageing. IGF-I is a polypeptide of 7.5 kDa, structurally related to insulin and produced as a result of hypothalamus-pituitary-liver axis activation. The hypothalamus produces growth-hormone-releasing hormone (GHRH), which stimulates the pituitary to release GH thus stimulating the liver to produce IGF-I. IGF stimulates muscle repair and muscle mass hypertrophy after damage, for example, from overload or stress. Increased expression of IGF leads to increased muscle power and mass making IGF a potential target for doping. The effects of IGF-I on muscle growth have not been tested on humans, but in IGF-I-deficient patients, insulin resistance, growth disorders and cardiovascular illnesses have all been documented. Transgenic mice have been used to test the effects of IGF-I. They showed 20-50 percent larger muscle mass than regular mice and no age-induced muscle degradation. The lifespan of these mice was decreased by 50 percent, possibly due to lower levels of antioxidative molecules, or cardiac hypertrophy. Although IGF-I is on the WADA prohibited list, it is available on the internet and anecdotal evidence proves IGF-I abuse. The clear benefits of IGF – muscle growth and endurance – are desirable in many sports. The local effect of IGF allows for selective muscle growth; however, it is not expected to be one of the first targets for gene doping. The health risks of IGF gene doping, in particular, the possible clinical consequences of IGF-overexpression such as cancer and cardiac hypertrophy, are significant [13011].

The liver is also the organ that secretes most insulin-like growth factor-1 (IGF-1) which is stimulated by growth hormone. It has several effects on skeletal tissues including skeletal muscle hypertrophy and blocking muscle atrophy and it is also protective for cartilage cells. Interestingly, when the IGF-1 gene was combined with regions from the avian skeletal α-actin gene, expression of IGF-1 in a transgenic mouse resulted in muscle hypertrophy and
increase in expressed IGF only occurred within the muscle and with no increase in systemic concentrations of IGF-1. The potential for increasing muscle mass and strength with IGF-1 and the knock on influence of training has been examined. In one study IGF-1 over-expression following AAV vector delivery increased muscle size in untrained rats and a further increase in muscle mass was observed when rats were also resistance trained (ladder climbing). Furthermore when rats were detrained there was slower loss of muscle mass in rats that were treated with the IGF-1 vector [13011].

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**Growth hormone (GH)**

Instead of applying IGF-based gene doping directly, it is possible to increase the production of IGF indirectly by aiming gene doping at the endogenous production of GH, that is, significantly more accessible. GH is mainly produced by the anterior pituitary gland. The pulsatile regulation of the various GH isoforms differs in men and women and is controlled by the GHRH, which fluctuates with sleep, exercise, hypoglycaemia, age, gender, amino acid availability and low levels of IGF-I. The effects of GH are regulated by GH-binding proteins. Since GH increases muscle strength, it could be used to increase athletic performances in sports where strength is important. In endurance sports when energy is scarce, GH promotes the use of lipids as fuel to conserve protein storage. Despite a 1989 ban by the IOC on GH, there is evidence of GH being used as doping. A recent survey of 10th-grade boys in the USA showed that 5 percent had taken GH and 1.2 percent of college athletes admitted to have used GH in the last year. GH-gene therapy tests in mice, rabbits, sheep and pigs have been performed with varying results. In GH-deficient mice, a 48 percent growth in the injected quadriceps was found after 60 days. The main concerns for GH use are the lack of control in expression and disruption of functional genes. No results of gene therapy with GH in humans have been published to date. Since the results of animal studies are far from convincing and the effects of GH are less targeted than IGF and other proteins, GH is not likely to be used as a target for gene doping [13011].

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significantly more accessible. GH is mainly produced by the anterior pituitary gland. The pulsatile regulation of the various GH isoforms differs in men and women and is controlled by the GHRH, which fluctuates with sleep, exercise, hypoglycaemia, age, gender, amino acid availability and low levels of IGF-I. The effects of GH are regulated by GH-binding proteins. Since GH increases muscle strength, it could be used to increase athletic performances in sports where strength is important. In endurance sports where energy is scarce, GH promotes the use of lipids as fuel to conserve protein storage. Despite a 1989 ban by the IOC on GH, there is evidence of GH being used as doping. A recent survey of 10th-grade boys in the USA showed that 5 percent had taken GH and 1.2 percent of college athletes admitted to have used GH in the last year. GH-gene therapy tests in mice, rabbits, sheep and pigs have been performed with varying results. In GH-deficient mice, a 48 percent growth in the injected quadriceps was found after 60 days. The main concerns for GH use are the lack of control in expression and disruption of functional genes. No results of gene therapy with GH in humans have been published to date. Since the results of animal studies are far from convincing and the effects of GH are less targeted than IGF and other proteins, GH is not likely to be used as a target for gene doping [13011].

Myostatin
Myostatin is an interesting protein as it is an inhibitor of muscle growth. In a published case a boy with inactive myostatin displayed hypertrophic limb muscle development, and at 4.5 years was able to hold two 3 kg dumbbells in horizontal suspension with extended arms. Recent work in racehorses has also linked different polymorphisms of the gene to suitability to sprint or stamina races. By preventing the action of myostatin it may be possible to increase muscle growth and one way to achieve this could be with another endogenous protein called follistatin. Myostatin binds to activin type II receptors (Act RII), particularly Act RIIA, and this interaction can be inhibited by follistatin, an activin-binding protein. When follistatin was expressed in quadriceps of non-human primates following delivery with an AAV1 encoding vector an increase in muscle size of 15 percent was observed while strength, measured in twitch force was increased by 26 percent and these changes persisted for the 6 month period of the experiment [13011].

In 2004, a German boy born with muscular thighs and strong upper arms was diagnosed with a myostatin gene deficiency. As a result, the antidoping community’s attention was then directed towards the effects of myostatin blocking. In cows, a myostatin mutation leads to downregulation of myostatin, which increases muscle growth. “Double muscled cattle” or “Belgian Blue cattle” present with significantly more muscle mass than ordinary cattle. These two examples made it clear that myostatin inhibition is yet another way to increase muscle mass, but it is more specific than the use of IGF or GH. As such, myostatin inhibitors are of interest to athletes who need muscles rather than speed; however, myostatin inhibitors are on the WADA-prohibited list. Despite the risks of inhibiting myostatin, which include reduced cardiac and respiratory functioning, the inhibitors can be purchased on the internet. It is thought that myostatin is involved in sarcopenia (age-related muscle loss), although how this occurs exactly is unclear. Some tests have found increased myostatin protein and mRNA expression in aged human and rats; others find no difference. Myostatin is overexpressed in muscle atrophy when there is immobilisation, HIV infection, sepsis, burn or glucocorticoid excess, or specific skeletal muscle degeneration diseases. These findings may lead to a new treatment for muscle atrophy using gene therapy to inhibit myostatin. Myostatin is underexpressed in Duchenne and Becker muscular dystrophy, probably as an adaptive response to increased muscle growth. Myostatin overexpression can induce cachexia and increased levels are associated with obesity and diabetes. Since the actions of myostatin inhibit muscle growth, blocking myostatin is a potential doping target. Various in vivo methods of inhibiting myostatin are available, such as [13011]:
using the myostatin propeptide, which binds to myostatin to prevent it from having an effect. Although wildtype myostatin propeptide is unstable in vivo, it can be altered to extend stability.

- using neutralising antibodies. Research in mice showed less sarcopenia-related muscle loss when antibodies were injected.

- applying follistatin in animal gene therapy studies to inhibit myostatin. Follistatin is a glycoprotein that binds to myostatin preventing myostatin from binding to its receptor.

- stimulating overexpression of a gene coding for a myostatin protein without its cleavage site to inhibit the production of myostatin.

Gene therapy to inhibit myostatin is usually based on the adeno-associated virus (AAV) vector technology, since muscle cells are one of the natural hosts for AAVs. There is long experience with all above-presented forms of myostatin gene therapy (except for antibodies) in animals; no clinical tests have been performed on humans. Athletes might be tempted to use a myostatin-inhibiting form of gene doping. The effects of myostatin are significant, but the lack of experience and the poor controllability of the various methods of myostatin blocking make it hard to say whether it is already being misused [13011].

**Vascular endothelial growth factor**

Increasing blood flow through a muscle postpones fatigue. One protein regulating muscle blood flow is VEGF or VEGF-A, also known as the vascular permeability factor. Autocrine VEGF released by endothelial cells, regulates vessel homeostasis by acting as a survival factor for endothelial cells. Paracrine VEGF produced by any hypoxic cell stimulates vessel branching. New capillary branches need additional hormones to become fully grown stable vessels. When VEGF reaches high levels in blood vessels, the blood vessel responds with increased permeability and vasodilation. Since VEGF increases neovascularisation of ischaemic tissue, it might help patients with heart diseases. On the other hand, high dosages of VEGF can cause vessel leakage and abnormalities as well as tumour tissue growth. Gene therapy targeting VEGF-mediated angiogenesis has been tested in mice, rats, rabbits and dogs with generally positive results. In a 10-year follow-up study on humans, VEGF gene therapy was found to be safe; therefore, VEGF might be of interest to athletes combating exhaustion. Since VEGF increases blood perfusion in muscles, heart, liver and lungs, it is likely to increase endurance. However, the risks of VEGF use mentioned above remain unmeasured and uncontrolled. Controlling gene expression by adding a hypoxia-response element, for example, EPO, might make VEGF safer. VEGF is a likely candidate for gene doping; however, an immune response against VEGF has been detected using affinity-based biosensors and this is likely to make detection possible soon. VEGF is on the WADA prohibited list [13011].

**Fibroblast growth factor**

VEGF production is also modulated by a specific fibroblast growth factor-2 (FGF2). FGF2 works partially in a synergistic manner with VEGF, producing some of the same intracellular effects. VEGF induces FGF2, which vice versa can induce VEGF expression. Inhibition of either VEGF or FGF2 shuts down angiogenesis. FGFs have multiple functions, some of which could be used in doping. The family of FGFs includes 22 growth factors, produced by a variety of cell types. The angiogenic effects of FGFs play an important role in muscle repair following exercise through the revascularization process during muscle regeneration. The modern clinical application of the principle of angiogenesis can be divided into two main areas: antiangiogenic therapies and proangiogenic therapies. Whereas antiangiogenic therapies are being employed to fight cancer and malignancies, which require an abundance
of oxygen and nutrients to proliferate, proangiogenic therapies are being explored as options to treat cardiovascular diseases. One of the first applications of proangiogenic methods in humans was the use of FGF-1 for the treatment of coronary artery disease. Clinical research in therapeutic angiogenesis is ongoing for a variety of atherosclerotic diseases, like coronary heart disease, peripheral arterial disease or wound healing disorders. The risks of exogenous FGF include the possibility of increasing blood supply for tumours, or stimulating pathogenic heart remodeling [13011].

Adenovirus vectors and plasmids containing genes for FGF2 and FGF6 have been tested in human skeletal muscles and significantly increased muscle repair. Phase II studies showed proteinuria as an effect of abnormal capillary network formation. Most studies combine FGF gene therapy with IGF, PDGF or VEGF. The synergistic effects of FGF with those proteins have been shown, but FGF alone has not been proven to be clinically effective. Most interesting for athletes is that FGFs increase muscle regeneration and neovascularisation. A combination of FGFs is most promising, especially for athletes recovering from injury and exercise. It is most likely that the first use of FGF-based gene doping will be in combination with another protein. All FGFs and FGF-based gene doping are prohibited by WADA [13011].

Preventing pain (endorphin and enkephalin)

Conquering pain is essential to push your body to new levels of performance. The potential to reduce the perception of pain, during exertion may allow athletes to push for longer and perform better. Gene therapy for the treatment of pain is currently being examined. Research has utilized replication deficient herpes simplex virus (HSV) encoding preproenkephalin delivered into the paws of mice. The HSV is neurotropic, able to enter nerve endings at the injection site and travel retrogradely to neuronal cell bodies in the dorsal root ganglia where transgene is expressed into preproenkephalin which reduces the response to noxious stimuli. The preproenkephalin is a precursor for enkephalin production which acts on opioid receptors as a naturally occurring analgesic. A phase 1 trial, using this method, in patients with intractable pain caused by cancer has shown safety of the approach but further study is necessary to determine its effectiveness. Although gene therapy holds promise for those suffering with chronic pain, the pain experienced by athletes is often more intermittent and is confined to periods of exertion. If pain relief is to become a target for gene doping, the therapy would have to desensitize the patient to pain during these times of high intensity, not just at a constant baseline level [13011].

A completely disparate approach for improving athletic achievements is diminishing the sensation of pain. This would specifically allow combat-sport competitors to achieve higher goals. For athletes in general, numbing the sensation of extreme exhaustion is beneficial; thus, analgesics are the most frequently used therapeutic class of drugs. Most analgesics are permitted by WADA, but opiates are prohibited as they have addictive properties that can lead to abuse. Chronic pain affects a large part of the general population and gene therapy with an endorphin or enkephalin may present a promising new approach for treatment. Endorphins and enkephalins delay fatigue and increase endurance. During exercise they diminish lactic acid-related pain and pain caused by earlier injuries. Multiple gene therapy studies aimed at combating pain have been conducted, generally with positive results. Gene therapy allows for local and specific treatment of pain, with few side effects and a low risk for abuse. Since herpes simplex virus (HSV) targets neurons specifically, this is the virus generally used for gene therapy for pain. Clinical trials using endorphin and enkephalin in HSV vectors are being performed in humans, but so far are restricted to cancer-induced pain. The pain-reducing effects of both endorphin and enkephalin seem useful for athletes and early tests in humans are in progress. Given the fact that the brain is targeted, it may be
difficult to detect endorphin or enkephalin gene doping in only blood or urine. It should be clear though, even for those without a biomedical education, that experimental medicines acting only on a partially understood brain system, pose a serious risk. Given the ambiguous doping qualities of endorphins and enkephalins it is rather unlikely that they are being used today for this purpose [13011].

alpha-Actinin 3

In 2003, the association between athletic performance and alpha-actinin 3 (ACTN3) genotype (instead of ACTN2) was demonstrated. ACTN3 is mainly produced by skeletal muscle, while actin and myosin are responsible for muscle contraction. ACTN3 binds sarcomeres at the Z-lines. Although it was long thought that ACNT3 was only important for muscle structure, it is now clear that it is also important for muscle metabolism. ACTN3 deficiency does not cause muscle disease, but rather it impairs power performance by shifting the characteristics of fast-type muscles to slow-type muscles. When there is a deficiency of ACTN3, part of the action of ACTN3 is taken over by ACTN2. ACTN3 expression increases strength (although androgens have a stronger effect than ACTN3113), while ACTN2 expression increases endurance. No exogenous forms of ACTN3 or substances that influence ACTN3 transcription are known. Sixteen percent of humans worldwide have a polymorphism in both their ACTN3 genes that causes a deficiency, and in European and Asian populations this can be up to 50 percent. It has been shown that female sprinters have a higher frequency of a functioning ACTN3 gene than the average population. Since lacking the gene does not cause disease, it is not a lucrative topic of research. No trial with an ACTN3 gene therapy product has been published, although there are a few animal studies on knock-out mice. Mice missing the ACTN3 gene weigh less than wild-type mice and have smaller muscles and less strength. On the other hand, they were able to run 33 percent longer than wild-type mice and recovered faster from fatigue. If translated to the sports arena, this indicates that increasing ACTN3 copies may be used in order to dope sprinters and diminishing ACTN3 copies as a means to stimulate endurance in marathon runners. Although both the risk of abuse and the chance of being caught would be small, no gene therapy products for ACTN3 have been tested, not even in animals. This means that currently ACTN3 is an unlikely candidate for gene doping purposes [13011].

Peroxisome proliferator-activated receptor-delta

Another genetic predisposition for achievement in the elite sporting world is the gene coding for peroxisome proliferator-activated receptor-delta (PPAR-delta). PPAR-delta – also known as PPAR-beta or NR1C2 – is a protein for regulating the oxidation of fatty acids. PPAR-delta also increases mitochondrial activity and muscular glucose uptake. Overexpression of PPAR-delta decreases the accumulation of triglycerides in muscle cells and increases the oxidative capacity in muscle fibres. This results in increased endurance and an enhanced response to endurance exercise. Both endurance and power training strongly increase the production of PPAR-delta. Elite athletes have more PPARd mRNA and protein than the general population. PPAR-ddelta agonists could be used for doping purposes and products claiming to boost performance with PPAR-delta are already for sale on the internet. MBX-8025, GW742 and GW1516 (also known as GW501516) are ligands for PPAR-delta. They are being used in studies with patients who are obese or have diabetes mellitus type II or atherosclerosis. GW1516 reduced the low-density lipoprotein and triglyceride plasma concentration, and increased fatty acid oxidation. GW1516 has passed phases II and IV clinical trials for dyslipidaemia and is detectable with mass spectrometry up to 4 days after intake. Although GW1516 may be abused, the abuse might not go unpunished. Anticipating possible abuse, the WADA put PPAR-delta agonist GW1516 and PPAR-delta-AMP-activated
protein kinase on the doping list in 2009. PPAR-delta could also be targeted with gene therapy. PPAR-delta has been delivered to various cell types using an adenoviral vector; however, effectiveness differed according to cell type. Gene doping using PPAR-delta is unlikely to be used soon, since it has only been tested in cells and not in vivo [13011].

**Cytosolic phosphoenolpyruvate carboxykinase**

An even stronger effect on endurance than PPAR-delta is found with cytosolic phosphoenolpyruvate carboxykinase PEPCK-C. In one particular study, wild-type mice were exhausted after running 0.2 km and the transgenic mice overexpressing PPAR-delta after 1.5 km; but the transgenic mice overexpressing PEPCK-C ran for more than 4.9 km. PEPCK-C regulates gluconeogenesis in the liver and kidney, and glycerooneogenesis in the liver and adipose tissue. Overexpression of PEPCK-C leads to hyperglycaemia. On a normal diet, PEPCK-C overexpression induces insulin sensitivity; on a fat-rich diet it causes insulin resistance. Despite all the evidence of PEPCK-C’s importance in gluconeogenesis, (and, thus, diabetes), no gene therapy product targeting this protein has been investigated. However, silencing vectors and decreasing PEPCK-C levels did prove to be effective in diabetic animals. Its role in skeletal muscles is not clear yet, but it is hypothesised that an increase in triglycerides leads to improved athletic performance. There are two forms of PEPCK-C: one functions in the mitochondria and one in cytosol. The PEPCK-C in cytosol is the most relevant for athletes, since the effects in trials with mice demonstrated convincing benefits for endurance. No specific PEPCK-C stimulating agent is yet known. Thiazolidinediones and glucocorticosteroids stimulate PEPCK-C production, but not specifically. Glucocorticosteroids are on the WADA-prohibited list; however, to date, no gene therapy product aimed at PEPCK-C specifically is known. Since the expression of the PEPCK-C gene would be tissue-specific, detection would be nearly impossible; thus, in addition to the significant effect of PEPCK-C, despite the lack of experience, PEPCK-C is a likely doping target [13011].

**Gene doping with intracellular molecules**

Gene therapy requires genetic material to be delivered to the cell nucleus for the therapeutically encoded genes to be expressed by the transcriptional machinery of the cell. Depending on the encoded gene product it will either be secreted from the cell or remain intracellular. It is this potential to deliver a gene whose products remain inside the engineered cells that provides opportunities for gene doping that will be extremely difficult to detect and is not feasible with delivery of recombinant proteins which act outside the cell. Candidate genes that could encode intracellular proteins include transcription factors and activators, enzymes or RNA molecules [13011].

**Transcription factors**

Type 1 muscle fibres have many mitochondria and are richly supplied by blood capillaries which allow large amounts of ATP to be produced under oxidative metabolism which means that these fibres are fatigue resistant. Type II fibres have lower levels of mitochondria and use glycolytic metabolism to produce ATP and are therefore more susceptible to fatigue. The fast manner in which the ATP is produced in type II fibres results in quick, more powerful contractions which are more important in sports such as sprinting. It is well known that endurance exercise induces phenotypic changes in skeletal muscles that lead to enhanced exercise capacity and improved metabolic homeostasis. More recently peroxisome proliferator-activated receptors (PPARs), which are nuclear receptor proteins that function as transcription factors, have been shown to have a role in regulating the fibre type within a muscle. Generation of transgenic mice in which an activated form of peroxisome proliferator-activated receptor delta (PPAR-delta) was expressed in skeletal muscle, induced an
increased number of type I muscle fibres. It was noted that these mice had an increased numbers of type I fibres compared with controls, both in normal type I rich muscle, such as soleus, and mixed type II muscle, such as gastrocnemius. These mice could also run further and for longer compared with controls, suggesting that the type I fibres generated from PPAR-delta gene expression produced a beneficial effect on the mice by increasing physical performance. Similarly, peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1delta) is a transcriptional co-activator which, when over expressed in muscle, promotes a fibre switch towards oxidative type I slow fibres. Recently is has been shown that this effect is mediated via the transcription factor hypoxia-inducible factor (HIF) 2alpha. The lack of oxygen in muscle during vigorous exercise causing hypoxia is also known to induce HIF-1alpha, a transcription factor that induces expression of target genes including the angiogenic factor VEGF and EPO. These HIF transcription factors are degraded by prolyl hydroxylase enzymes under normoxic conditions but these enzymes are inactive in hypoxia permitting HIF activity. It is however possible to engineer a stable version of HIF-1alpha by point mutation of the proline residues that are subject to prolyl hydroxylation. In the mutated form HIF-1alpha is constitutively active and can be delivered to muscle genetically to enhance vascularization and potentially promote erythropoiesis. In theory, synthetic transcription factors could also be generated that have similar effects using zinc finger domains targeted to the VEGF locus which when expressed in vivo in mice induced angiogenesis [13011].

**Enzymes**

An example of an intracellular enzyme that could be used in gene doping to reduce pain is GAD which controls production of the inhibitory neurotransmitter GABA from glutamate, which has an analgesic effect. Expression of GAD from an HSV vector into the dorsal root ganglia of rats with T13 hemisections showed a reduction in pain perception below the level of the lesion [13011].

**Small inhibitory RNA (siRNA)**

Endogenous gene expression can be regulated at the level of micro RNA (miRNA) molecules which target messenger RNA (mRNA) molecules. Synthetic siRNA molecules utilize the endogenous machinery of the cell to inhibit protein translation in a similar manner to miRNA. In addition siRNA molecules can also be produced long-term inside cells as short hairpin RNA molecules that are processed inside the cell into siRNA. Both these types of RNA molecule could be utilized in gene doping strategies where inhibition of a protein's synthesis could enhance performance. An obvious target is myostatin and studies using either siRNA or shRNA targeting myostatin mRNA have demonstrated an increase in muscle size and mass [13011].

**Options for gene doping**

Along with enormous research efforts regarding gene-based therapies, the options to misuse the acquired knowledge for illicit performance enhancement increased at an almost equal level. Despite the fact that most of the therapeutic strategies employing, for example, RNA interference- or in vivo gene transfer-based approaches are still undergoing clinical trials, the potential for misuse has long been identified and various countermeasures were initiated. Particularly the detection of transgenic DNA, characterized by intronless DNA sequences from conventional blood samples have been studied using mouse or macaque models. Proof-of-concept was obtained by sampling blood of mice that underwent intramuscular adeno-associated virus (AAV)-mediated gene transfer with human VEGF-A in which the transgene was detected for 28 days in all tested animals (n=6) and up to 56 days in four out
of six rodents. Focusing on the EPO transgene, a similar real-time PCR-based methodology was presented which in contrast to the described approach above aims primarily for the analysis of white blood cells of non-human primates. Following the injection of the recombinant AAV (rAAV) vector expressing cynomolgus macaque EPO leucocytes were collected and tested for collateral transfection. Viral vector backbone as well as transgene DNA was identified for up to several months (varying with animal and serotype of the rAAV). In addition, it was shown that serum and urine represent viable matrices offering detection windows between few days and weeks. In continuation of earlier approaches suggesting the use of affinity-based biosensors targeting specific non-human plasmid regions (e.g. the enhanced green fluorescent protein (EGFP) reporter gene and the Cytomegalovirus (CMV) promoter sequence) as indicators for transgenesis events, the use of surface plasmon resonance imaging (SPRi) was described. Under the definition of gene doping of WADA's Prohibited List of 2011, the use of substances influencing the gene expression are banned (paragraph M3.3). Among these the peroxisome proliferator-activated receptor (PPAR)delta agonists such as GW1516 as well as the adenosine monophosphate (AMP)-activated protein kinase (AMPK) axis agonists (e.g. AICAR) are listed. Clinical trials of these experimental drugs are not completed, however, Internet-based suppliers offer these substances as “research chemicals” which were acquired and analyzed to ascertain authenticity, purity, quantity, and formulation using high resolution/high accuracy mass spectrometry. In both cases of GW1516 and AICAR, the ordered compounds were delivered under the disguise label of “amino acids” as an orange suspension (GW1516) and a colourless powder (AICAR). The quantity/concentration of the substances was much lower than indicated but both drug candidates were identified with correct structure and composition, thus outlining the ease of availability of these banned drug candidates to cheating athletes [12016].

**Gene doping targets**

Depending on the desired effect and the type of sport and athlete, gene doping might enhance performance. Athletes who compete in endurance sports, like marathons and long-distance swimming, may look to gene therapy to boost their oxygen supply or delay the sense of fatigue. Sprinters and weight lifters, who mainly need power, may consider gene therapy to increase muscle mass or improve their injury recovery time. Boxers would appear to be most interested in improved pain tolerance from gene doping [13011].

One of the most difficult steps in gene therapy is delivering the gene into host cells. Three major techniques used for delivering genes are injecting naked DNA, viruses or modified cells. Initially, a desired gene can be produced in bacterial plasmids and then purified. Next, the gene can be directly injected into the target tissue. Unfortunately, direct injection of DNA is not very effective due to limited uptake and nuclear translocation (although electroporation of the target tissue increases the uptake) However, it is safer than using viral vectors, since there are generally fewer immune responses, and considerably cheaper than other gene-transfer options. Genetic material can be delivered to a target tissue by using a viral vector. Viruses have evolved to efficiently transfecct cells with their genetic information and multiply, which makes them ideal for use in gene therapy. To prevent the virus from replicating, the viruses are tamed (all DNA or RNA coding for proteins allowing the virus to multiply and escape infected cells are removed) and therapeutic genes are inserted. Because of this inhibited replication, the viruses are less immunogenic. The modified virus may be injected intravascularly or directly into a target tissue, or inhaled. Injection into the target tissue limits gene expression to the injection site, whereas intravascular injection usually results in systemic expression. Inhalation is used if the lungs are the target tissue. The viral vector contains a promoter that allows the inserted gene to be transcribed and translated, thus
yielding the desired protein. For ex vivo gene therapy, stem cells are removed as in the case of patients with severe combined immune deficiency (SCID) and a therapeutic gene is introduced in vitro. The genetically modified stem cells are then injected back into the patient’s bone marrow. This can be done using plain DNA (with or without liposomes), a viral vector with electroporation or with a gene gun. The technique allows for limited screening and sorting of the cells before reinjection, which increases efficacy and safety. Disadvantages are low efficiency and increased cost. Depending on the target tissue, the gene and the desired duration of transgene expression, multiple vectors can be used [13011].

Of the three gene therapy delivery methods described, in vivo viral gene transfer is the most successful method for now. In general, the benefits are efficacy and low cost; downsides are immune responses and poor controllability of integration and expression. Ex vivo viral gene transfer can be used to insert a gene to produce a desired protein, or increase or inhibit the transcription of an already present gene by influencing promoters. On the other hand, gene expression can also be prevented using antisense RNA sequences. RNA sequences bind to the original gene, prevent translation and cause destruction by the RNase H or the siRNA pathway. Even splicing patterns can be altered by blocking splicing recognition sequences, thus allowing for the inclusion or exclusion of specific exons [13011].

**Direct injection of DNA into the target tissue**
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**Introducing genetic material using a virus**
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**Ex vivo gene therapy**
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poor controllability of integration and expression. Ex vivo viral gene transfer can be used to insert a gene to produce a desired protein, or increase or inhibit the transcription of an already present gene by influencing promoters. On the other hand, gene expression can also be prevented using antisense RNA sequences. RNA sequences bind to the original gene, prevent translation and cause destruction by the RNAse H or the siRNA pathway. Even splicing patterns can be altered by blocking splicing recognition sequences, thus allowing for the inclusion or exclusion of specific exons [13011].

**Candidate genes for athletic gene doping**

Any physiologic process involved in producing a motor action or assisting in the implementation of a motor movement could be a candidate for gene doping. The physiologic processes of pulmonary respiration, cardiovascular circulation, oxygen delivery, striated muscle growth/efficiency/repair, and even neuromuscular coordination could be altered to give an athlete an edge over his or her competition. Although more esoteric, neurophysiologic processes, such as mental alertness, motivation, and central nervous system recovery, also might be amenable to gene doping, the list of physiologic processes related to athletic competition is long and likely only limited by current understanding of exercise physiology and exercise psychology. Even now, in the exploratory phase of gene therapy/gene doping, there are obvious candidates for the aspiring gene cheat. These primary gene candidates exist as targets of biomedical researchers looking for legitimate disease treatments [07046].

**Hematopoietic/vascular systems**

The classic example of a genetic alteration to enhance athletic performance occurred naturally in 1964. A Finnish skier, Eero Mantyranta, dominated Olympic Nordic skiing. Studies later demonstrated that Mantyranta benefited from a natural mutation in the EPO gene that produced a greater number of RBCs, with a concomitant increased oxygen-carrying capacity. This skier possessed what every blood doper in history tried to achieve – a physiologic advantage in delivering more oxygen to various tissues, including muscles. rEPO (epoetin and darbopoetin) is a much-abused injected recombinant protein that increases RBC production, leading to increased oxygen-carrying capacity and oxygen delivery to tissues. The abuse of rEPO is epidemic in cycling, leading to frequent controversy, tedious forensic investigations, and serious side effects, including death. Using gene doping, an additional EPO gene could be delivered to the athlete by way of a viral vector. Once in the athlete, the gene would express much more EPO than normally produced, even with training. The desired result would be an increase in the oxygen-carrying capacity of the blood, thus bestowing a clear competitive advantage in endurance sports. Laboratory experiments have been successful in injecting the gene into monkeys and mice. Although the procedures successfully raised hematocrit, severe side effects, including a paradoxical anemia, resulted [07046].

**Hypoxia inducible factors**

The hypoxia inducible factor (HIF) family of proteins modulates the activity of genes in low-oxygen environments. Various HIFs increase production of RBC, as well as increase cellular energy use. Enhancement of these proteins clearly would benefit the aerobic athlete [07046].

**Vascular endothelial growth factor**
Clinical studies are currently ongoing for vascular endothelial growth factor (VEGF), a gene product that encourages development of new blood vessels. This genetic manipulation would be advantageous to patients suffering from coronary artery disease. The gene-doping athlete would benefit from a putative increase in vasculature and more delivery of oxygen and nutrients to the peripheral tissues [07046].

**Actin-binding peptides**

The proteins actin and myosin form the machinery of muscle contraction. The family of actin-binding proteins in humans includes alpha-actinin alleles ACTN2 and ACTN3. Alpha-actinins maintain the structure of the myofibrillar array and regulate myofiber contraction. A defect of ACTN3 regulation occurs when the ACTN3 gene codes for a premature stop codon; ACTN2 seems to compensate in these persons. Correlation studies found evidence that the ACTN2 endows muscular endurance traits upon athletes. Conversely, elite sprinters benefit from more copies of ACTN3. Depending on the event of the competitor, gene doping with the appropriate ACTN allele could enhance endurance capacity or sprint effectiveness [07046].

**Angiotensin-converting enzyme**

Physicians are well aware of angiotensin-converting enzyme (ACE) inhibitors used for the treatment of hypertension; however, the ACE gene, like the ACTN gene, codes for proteins that seem to endow different exercise capacities. Research suggests that the ACE-I allele endows advantages in endurance, which would be useful for distance runners. The ACE-D allele seems to be associated with elite sprinting performance. Again, a gene-doping athlete could inject the appropriate gene to influence better performance in his or her event, be it a sport featuring short bursts of speed and power or a sport in which endurance is the key to success [07046].

**Insulin-like growth factor**

Several sophisticated studies, most notably at the University of Pennsylvania, targeted IGF-1, a peptide, in conjunction with HGH, intimately involved with muscle growth, repair, and power. These studies demonstrated eloquent ways in which IGF-1 controlled mammalian muscle development and demonstrated that targeted gene therapy could successfully produce hypertrophied and powerful muscles in laboratory mice. The primary investigator, Lee Sweeney, designed the procedure carefully so that the virus and the gene were not expressed systemically; by avoiding systemic distribution of the peptide, the researchers hoped to avoid the serious side effects of IGF-1, including myocardial hypertrophy and carcinogenesis. Thus, for therapeutic reasons, the researchers designed the gene therapy to be effective locally, in injected muscles. That particular feature also would benefit a doping athlete, because the transgene IGF-1 would not enter the systemic circulation where it might be detected by laboratory testing. In this University of Pennsylvania protocol, “fingerprints” of the gene therapy did occur; however, the theoretic aim of the study itself would present problems for antidoping agencies [07046].

**Myostatin**

Myostatin, known to be a negative regulator of muscle development, presents another candidate gene. This regulator protein seems to turn-off muscle growth. Substances that block myostatin or genes that produce ineffective myostatin proteins would allow superphysiologic muscle growth in terms of number and thickness of cells (as seen in certain breeds of cattle through a natural mutation). Not only do striated muscles hypertrophy
without myostatin regulation, but less fat is gained on the body of the animal. The manipulation of this regulatory protein has obvious advantages for the athlete [07046].

*Peroxisome proliferator-activated receptor delta*

The peroxisome proliferator-activated receptor delta (PPAR-delta) gene seems to be a prime candidate for gene doping. This gene codes for an increase in mitochondrial biogenesis and promotes an adapted muscle fiber transformation. The gene promotes an increase in type 1 muscle fibers (slow twitch). Elite athletes show an increase in PPAR-delta gene levels. A PPAR-delta gene was inserted into mice; it dramatically improved the animal's endurance capacity. It was concluded that “…these genetically generated fibers confer resistance to obesity with improved metabolic profiles, even in the absence of exercise. These results demonstrate that complex physiologic properties such as fatigue, endurance, and running capacity can be molecularly analyzed and manipulated” [07046].

*Endorphins*

In injuries, as well as in competition, pain limits athletic achievement. Athletes sustain countless painful injuries for which they consume an abundance of anti-inflammatory drugs and pain-relieving medicines. Likewise, the buildup of lactic acid during competition induces pain. Clearly, increasing the pain threshold – and alleviating the discomfort of nagging injuries – would improve performance. The introduction of genes producing analgesic endorphins and enkephalins would increase the pain threshold in an athlete, for pain experienced in competition as a result of lactic acid buildup and pain due to acute and chronic injury. Clinical trials are testing the efficacy of genes encoding these natural narcotic peptides for pain relief in humans [07046].

*Erythropoietin gene transfer*

Aviral gene delivery vector carrying the human Epo gene under the control of an O2-dependent hypoxia response element (Repoxygen; Oxford BioMedica) was earlier developed that might have been misused for Epo gene doping. Reportedly, however, the technique never proceeded beyond animal experiments. In addition, in vivo Epo gene transfer could probably be detected if applied by athletes, as an IEF study revealed unusual Epo glycosylation forms on allogeneic Epo transfer into skeletal muscle of cynomolgus macaques via adeno-associated virus. In the initial studies of adeno-associated virus-mediated allogeneic Epo cDNA transfer to macaques, severe anemia developed in many animals after a few months, which was probably the result of an immune reaction. Regarding the possibility of Epo gene doping in humans, strategies are under development to specifically amplify intron-less DNA sequences and PCR protocols allowing the detection of small amounts of transgenic DNA in blood. The tests take into consideration that transgenes are usually derived from the cDNA for the gene to be transferred and cDNA does not contain introns. In conclusion, Epo gene transfer is possible but medically little explored with respect to efficacy, safety, and immunogenicity. It seems less likely that any of the techniques has entered the sports scene [11116].

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macaques via adeno-associated virus. In the initial studies of adeno-associated virus-mediated allogeneic Epo cDNA transfer to macaques, severe anemia developed in many animals after a few months, which was probably the result of an immune reaction. However, in using a rapamycin dimerizer-regulated gene expression system, it was achieved controlled, long-term production (up to 6 years) of Epo in rhesus monkeys, with no apparent immune response. Regarding the possibility of Epo gene doping in humans, strategies are under development to specifically amplify intron-less DNA sequences and PCR protocols allowing the detection of small amounts of transgenic DNA in blood. The tests take into consideration that transgenes are usually derived from the cDNA for the gene to be transferred and cDNA does not contain introns. In conclusion, Epo gene transfer is possible but medically little explored with respect to efficacy, safety, and immunogenicity. It seems less likely that any of the techniques has entered the sports scene [11428].

Polymorphism

Performance enhancing polymorphisms (PEPs) are examples of natural genetic variation that affect the outcome of athletic challenges. Elite athletes, and what separates them from the average competitor, have been the subjects of discussion and debate for decades. While training, diet, and mental fitness are all clearly important contributors to achieving athletic success, the fact that individuals reaching the pinnacle of their chosen sports often share both physical and physiological attributes suggests a role for genetics. That multiple members of a family often participate in highly competitive events, such as the Olympics, further supports this argument. In a review, it was discussed what is known regarding the genes and gene families, including the mitochondrial genome, that are believed to play a role in human athletic performance [10265].

Sport as case study

One of the more volatile debates that surrounds enhancement in sport has been the application of gene transfer and genetic technology more generally. In 2003, the World Anti-Doping Agency instituted a policy prohibiting the use of “gene doping” and yet there is still considerable lack of clarity over whether it will ever be possible to detect all kinds of genetic enhancement. These debates have engaged the mainstream of bioethicists where controversies relating to human enhancement abound. Sport, it would seem, has become an exemplar case study for investigations into the ends of technology in society. One of the pivotal questions surrounding sports is whether the approach to doping needs radical transformation, as the age of enlightenment gives way to an age of enhancement [06168].

Risks and complications of gene doping

Genes of relevance to doping such as growth hormone, insulin-like growth factor I, and erythropoietin have been cloned, and so are readily available. They could be used as an alternative way to produce a range of performance enhancing agents. The risks of taking these substances in the form of traditional chemicals are known, and so decisions about risk versus benefit are straightforward. The same cannot be said for gene doping, as there continue to be many unknowns in this form of cellular intervention. Effects cannot be predicted, and so the sportsperson taking this route for cheating does not have control of the product. Random integration of vector sequences, for example, could produce complications such as acute leukaemia or other forms of cancer. Finally, unlike taking a drug, gene transfer
is not easy to reverse, and so any untoward effects may be long term. There is also a small risk of inadvertent gene transfer to germ cells with the potential for harm to be passed on to an athlete's children [06306].

Groups studying EPO gene transfer have demonstrated that a high percentage of monkeys, who were genetically modified to produce a foreign EPO gene from a virus vector, has developed lifethreatening anemias, probably as a result of an immune response to vector itself. Therefore, no matter how impressive the results may be in animal testing, the gene transfer technology is not yet characterized well enough to apply in human settings in which the goal is the enhancement of normal human traits rather than the correction of a human disease [06307].

Today, the risks for gene doping are much greater than the taking of traditional chemical products. Those involved in sport should be sufficiently informed of the risks, as well as likely future benefits of gene therapy. As the technology improves, many of the complications may be avoided, and so ongoing assessment of the potential for gene doping will be necessary. Detecting gene doping cheats will be possible using the standard assays as well as through the identification of gene vectors or their products. The bypassing of various metabolic pathways through the insertion of genes may lead to changes in gene expression profiles, and this may open up another approach to detecting gene doping [06306].

Examining the history of clinical pharmacology reveals that side effects of novel treatments can be unexpected and occasionally fatal. From thalidomide to valdecoxib, newly introduced medications, even with exhaustive preclinical trials, produce unanticipated untoward side effects. Consider the potential side effects of introducing a foreign gene, by way of a viral vector, into an organism's chromosomes. Gene therapy trials made headlines several times with unexpected and fatal side effects. Then 18, Jesse Gelsinger died in 1999 as the victim of an immune response to the virus used in a well-publicized gene therapy trial. That death shocked the biomedical world and resulted in regulation as well as multiple legal actions. Several patients who were treated for an X-linked hemophilia with a gene therapy protocol developed leukemia, an obviously unexpected side effect. A gene therapy trial of EPO in macaque monkeys produced such stimulation of RBC production that the monkeys' blood thrombosed. Moreover, many of the monkeys suffered anemia, the result of an immune response to the gene therapy. The overactive immune response attacked endogenous EPO as well as gene-stimulated EPO. A similar experiment using gene-therapy EPO revealed that the gene-induced EPO was slightly different from natural monkey EPO. Likewise, the use of transgene EPO in human gene doping might induce erythrocytosis to a dangerous level, with potentially lethal consequences. Although not generally considered a risk from gene therapy, the virus vector could infect other humans. Clinical trials monitor subjects for viral shedding. It would be unlikely that gene cheats would monitor their secretions for viral contamination. Furthermore, rogue gene-doping laboratories (like their steroid-synthesizing counterparts) would not implement proper preclinical trials. Thus, there may be a possibility of a modified infectious virus passing from a gene cheat to other persons. If the experience with anabolic steroids is any indicator, athletes generally ignore common dosing recommendations [07046].

**Experimental**

One paper presents studies that generated mouse models with outstanding physical performance, by manipulating genes such as insulin-like growth factor 1 (IGF-1) or phosphoenolpyruvate carboxykinase (PEPCK), which are likely to be targeted for gene
doping. The potential transition from super mice to super athletes was also discussed, in addition to possible strategies for detection of gene doping [10266].

**Risks and complications of gene doping**

Although there is as yet no definitive evidence of genetic manipulation in athletes, gene doping is a serious health concern as gene therapy is not safe from side effects, most of which are not predictable and are potentially deleterious. The onset of severe complications of gene transfection, such as insertional oncogenesis following inactivation of tumor suppressor genes or activation of proto-oncogenes, propagation and recombination of retrovirus or adenovirus vectors, and potential humoral and cellular immune responses against transgenic proteins, could be ethically justified for the treatment of patients with serious, life-threatening disorders, but it is an unacceptable risk for healthy individuals who seek to enhance athletic performance. Such treatment is virtually impossible to reverse; early benefits in terms of improved athletic performance could later turn into serious complications, from thrombosis to cancer. In the specific case of Epo gene therapy, the administration of recombinant adeno-associated virus serotype 2 vector containing feline Epo cDNA resulted in pure RBC aplasia or pathologic erythrocytosis, which could not be abolished by injection site removal. Finally, besides health concerns, “gene cheating,” as does doping in general, contravenes the ethics of sports and influences outcomes of competitions that frequently result in considerable economical benefit [10353].

Like all new medicines, gene therapy presents unsolved problems. Until they are solved, large-scale use of gene therapy in the clinic is ruled out. Contrary to gene therapy and by implication of its illegal character, gene doping is not bound to safety regulations [0011].

The risks associated with gene doping are substantial. Gene doping is unlikely to be performed under close medical supervision/monitoring and short term gain may well be achieved at the expense of longer term health problems for athletes. It is of paramount importance that athletes and coaches are educated about the potential risks of gene doping before we read tragic headlines involving sporting heroes [13011].

**Gene silencing**

One limit to the effectiveness of gene therapy is gene silencing; thus, even when the target tissue is infected, it might not express the inserted gene [13011].

**Immune reaction**

Both the virus used and the protein itself can cause an immune reaction. How to handle this reaction appropriately is not completely clear. The immune reaction against the protein can also induce a response against the endogenous protein, as happened with EPO in macaques resulting in anaemia [13011].

**Integration**

Though not all viruses integrate, those that do can present problems. Splitting up a tumour suppressor gene, or worse, increasing production of a proto-oncogene, can lead to cancer. It is estimated that about one in every 10,000 retroviral insertions might be dangerous, and one in every 10^9 could induce cancer [13011].
**Infection of germ cells**

The danger of the infection of germ cells with gene therapy also exists. This would transfer exogenous genes to future generations. Though it is explicitly prohibited to target cells that reproduce, and it is not likely gene therapy (which is not aimed at germ cells) would cause an infection of germ cells, this risk should be strictly monitored [13011].

**Expression**

Expression of gene therapy is hard to control, and overexpression could be dangerous. In addition to the effects of the protein itself, toxicity by accumulation is also dangerous. Also, if a cell producing the desired product is infected by another virus, this could lead to overexpression of the protein. Expression is controllable by an inducer drug (e.g. doxycycline, which is approved for human use), but since this is detectable, it is less likely to be used for gene doping [13011].

**Storage and usage**

Gene therapy would require good storage and it is questionable whether those wishing to abuse it are knowledgeable about proper handling procedures. Since professionals face difficulties with the non-linear dosage-expression relationship, it is likely those who have not been thoroughly educated could do worse. Even more dangerous would be an attempt to produce gene doping in rogue laboratories, leading to unsafe products [13011].

**Long term**

Gene therapy is a new technology. Only short-term studies have been conducted, which means that the long-term effects are not yet clear. There may still be problems with gene therapy products that simply have not been identified yet. It should be emphasised that the above-listed risks are only the foreseeable risks, as all the information was obtained in regulated and controlled settings. The unknown risks present a much larger problem, because they are far more difficult to anticipate [13011].

**Uses of gene therapy**

Gene therapy can be used to treat a variety of illnesses. It may be applied to weaken or kill cancer cells by triggering apoptosis, to enable target cells to produce a protein that otherwise has to be administered or to upregulate the production of a specific protein. Though a couple of gene therapy products have been marketed outside China to date, at least 1843 gene therapy trials have been conducted worldwide with thousands of patients suffering from cancer diseases, cardiovascular and neurological diseases and a range of other diseases. Early clinical trials in Europe and the USA had limited results and even fatalities were reported from gene therapy; however, examples of successful gene therapy include treatment of SCID-X1 and Leber's congenital amaurosis. The proof of concept of various transfer strategies in gene therapy shows that we are at least at the beginning of a gene therapy revolution for patients with monogenic diseases [13011].

**Potential strategies for detection of gene doping**

*Detection of erythropoietin gene doping*
Gene doping – or the abuse of gene therapy – will continue to threaten the sports world. History has shown that progress in medical research is likely to be abused in order to enhance human performance. In one review, it was critically discussed the progress and the risks associated with the field of erythropoietin (EPO) gene therapy and its applicability to EPO gene doping. It was presented typical vector systems that are employed in ex vivo and in vivo gene therapy trials. Due to associated risks, gene doping is not a feasible alternative to conventional EPO or blood doping at this time. Nevertheless, it is well described that about half of the elite athlete population is in principle willing to risk its health to gain a competitive advantage. This includes the use of technologies that lack safety approval. Sophisticated detection approaches are a prerequisite for prevention of unapproved and uncontrolled use of gene therapy technology. In this review, we present current detection approaches for EPO gene doping, with a focus on blood-based direct and indirect approaches. Gene doping is detectable in principle, and recent DNA-based detection strategies enable long-term detection of transgenic DNA (tDNA) following in vivo gene transfer [12478].

The practice of doping threatens fair competition in sports. With the very recent reports on successful gene therapies for several diseases, the likelihood for abuse of gene transfer techniques in elite sports is rapidly increasing. It is therefore very important to develop valid detection techniques for transgenic DNA (tDNA) with ultimate sensitivity and specificity. To date, three slightly different procedures have been reported to reliably detect tDNA with sufficiently high sensitivity. Two utilize a real-time PCR-based approach and one uses a primer-internal, intron-spanning PCR approach (spiPCR). The specificity and sensitivity of these techniques, however, is still a matter of debate. Based on spiPCR, here we present a novel one-tube nested PCR approach that minimizes the chances for cross-contamination and shows increased sensitivity compared to non-nested PCR techniques. To further reduce the occurrence of false-positives based on cross-contamination, a multi-functional 19bp extended erythropoietin standard (EPO) was cloned which can be easily differentiated from transgenic EPO DNA (tEPO) and can be used as an internal or external positive control in PCR-based applications. We found that one-tube nested PCR is superior in terms of sensitivity and specificity compared to conventional PCR, and shows similar sensitivity compared to real-time based PCR assays. Although it did not reach sensitivity of spiPCR, the one-tube nested PCR technique described here is less laborious, less expensive and much faster than spiPCR. This technique might therefore be useful as a pre-screening tool for gene doping in the future [12479].

Gene doping – or the abuse of gene therapy – will continue to threaten the sports world. History has shown that progress in medical research is likely to be abused in order to enhance human performance. In one review, it was critically discussed the progress and the risks associated with the field of erythropoietin (EPO) gene therapy and its applicability to EPO gene doping. It was presented typical vector systems that are employed in ex vivo and in vivo gene therapy trials. Due to associated risks, gene doping is not a feasible alternative to conventional EPO or blood doping at this time. Nevertheless, it is well described that about half of the elite athlete population is in principle willing to risk its health to gain a competitive advantage. This includes the use of technologies that lack safety approval. Sophisticated detection approaches are a prerequisite for prevention of unapproved and uncontrolled use of gene therapy technology. In the review, it is presented current detection approaches for EPO gene doping, with a focus on blood-based direct and indirect approaches. Gene doping is detectable in principle, and recent DNA-based detection strategies enable long-term detection of transgenic DNA (tDNA) following in vivo gene transfer [12480].

There are several theoretical ways of detect gene doping. At present, human applications with gene doping have not yet materialized. However, there is a potential of misuse of this technology in the future [12242].

2062
Direct methods
- since glycosylation of EPO differs in the skeletal muscle and endogenous EPO, Lasne’s method would be able to detect the same
detection of the gene delivery system in the body in case of gene doping: plasmids, viral vectors, liposomes, and protein-DNA conjugates
- labeling all EPO gene transfer products with genetic “barcodes”

Indirect methods
- “Hematological passport”: in this concept, hematological parameters are monitored sequentially in all the athletes and subject-specific references are generated. This would be useful in finding the genetic polymorphisms/mutations leading to increased endurance in a particular person (e.g. Finnish cross-country skier Eero Mantyranta who won two gold medals in the 1964 Winter Olympics was later identified to have a mutation in the EPO-R gene that caused sustained activation of EPO signaling. Mantyranta’s oxygen carrying capacity was increased by 25-50 percent)
- “Molecular passport”: Sequential determinations of the expression levels of certain EPO target genes by DNA microarray analysis could define athlete-specific reference ranges for the level of expression of these genes. Athletes with gene expression levels above or below their personal range would be considered suspicious for doping

With novel EPO molecules around the block, misuse of them in sports would be increasing and the challenge would be to provide easy and reliable detection strategies which can be used for mass screening [12242].

Some have suggested that adaptation of the aforementioned detection methods will also be suitable for the detection of genetic doping, for example, via the detection of changes in gene expression profiles that may occur secondary to the bypassing of metabolic pathways. However, others have highlighted several difficulties when attempting to apply pre-existing detection systems to genetic doping. First, for a transgenic protein (a protein produced from a transferred gene) to be detected, it must be distinguishable from its physiological counterpart. As we have already seen, one of the fundamental aims of gene therapy is to enhance 'normal' physiological production of various proteins, thus meaning that any detection on this basis would be very challenging. Substances such as erythropoietin are known to have very short half-lives, therefore this would thus further reduce the probability of their detection via a random blood or urine sample. The only present method that may enable site specific detection would be that of a muscle biopsy to test for injected viral particles. However, while deemed safe, any biopsy method is likely to be highly unpopular with professional athletes due to its invasive nature. As has been highlighted, even if such sampling were to be enforced, engineered viral products may be indistinguishable from their endogenous counterparts and it is unclear how long such particles would persist following their injection. Recent advances have suggested that a fine needle aspiration biopsy in combination with real-time polymerase chain reaction (PCR) techniques may offer a solution to this barrier, and is one of the areas to have received both encouragement and funding from WADA. Utilizing a nested PCR assay, the authors were able to detect the presence of transgenic DNA (VEGF-A cDNA) in blood samples taken from all six of the mice subjects to whom it was administered via intramuscular adeno-associated virus (AAV)-mediated gene transfer. Furthermore, the VEGF-A transgene remained detectable at 4 weeks in all six subjects; and, remarkably, was still detected in 4/6 of the mice at day 56 post administration. This method would avoid the difficulties of ascertaining possible gene doping via muscle biopsy; and it only required minute amounts of blood (20 microL). While the authors highlight that transgenic efficiency is considerably greater in mice, they remain confident that the technique can be successfully used to detect gene doping in humans and have thus far
developed target specific identification PCRs for several other key doping targets including EPO and IGF-1. A final means of detecting gene doping may come from the immunological detection of antibodies produced in response to injected viral particles. If this were to be initiated as a detection method, repeated testing of athletes’ immune responses to various viral agents would be necessary. Moreover, since viral gene delivery requires the injection of a bolus dose of relatively high concentration (several million particles) of recombinant virus, it has been predicted that some very aggressive immune responses would be witnessed; and that these would be accompanied by significantly higher titres of antibodies than those witnessed as a result of typical viral infections. Looking to the future, application of this detection method could be limited by the development of increasingly efficient vector systems that will not induce such immunological reactions. Finally, it has been suggested that the detection of gene doping may be possible through the barcode-like labelling of agents. While proven to be successful in agricultural industries and stated to be a prerequisite for the development of any new therapeutic agent designed to induce genetic alteration, it is questionable whether a commitment to such labelling would withstand the considerable financial gains likely to be rewarded to those willing to make a gene doping product available [11547].

Administration of prohibited substances to enhance athletic performance represents an emerging medical, social, ethical and legal issue. Traditional controls are based on direct detection of substances or their catabolites. However out-of-competition doping may not be easily revealed by standard analytical methods. Alternative indirect control strategies are based on the evaluation of mid- and long-term effects of doping in tissues. Drug-induced long-lasting changes of gene expression may be taken as effective indicators of doping exposure. To validate this approach, it was used real-time PCR to monitor the expression pattern of selected genes in human hematopoietic cells exposed to nandrolone, insulin-like growth factor I (IGF-I) or growth hormone (GH). Some candidate genes were found significantly and consistently modulated by treatments. Nandrolone up-regulated AR, ESR2 and PGR in K562 cells, and SRD5A1, PPARA and JAK2 in Jurkat cells; IGF-I up-regulated EPOR and PGR in HL60 cells, and SRD5A1 in Jurkat; GH up-regulated SRD5A1 and GHR in K562. GATA1 expression was down-regulated in IGF-I-treated HL60, ESR2 was down-regulated in nandrolone-treated Jurkat, and AR and PGR were down-regulated in GH-treated Jurkat. This pilot study shows the potential of molecular biology-based strategies in antidoping controls [07397].

The WADA initiated research to prepare for the world of gene doping. Research scientists suggest several biologic/laboratory tests that could operate to expose gene-doping cheats. The usual parameters of laboratory tests – sensitivity, specificity, validity, and reliability – would need documentation to allow such innovative tests to withstand the certain legal scrutiny when elite athletes test positive for gene cheating [07046].

**Muscle biopsy**

A biopsy of suspected muscle tissue could reveal viral vehicles or evidence of altered genes; however, that possibility presents a invasive and low-yield antidoping measure [07046].

**Blood monitoring**

Proteins and hormones produced by doped genes could be exactly like endogenous proteins. Thus, it may be extremely difficult to detect the difference between the endogenous gene product and the doped-gene product; however, serial monitoring of blood parameters may reveal suspicious elevations of key biologic substances that indicate gene doping. For instance, the dramatic increase in hematocrit, in conjunction with several other hematological
parameters, could tip off a regulatory agency that an athlete used a gene-doping technique to improve oxygen delivery to muscles [07046].

**Genetic activity tests**

Interesting developments could use patterns of gene activity or gene products to detect abnormal gene activity. Detection of these patterns uses cutting-edge microchip gene array technology or nanotechnology breakthroughs. The monitoring or visualization of gene activity or gene products through the expression of DNA and RNA by a sophisticated microchip array could monitor thousands of genes, enabling the laboratory to use a sophisticated detective tool for gene doping [07046].

**Protein fingerprints**

In this process, similar to gene microarray testing, hundreds of biologic proteins could produce a "protein fingerprint" or a "genetic map" of the biochemistry of individual athletes. Suspicious alterations of such an individualized fingerprint or map would alert sporting authorities to possible gene doping [07046].

**Genetic barcodes**

It may be possible to label the transgene products with a genetic "bar code"; however, this tactic would require the cooperation of a broad array of professionals from the research scientists to the pharmaceutical houses to the administering physicians [07046].

**Laboratory techniques for detection of gene doping**

With a feasibility study a first step towards a new monitoring system for hormonal treatments was done. Screening of regulation and function of anabolic sex steroids via modified gene expression of mRNA in various tissues could be a new approach to trace treatments with unknown drugs or newly combined cocktails. In the study, uterus, liver and muscle tissue from 24 cycling heifers were taken after the animals were treated either with Melengestrol Acetate (MGA), Finaplix-H (200 mg Trenbolone Acetate) or Ralgro (36 mg Zeranol) for 56 days. In every treatment group always two heifers were given 1-fold, 3-fold and 10-fold doses of the standard preparation, the control group without any treatment consisted of two animals. The different tissue gene expression profiles were investigated via the candidate gene approach. Totally 57 candidate genes were selected according to their functionality by screening the actual literature and composed to functional groups: angiogenesis, apoptosis, cell cycle, endocrine factors, energy metabolism, inflammatory factors, muscle function, oncogenes, protein metabolism and transcription factors. Gene expression was measured using quantitative real-time RT-PCR (qRT-PCR) technology. From 24 tested candidate genes in the liver, 17 showed a significant regulation. Eight genes were influenced by MGA, 9 by Finaplix-H, and 4 by Ralgro. For the muscle tissue 19 genes were tested with the result that in the neck muscle 11 genes were regulated and in the hind limb muscle 8 genes. In the neck 5 genes were affected by MGA, 6 by Finaplix-H and 3 by Ralgro. Only 2 genes were influenced by MGA in the hind limb muscle. Finaplix-H affected 6 and Ralgro 4 genes. In the uterus 29 target genes were tested and 13 were significantly influenced by the anabolic sex steroids. Under Finaplix-H treatment eight target genes were regulated and Ralgro and MGA showed a significant regulation in four target genes. The highest gene expression changes under anabolic treatment were observed in the uterus. The analyzed genes showed significant regulations but further studies, testing different animal husbandry conditions will
be needed to identify meaningful expression patterns for the different tissues. With the investigation of the regulation and possible function of anabolic sex steroids via gene expression, a preparatory work for the development of an expression pattern for drug screening was made [06311].

It was described a direct detection approach for gene doping that gives a clear yes-or-no answer based on the presence or absence of transgenic DNA in peripheral blood samples. The procedure is based on the specific detection of intronless DNA by using a single-copy PCR protocol using a priming strategy that is specific for DNA sequences that could be abused for gene doping purposes. The priming strategy is targeting the transgene itself, as this sequence part is the minimal prerequisite to achieve a doping effect. In contrast to this, other non-human sequence parts delivered by gene transfer, such as viral sequences or promoter sequences, may vary depending on the technique used. In one study, it was provided experimental evidence that transgenic DNA can be detected for several weeks in the peripheral circulation of mice that received intramuscular adeno-associated virus (AAV)-mediated gene transfer, representing one of the most likely candidate vector systems to be used in gene doping practice. Transgenic DNA could be detected in minute amounts of blood samples, thus allowing minimally invasive sample retrieval, a fundamental prerequisite to legitimate doping testing. To make our detection strategy amenable for routine testing, it was implemented a robust sample preparation and processing protocol that allows specific and reproducible analysis of human blood samples to screen for six prime gene doping candidate genes: erythropoietin (EPO), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factors A and D (VEGF-A, -D), human growth hormone 1 (GH-1) and follistatin (FST). The detection strategy was verified in a mouse model, giving positive signals from minute amounts (20 microL) of blood samples for up to 56 days following intramuscular adeno-associated virus-mediated gene transfer, one of the most likely candidate vector systems to be misused for gene doping. To make our detection strategy amenable for routine testing, it was implemented a robust sample preparation and processing protocol that allows cost-efficient analysis of small human blood volumes (200 microL) with high specificity and reproducibility. The practicability and reliability of the detection strategy was validated by a screening approach including 327 blood samples taken from professional and recreational athletes under field conditions [10360].

The non-therapeutic use of genes to enhance athletic performance (gene doping) is a novel threat to the world of sports. Skeletal muscle is a prime target of gene therapy and we asked whether we can develop a test system to produce and detect gene doping. Towards this end, it was introduced a plasmid (pCMV-FAK, 3.8 kb, 50 microg) for constitutive expression of the chicken homologue for the regulator of muscle growth, focal adhesion kinase (FAK), via gene electro transfer in the anti-gravitational muscle, m. soleus, or gastrocnemius medialis of rats. Activation of hypertrophy signalling was monitored by assessing the ribosomal kinase p70S6K and muscle fibre cross section. Detectability of the introduced plasmid was monitored with polymerase chain reaction in deoxyribonucleic acids (DNA) from transfected muscle and serum. Muscle transfection with pCMV-FAK elevated FAK expression 7- and 73-fold, respectively, and increased mean cross section by 52 and 16 percent in targeted muscle fibres of soleus and gastrocnemius muscle 7 days after gene electro transfer. Concomitantly p70S6K content was increased in transfected soleus muscle (+110 %). Detection of the exogenous plasmid sequence was possible in DNA and cDNA of muscle until 7 days after transfection, but not in serum except close to the site of plasmid deposition, 1 h after injection and surgery. The findings suggest that the reliable detection of gene doping in the immoral athlete is not possible unless a change in the current practice of tissue sampling is applied involving the collection of muscle biopsy close to the site of gene injection [12481].
With the continued development of gene therapy the potential for gene doping increases. Detection of gene doping is difficult because expressed proteins can be identical to endogenous versions of the same protein. Where gene encoded proteins are secreted from cells it may be possible to at least detect them in biological samples and in some cases it may be possible to differentiate between the endogenous molecule and ‘doping’ protein by differences in glycosylation as previously demonstrated with recombinant and endogenous EPO. If doping genes express proteins or RNA molecules that remain inside the engineered cell or are retained at their site of production, then detection will only be possible by directly sampling tissue from the doping site. Without knowledge of a precise gene doping site then multiple samples (biopsies) will be required for detection which will not be tolerated by athletes and authorities. There are other possibilities for detection of gene doping such as an immune response to the delivery vector or monitoring the metabolic profile in biological samples but these will only provide an indirect indication of gene doping and not conclusive proof. Detection of gene doping could be further complicated if the doping gene is expressed from a regulated expression system which could mean that expression can be switched “on” and “off”. The way in which the switch is controlled could be through use of another chemical such as an antibiotic, a combination of chemical regulation and endogenous demand or in response to the demands of training and performance alone. An example of this is Repoxygen™ developed by Oxford Biomedica in 2002 which comprises of a viral-vector with a human EPO gene expressed from a promoter that is activated by HIF transcription factors and therefore production of EPO is self-regulated, only being induced (when required) under hypoxic conditions. This product delivered by intramuscular (i.m.) injection has been assessed in experimental models for the treatment of anaemia. Gene doping with self-regulated genes of this sort will be even more difficult to detect [13011].

The development of gene therapy has not been a smooth journey to date. There have been some major setbacks along the way which have usually been a consequence of the vector used to deliver the therapeutic gene. Pre-existing immunogenicity in patients is caused by prior exposure to infectious forms of the virus and this can be a fundamental problem which limits effectiveness of gene delivery and results in immune killing of transduced cells. High doses of adenovirus delivered to one individual caused a massive inflammatory response with an “immune revolt” where the immune system targeted vital organs leading to his death. An area that has scarcely been explored in gene therapy trials is the re-administration of vectors which may well be attractive in gene doping but is likely to pose additional danger. Retroviruses deliver genes permanently into the genome and this can also have severe consequences. The treatment of X-linked SCID patients through ex vivo modification of BMSCs was initially effective but a number of children later developed T cell leukemia as a consequence of gene insertion activating the expression of oncogenes including LMO2 [13011].

Detection of gene doping is significantly more difficult than detection of doping with pharmaceuticals. This might make gene doping more attractive for athletes considering cheating. Currently, no specific test to detect gene doping has been approved by the WADA or used by a WADA-accredited laboratory. Any detection method would have to comply with at least the following requirements. First, the doping detection method must be adequately selective to detect cheating athletes. Second, it should be accessible and easy to use on a large scale, while remaining reliable. Finally, it should be fast, as convicting an athlete years after the crime is not desirable (although legally possible up to 8 years after a doping violation has occurred). As stated earlier, athletes who engage in doping generally use pharmaceuticals to improve their performances, so the first gene dopers are likely to have had early access to gene therapy products. Detection efforts to identify gene doping by athletes should initially explore the current uses of known gene therapy for disease treatment. Some detection methods might also help to determine the efficacy of gene
therapy for disease while in development. Generally, detection methods can be divided into two groups: direct and indirect. Direct methods test for an illegal substance, or the genetic material or virus that delivered it. Indirect methods use the effect, immune response, differences in expression or metabolic changes for detection. The direct detection of an illegal substance is preferred over indirect testing for legal reasons, but unfortunately, illegal substances are metabolized or cleared too quickly to be detected, in general [13011].

Vector biodistribution and clearance studies are essential in the development of gene transfer medicine. To provide reliable and accurate data, protocols for vector analysis must be optimized and validated. It was addressed several parameters affecting the detection of gene therapy vectors in blood. Using an in vitro system based on plasmid DNA incorporating, as a transgene, complementary DNA for human erythropoietin gene, we developed and validated a suite of real-time PCR assays for the transgene splicing sites. The most sensitive assays detected the transgene present at 0.011 percent of the copy number of the endogenous erythropoietin gene in human genomic DNA at 100 percent specificity. Plasmid linearization incorporated with PCR resulted in an increase in assay sensitivity up to 4.5-fold without compromising analysis workflow. This allowed detection of five copies of transgene in a background of 0.4 microg of genomic DNA (or 0.0035 % detectable transgene copies relevant to copies of the endogenous gene). Finally, desktop assessment of 18 DNA extraction protocols was undertaken and 5 kits were evaluated experimentally for extraction of nonviral vectors from blood. Three kits reliably detected 80 copies of the transgene in a milliliter of blood. Adoption of the described protocols will enable more reliable vector analysis in gene therapy and will assist in accurate interlaboratory comparison. The methodology will also facilitate detection of gene doping in sport, a potential new form of misuse of gene transfer technology [13757].

Athletes who illicitly use drugs to enhance their athletic performance are at risk of being banned from sports competitions. Consequently, some athletes may seek new doping methods that they expect to be capable of circumventing detection. With advances in gene transfer vector design and therapeutic gene transfer, and demonstrations of safety and therapeutic benefit in humans, there is an increased probability of the pursuit of gene doping by athletes. In anticipation of the potential for gene doping, assays have been established to directly detect complementary DNA of genes that are top candidates for use in doping, as well as vector control elements. The development of molecular assays that are capable of exposing gene doping in sports can serve as a deterrent and may also identify athletes who have illicitly used gene transfer for performance enhancement. PCR-based methods to detect foreign DNA with high reliability, sensitivity, and specificity include TaqMan real-time PCR, nested PCR, and internal threshold control PCR [13758].

**Direct methods**

Measuring the *plasma levels* of a protein would not be an accurate method for detecting gene doping. Some endogenous mechanisms to control expression prevent high plasma levels and the plasma levels of some proteins are too low to detect. Also fluctuation in physiological levels of a protein complicates this method. Measuring various isoforms of a protein would be helpful. When an exogenous protein inhibits production of the endogenous variant, a difference in the isoform ratio would be detected; thus, detection would be possible for gene doping strategies targeting EPO and GH [13011].

A *biopsy* of an infected area would provide a sample in which the virus or the exogenous gene might be detectable in an athlete. Gene transfer has been shown to be detectable in a biopsy for up to a decade. Since knowledge about the injection site is required, and biopsies are generally considered to be too invasive, methods using only blood, urine, serum, hair,
The presence of a virus might be detectable in the bloodstream, so blood samples could be tested with PCR to detect DNA or RNA or with other methods to test for viral proteins. The difficulty with this technique is timing; the persistence of viruses varies from hours to months. Testing for a virus in urine (persistence over several weeks) or saliva (persistence over several days) might be better. The downside of this approach is a possible false positive, for example, an athlete who is infected with a normal virus.

The genetic material commonly used in gene doping is complementary DNA (cDNA), which lacks introns; therefore, it can be discriminated from genomic DNA with PCR. In mice injected intramuscularly with AAV-mediated gene therapy, PCR allowed detection in the blood for several weeks, though in another test it was undetectable in blood after half an hour. However, PCR is less useful for detecting doping using genes with introns because it presents problems with alternative splicing and efficacy. In addition, it is conceivable that once a PCR detection method is introduced, gene-doping products based on genomic DNA will become available quite soon.

Since each cell type differs in post-translational modification, endogenous proteins would be distinguishable from the ones produced by gene doping. This is what caused the autoimmune reaction against EPO in macaques, resulting in anaemia. Detection would be possible with isoelectric focussing. The method is useful until viruses target cells more specifically, or specific promoter regions are developed. However, it is possible that these target cells or promoter regions might also be detectable in the future.

Genetically modified agricultural products have a genetic barcode, to help with identification. This could be done for gene therapy too, which would make the gene therapy products detectable with PCR. This approach requires global coordination in the pharmaceutical industry, which in the past has been proven to be difficult to achieve, and is likely to become practically irrelevant once gene-doping products are produced without barcodes. Creating barcodes for identification could stimulate rogue laboratory production practices that eliminate barcodes.

**Indirect methods**

Every virus induces a specific immune response in the host. Plasmid vectors or the produced proteins can induce immune responses that can be detected and distinguished from common immune responses. However, distinguishing common virus reactions from immune reactions remains a problematic issue.

Use of gene doping will probably change the transcription of other proteins as well. By tracking selected protein levels (proteomics) and gene transcription rates in a biomedical passport, dopers can be caught. One risk of this method is the chance of a false positive or false negative, since changes in training or injuries can also induce changes in metabolism. Most research on possible detection methods of gene doping uses this approach, also because this approach is potentially quite useful to determine the efficacy of gene therapy trials; but the validity is as yet unproven.

**Transgene and nonviral vectors in blood**

Vector biodistribution and clearance studies are essential in the development of gene transfer medicine. To provide reliable and accurate data, protocols for vector analysis must
be optimized and validated. It was addressed several parameters affecting the detection of gene therapy vectors in blood. Using an in vitro system based on plasmid DNA incorporating, as a transgene, complementary DNA for human erythropoietin gene, we developed and validated a suite of real-time PCR assays for the transgene splicing sites. The most sensitive assays detected the transgene present at 0.011 percent of the copy number of the endogenous erythropoietin gene in human genomic DNA at 100 percent specificity. Plasmid linearization incorporated with PCR resulted in an increase in assay sensitivity up to 4.5-fold without compromising analysis workflow. This allowed detection of five copies of transgene in a background of 0.4 microg of genomic DNA (or 0.0035 % detectable transgene copies relevant to copies of the endogenous gene). Finally, desktop assessment of 18 DNA extraction protocols was undertaken and 5 kits were evaluated experimentally for extraction of nonviral vectors from blood. Three kits reliably detected 80 copies of the transgene in a milliliter of blood. Adoption of the described protocols will enable more reliable vector analysis in gene therapy and will assist in accurate interlaboratory comparison. The methodology will also facilitate detection of gene doping in sport, a potential new form of misuse of gene transfer technology [13759].

**PCR-based detection of gene transfer vectors**

Athletes who illicitly use drugs to enhance their athletic performance are at risk of being banned from sports competitions. Consequently, some athletes may seek new doping methods that they expect to be capable of circumventing detection. With advances in gene transfer vector design and therapeutic gene transfer, and demonstrations of safety and therapeutic benefit in humans, there is an increased probability of the pursuit of gene doping by athletes. In anticipation of the potential for gene doping, assays have been established to directly detect complementary DNA of genes that are top candidates for use in doping, as well as vector control elements. The development of molecular assays that are capable of exposing gene doping in sports can serve as a deterrent and may also identify athletes who have illicitly used gene transfer for performance enhancement. PCR-based methods to detect foreign DNA with high reliability, sensitivity, and specificity include TaqMan real-time PCR, nested PCR, and internal threshold control PCR [13760].

**Regulation of gene doping**

Regulatory oversight is stricter for gene therapy trials than for most clinical trials due to the potential risks. Since the first documentation of fatalities during gene therapy, regulations have been tightened, primarily in Europe and the USA. In the USA and Europe gene therapy is only allowed in cells that do not reproduce, preventing gene therapy from affecting following generations [13011].

The ethical criteria for a drug or medical technique to be included on the WADA prohibited list are "scientific evidence, proven pharmacological effect or experience that substances or methods included have the potential to enhance or enhances sport performance." Two arguments are used for inclusion on the list: the substance or method may be harmful or cause a health risk to the athlete and the use of doping violates the spirit of sport, as defined by WADA criteria. Essentially, the substance or technique is outside "fair play," which could be construed as "cheating." The WADA tenets have been criticized as ambiguous. Clearly, any medical intervention can be a health risk; athletic competition itself is a health risk. The key factor in determining the ethical use of a drug in athletic competitions rests on the point of fair play. When is a drug not given or taken for a therapeutic purpose, but for a purpose of obtaining an unfair competitive advantage? That ethical battle continues every day in many
sporting venues. With the advent of gene therapy, the focus of the debate will turn from drugs to transgene products; however, the key element of therapeutic versus manipulative will remain unchanged [07046].

The ethics of gene doping

Is it ethical to test people, particularly children, for athletic potential? Perhaps such tests could be useful to elite athletes looking to tailor their training to their bodies. Even a tiny advantage at that level can be significant. But for almost all amateur athletes, even very good ones, there appears to be little to gain from possessing genetic information about their metabolisms or muscle fibres. However, several biotech companies, however, would beg to differ. One of them is Colorado-based Atlas Sports Genetics, which in 2008 began selling a test based on the ACTN3 gene that it claimed could determine if you were better suited for sports requiring speed/power or for those requiring endurance. In June 2010, Richmond, Virginia-based AIBioTech launched a set of genetic tests that not only look for athletic potential, but also for possible health risks that are exacerbated by strenuous physical activity. Shortly after these types of tests came to market, concerns were raised about the possible negative effects on children. Some critics worry that favourable genetic data will only lend support to the irrational dreams of any parent who believes the three-year-old across the dinner table will someday become a scholarship-snagging, endorsement-earning superstar with a million-dollar contract to shoot, kick or throw a ball. If anything, steering a child toward a particular sport or activity based on genetic data will actually limit their future opportunities, not enhance them. In a paper exploring genetic testing and sports medicine ethics, it was raised several other ethical issues associated with using genetic markers to predict athletic ability [Sports Med 2009; 39: 339-44]. There are confidentiality issues, for instance. What if a test for athletic ability also indicated a predisposition to a particular illness, such as Alzheimer disease? Who has a right to that information? Potential problems could also arise if professional athletes were compelled to undergo genetic testing. Perhaps, however, such problems are unfounded speculation. Many of the ethical concerns swirling around genetic testing in sports have no basis in reality, says the vice president of science and technology for AIBioTech, which offers a basic set of genetic tests under the Sports X Factor banner to help athletes “perform better and safer” for USD 200 [12485].

Gene transfer technology in sport is prohibited largely for its being a form of experimental science. The most likely applications of gene transfer to sports involve manipulation to enhance endurance capacity or muscle mass. Currently, research implicated for gene doping includes modifications to growth factors, such as IGF-1, recombinant EPO, and the so-called ACE gene. Ethically, its application to sport is considered by officialdom as unacceptable since there is no protocol for such use, nor standards of efficacy or safety. To this extent, any attempt to genetically modify athletes would currently be seen as medical malpractice. As such, any argument in favor of gene doping will need to address the broader question about the limits of medicine, which will involve tackling fundamental matters of medical ethics. Specifically, an argument will be required to justify treating healthy humans (athletes) with medical technology. The emergence of gene doping should mark a new paradigm for antidoping policy makers, because it presents a new landscape of ethical issues, political views on enhancement and concerns. This position does not suggest genetic exceptionalism, but speaks specifically to the moral opinions surrounding genetics, which are rather more unresolved than one might say for doping generally. The moral tension arising from the application of genetic engineering to sport reflects the crisis of authenticity in contemporary society, specifically, the demise of the natural human and the widespread ambivalence or anthropic bias over this. Fair play and health are secondary matters in this debate and, yet, they dominate, in part because they lend themselves to an artificial, but sincere moral...
intuitionism and paternalism that remains part of elite sporting culture [06168].

Considerable clarification is needed on what constitutes the genetically modified athlete. Currently, sports authorities are interested only in the somatic cell doper, who themselves consent to using gene transfer to gain an edge over a competitor. Yet it is unclear what would happen if an individual is made to be "better than well" through the same kind of use in a therapeutic context. Alternatively, are we interested in the athlete who has been born from parents that have, themselves, been modified? Last, does the ethical debate take into account the child born from parents who select a form of enhancement for their child, or perhaps select their preferred embryo on the basis of its propensity for elite sports competition? In 2004, the first genetic test for performance was made commercially available. One year later, the WADA (World Anti-Doping Agency 2005) announces in its Stockholm Declaration on gene doping that such tests are to be discouraged [06168].

It seems unlikely that the world of sports will remain untouched by the potential for gene-based enhancement to ensure victory in competition. In fact, sport represents one of the early and most obvious areas of human activity in which serious attempts at genetic enhancement are likely to be made, and made fairly soon. Because the tools of gene transfer are now slowly but surely being applied successfully to the treatment of life-threatening disease, there will increasingly be temptations to apply the same methods to many other human traits that represent less severe disease and are not disease-related at all but rather constitute normal human functions that some will wish to augment or “improve.” For instance, one such study involved the sustained expression of a gene coding for the production of the hormone erythropoietin (EPO) for delivery through the circulation to blood-forming tissues for treatment of the anemia associated with chronic kidney disease. Another study proposed the introduction of a gene encoding the insulin-like growth factor-1 (IGF-1) into muscle undergoing degenerative changes as a result of a nerve entrapment disorder. Still another proposal involved gene transfer for the correction of erectile dysfunction. The move from clear therapy to more and more clearly “enhancing” gene transfer applications should come as no surprise in a society that already accepts and even seeks improvement or enhancement of many human traits through drugs and cosmetic surgery [06307].

A gene transfer approach aimed at enhancing athletic capability, to be carried out safely and ethically, would have to conform, like all gene transfer manipulations in human subjects or patients, to the standards of ethical performance of experimental clinical research in humans. Any gene transfer in sports would also have to submit to the oversight and regulatory function of a variety of local and federal bodies that oversee all human gene transfer clinical trials and that have the responsibility of evaluating the important technical, policy, and ethical issues inherent in all human clinical research. All established codes of human medical experimentation require that the known risks and possible adverse consequences of gene transfer studies should be fully and honestly presented to patients and research subjects, and it is only after full disclosure and informed consent that a decision can be made to proceed with the study. It is also required that the anticipated benefit outweighs the known or likely harm that can be caused to the participants. Given our understanding of the science of gene transfer in humans, such studies would at best be very difficult and hazardous to the subject and therefore, unlikely for the foreseeable future to receive approval from the oversight bodies. For all of these reasons, it is impossible at the present time to perform such illicit applications ethically, safely, and honestly in ways consistent with all of the relevant tenets of human research [06307].

Only a handful of the more than 700 clinical gene therapy studies that have already taken place under truly rigorous scientific conditions have achieved a clear therapeutic result while several have caused death and other harm to participants. It is not too complicated merely to
imagine approaches to gene transfer, but carrying out an effective and safe study has proven to be excruciatingly difficult and filled with unknowns. Therefore, effective but also safe application to nontherapeutic settings such as sports might seem straightforward but is guaranteed to be frustratingly difficult. Nevertheless, the successes already achieved with immune deficiencies and imminent likely successes with other diseases establish the principle that one can alter human disease traits by genetic means and open the door widely to modification of a variety of normal human nondisease traits [06307].

Bioethical concerns

One paper provides an overview of the ethical issues pertaining to the use of genetic insights and techniques in sport. Initially, it considers a range of scientific findings that have stimulated debate about the ethical issues associated with genetics applied to sport. It also outlines some of the early policy responses to these discoveries from world leading sports organizations, along with knowledge about actual use of gene technologies in sport. Subsequently, it considers the challenges with distinguishing between therapeutic use and human enhancement within genetic science, which is a particularly important issue for the world of sport. Next, particular attention is given to the use of genetic information, which raises questions about the legitimacy and reliability of genetic tests, along with the potential public value of having DNA databanks to economize in health care. Finally, the ethics of gene transfer are considered, inviting questions into the values of sport and humanity. It argues that, while gene modification may seem conceptually similar to other forms of doping, the requirements upon athletes are such that new forms of enhancement become increasingly necessary to discover. Insofar as genetic science is able to create safer, more effective techniques of human modification, then it may be an appealing route through which to modify athletes to safeguard the future of elite sports as enterprises of human excellence [12476].

Definition of enhancement

Patented genetic technologies such as the ACTN3 genetic test are adding a new dimension to the types of performance enhancement available to elite athletes. Organized sports organizations and governments are seeking to prevent athletes’ use of biomedical enhancements. One paper discusses how these interdiction efforts will affect the use and availability of genetic technologies that can enhance athletic performance. One paper provided a working definition of enhancement, and in light of that definition and the concerns of the sports community, reviews genetic enhancement as a result of varied technologies, including, genetic testing to identify innate athletic ability, performance-enhancing drugs developed with genetic science and technology, pharmacogenetics, enhancement through reproductive technologies, somatic gene transfer, and germ line gene transfer [12477].

Genetic enhancement

These kinds of studies would obviously represent a departure from gene therapy as it has come to be recognized as an approach to preventing or correcting disease. In all countries in which gene therapy is being pursued under the all the required review and regulation, all gene transfer studies carried out until now have been aimed at conditions that would be universally seen as diseases. However, the loss of muscle mass and function as a part of human aging can be considered to be a completely normal phenomenon and therefore accepted with no need for “treatment.” On the other hand, some could maintain with good reason that genetic methods to improve muscle function, as developed in studies of terrible muscle diseases such as muscular dystrophy, would increase quality of life for millions of
people in the same way that treatment of other aspect of human aging are all acceptable parts of modern medicine. It would not be a great conceptual or technical leap then to apply the same technologies to completely normal human traits such as muscle function or blood production in normal young athletes for the specific purpose of improving athletic prowess. In that way, the world of sports may be one of the provinces of human activity to become an early target for the application of existing and future advances in the field of human gene therapy for the enhancement of nondisease human traits. Most of the more thoughtful discussions have concluded that there are severe moral objections to such efforts, even if and when the technical limitations that we now face are overcome and such changes might be produced safely without harm to the subject or, for that matter, to future generations. These have also forced a new look at the role of a sport in a society, the very concept of a sport, and its apparent evolution from a rule-based noble and romantic striving for individual achievement to potentially grotesque uncontrolled biotechnology.

Athletes need and deserve access to the best medical procedures and methods to repair their injured muscle, tendons, bones and all other tissues just as all injured patients need and deserve the best in medical repair technology. Athletes must not be deprived of the best medicine available to all others. However, it seems very likely that some methods for treatment of injuries or illness in athletes result inadvertently not only in restoration of the original normal function but that produce an enhanced physical function: stronger muscles, ligaments, and tendons less vulnerable to further injury, and so on. That is probably just as true of currently available methods of injury repair, but enhancing physical function is usually not a significant goal in treatment of injuries and illness by existing methods. Well-treated athletes are not excluded from competition because previous therapy for an injury or an illness has left them with possibly enhanced athletic capability. However, it is now becoming possible that enhanced function might not be merely an unintended consequence of treatment but in fact may become a part of the goal of the "treatment." For instance, it has been demonstrated in animal studies that treatment of some joint and musculoskeletal injuries and illness with growth factors such as IGF-1 markedly increases the degree and speed of tissue repair. If similar gene-based techniques were also to be proven to be effective in humans, it would seem to be of great benefit to offer such therapies to all injured people, including injured athletes. Could athletes ethically be deprived of such essential medical procedures? Of course not. But such an athlete's competitors might be placed in a position of unfair disadvantage. Even more troublesome, of course, is the possibility that prophylactic treatment of an injury-prone muscle in an athlete with such a factor for the direct purpose of providing such the benefit of improved athletic capability could readily become feasible. Such an application could certainly be consistent with the best principles of preventive medicine but would certainly cause discomfort in the sports context. And by sport we should not restrict ourselves only to human athletes. Race horses and other animal athletes could just as easily become the subjects for such therapy-enhancement applications.

The thin line between therapy and enhancement

One of the areas in which troublesome scientific, policy, and ethical issues related to gene transfer applications in humans is the indistinct border between therapy and enhancement. What trait is being modified? Is it a disease or the cause of a severely compromised quality of life? Is the modification justified? There are certainly human conditions that most of us – with the exception of adherents to some religious principles – would agree are "diseases" and require intervention. There are other physical traits that are not so clearly "diseases" but that many in our society nonetheless might wish to modify for the purpose of improving "quality of life" or even for cosmetic reasons. Most human characteristics span a broad range from obviously "defective" to more-or-less "normal," and most of our modern societies have

2074
already accepted the notion that the edges of this normal distribution that border on the “abnormal” or “defective” are targets for modification. For instance, there is a very wide variation in the height of human beings; some are very tall, some are very short. In some cases, such extremes result from demonstrable defects in growth factors, such as growth hormone, leading to pathological shortness. These children are often treated with growth hormone to add valuable inches to their eventual height. In many other instances, people are short in the absence of any demonstrable abnormalities in these or other growth factors. They are short because their parents are short, through whatever mechanisms that may reflect. Such a trait is just as clearly inherited as is an inborn genetic deficiency of growth hormone, but the resulting short stature might not be seen by most of us to be abnormal or require therapy. Humans also often show a loss of memory and intellectual acuity during “normal” aging. In the extreme forms of these problems found Alzheimer’s disease, we have taken the extreme nature of the changes to represent pathology and real disease, and a great deal of research is being aimed at treatment for this disorder [06309].

Influence on mood and behavior
Even complex social behaviors are now slowly coming to be understood as reflections of mixed genetic and environmental influences. For instance, mice having a deficiency in the gene known as MECP2 have been developed as a model for the human autism-like disorder called Rett’s syndrome. Mice with this genetic deficiency show deficiencies in a variety of behavioral traits important to mice-nesting behavior and reduced decisiveness in interacting with other mice. A study in mice demonstrated that defects in the gene making the protein stathmin seemed to make timid mice much more daring and less intimidated by novel situations, possibly by interfering with nerve cell connections in the part of the brain called the amygdala, a center thought to be important in fear responses. And so it seems likely that, as this kind of research gathers speed, more genes will be found with powerful effects on complex social behaviors and thereby make those genes susceptible to manipulation and possibly make these and many other normal human behaviors available for drug- and possibly genetic intervention [06309].

We also nowadays have medicine cabinets full of drugs designed to improve our moods and memory, enhance our sexual performance, and modify aspects of our personality and temperament. If drugs are acceptable for these purposes, why not genetic approaches to exactly the same conditions? It might be difficult to develop convincing and logically consistent arguments against such applications of gene transfer [06309].

Repair of injury
The effective management of injury and the repair of damaged tissues are central to athletic training and competition. A great deal of research is being carried out to develop methods to speed and improve tissue repair by introducing genes into injured muscles, tendons, joints, etc that stimulate growth and repair. In fact, studies in animal models have shown that the gene transfer into such damaged tissues can speed repair and regrowth of some injured tissues: muscle, tendon, and cartilage. Certainly, if these kinds of treatments are found to be effective in human injuries, athletes should not be deprived of the therapeutic benefits of gene-base repair of legitimate athletic injuries. However, one can also imagine a temptation to perform preventive treatment of vulnerable or repeatedly injured tissues not to treat but to prevent future injury [06309].

Misuse of genetic science: the reemergence of eugenics
Through the genetics revolution that we have been witnessing during the past several decades, we have come to realize not only that most human disease is determined at least partly by genetic components but also that most normal human traits are similarly affected to a greater or lesser degree by genetic influences. These include our most complex and
mysterious traits – our personalities and cognitive and intellectual capacities. Of course, most of the genes involved in these functions have not yet been identified but we know that they are there. We do not yet know what they are or what they do, but in time we will find them and increasingly understand them. To the extent that enhancement in sports might easily represent one of the earliest opening scenarios in the broad problem of gene-based enhancement, what occurs in the world of sports is likely to define the ways in which our society approaches and solves the problems arising from the deliberate genetic modification of human traits in general [06309].

**Specific ethical questions**

Sports medicine ethics is neither a well established branch of sports medicine nor of medical ethics. It is therefore important to raise to more general awareness some of the significant ethical implications of sports medicine practices. The field of genetics in sports is likewise in its infancy and raises significant ethical concerns. It is not yet clear how genetics will alter our understanding of human potential and performance in sports. While a number of professional medical bodies accept genetic interventions of a therapeutic nature, we argue that the use of genetic technologies to predict sports potential may well breach both the European bioethics convention and North American anti-discrimination legislation, which are designed to support important ethical ideals and the ongoing commitment of the physician to the welfare of their patient. We highlight further ethical problems associated with confidentiality and consent that may arise in genetic testing as opposed to more conventional methods of testing in sports medicine. It was concluded that genetic testing in sport that is not strictly limited to the protection of the athlete against harm, should be viewed in a very sceptical light by sports medicine professionals [09382].

In genetic preselection, the genome would be scanned, allowing parents to choose the most genetically athletically gifted offspring to survive. This process is a sophisticated twenty-first century variant of the ancient Spartan child-selection process. Although no reports exist of parents scanning the genome of their prospective children, there are reports of a sporting organization using a limited genome scan to select prospective athletes or to genetically tailor training [07046].

Although gene doping sounds like a science fiction plot, the physician should not underestimate the capacity of humans to find an edge in competition—legal or illegal. As athletes, professionals, parents, and coaches, the authors have experienced numerous examples of cheating in sports. From the simple falsification of player records to the importation of foreign athletes to the use of anabolic steroids and PEDs, athletes, coaches, boosters, parents, and physicians will bend the rules of fair play. The greater the stakes, the higher the rewards; the temptation to cheat becomes more alluring. If gene therapy becomes reality in humans – and the technique is poised to become clinically useful – those participants who hold no moral compunctions against cheating fellow competitors will use the technique. At some point in time, performance-enhancing genetics will be a reality; as professionals, be forewarned and be prepared [07046].

Without a doubt, tinkering with an athlete’s genes has enormous potential to increase performance – initial results from basic research in this area are impressive. Transgenic mice created in an effort to understand muscle growth and muscle disease were soon baptized “Schwarzenegger mice” owing to their enormously increased strength and muscle mass. Genes such as insulin-like growth factor 1 (IGF1) and myostatin, which regulate muscle mass, are obvious targets to increase performance in sporting disciplines in which sheer strength is required. Similarly, genes that stimulate blood production or increase the oxygen-carrying capacity of blood, as well as those that regulate mitochondrial energy production
and energy use, are of interest to endurance athletes. And manipulating pain perception by genetically modifying the release of endorphins in athletes' brains might be the ultimate winning combination. Irrespective of the gene of interest, the advantages over conventional doping are obvious: as the gene product is the same as the endogenously produced protein, it is much harder to detect by current methods than any injected or ingested substance. Scientists are becoming aware of this potential for abuse. Indeed, gene doping carries considerable health risks. A certain problem is that genetic enhancements would not be as tightly regulated as other endogenous processes. Once the gene is introduced to the body, in general it is turned on and so far it has not been detected a good regulatory system to turn it on and off. The highest risk for the athlete is therefore "overdosing". It is the same for EPO, IGF1 or other target genes. However, it is nearly impossible to stop people who really want to cheat. All students with some training in molecular biology can isolate these genes and produce them in bacteria as DNA. If someone really wants starting material for gene doping then it is easy to get. WADA reacted proactively by banning all forms of gene doping after scientists warned them about the possibility. The challenge, however, is to develop effective ways to prove gene doping. Conventional methods would fail to detect an inserted gene that was isolated from the athlete's own body because its product is indistinguishable from the naturally produced form. WADA has therefore established vigorous research programs to develop new detection methods. It is a global approach to characterize doping from the point of view of disturbance of the homeostasis of the system [07001].

Regardless of whether and how athletes use gene doping, genetic and genomic research has already changed the nature of sport. For example, DNA and protein profiling could be used to identify specific gene variants to predict and select athletes for certain sports. It is known for sure that there are at least 50 alleles that you don't find in the general population. If gene therapy becomes sufficiently safe to be used not only as a medical treatment but also for normal enhancement purposes, it will raise the question of whether gene doping should remain forbidden. There is already a grey zone of performance enhancements that are legally used in sports because they are accepted as standard medical treatments. Professional golfers, for example, have subjected themselves to laser eye surgery to enhance their vision. Sport is cutting edge, because technological advancements will get experimented with there first. Ethicists have already envisaged future scenarios in which genetic enhancement would be common. Gene transfer methods were developed for therapeutic use, but there are further uses now. The examples of LASIK (laser-assisted in situ keratomileusis) or therapeutic muscle treatment show how grey zones might eventually become slippery slopes that question values of fair play and joy in sport. The risk is that the athletes will be treated like pieces of meat. They are created for port activity and only for that. If commercialism pushes this so strongly, the core values about celebrating human effort and the joy of the effort and the love of the game will be lost [07001].

Many genetic researchers and academics have raised questions about the scientific validity of these genetic tests aiming at performance evaluation, claiming they have little predictive value. Some critics have also raised another concern: Is it ethical to test people, particularly children, for athletic potential? Perhaps such tests could be useful to elite athletes looking to tailor their training to their bodies. Even a tiny advantage at that level can be significant. But for almost all amateur athletes, even very good ones, there appears to be little to gain from possessing genetic information about their metabolisms or muscle fibres. Some people worry that fanatical parents will push their children too hard to excel in a particular sport based on the results of genetic tests for athleticism. Several biotech companies, however, would beg to differ. One of them is Colorado-based Atlas Sports Genetics, which in 2008 began selling a test based on the ACTN3 gene that it claimed could determine if you were better suited for sports requiring speed/power or for those requiring endurance. In June 2010, Richmond, Virginia-based AlBioTech launched a set of genetic tests that not only look for athletic
potential, but also for possible health risks that are exacerbated by strenuous physical activity. Shortly after these types of tests came to market, concerns were raised about the possible negative effects on children. Some critics worry that favourable genetic data will only lend support to the irrational dreams of any parent who believes the three-year-old across the dinner table will someday become a scholarship-snagging, endorsement-earning superstar with a million-dollar contract to shoot, kick or throw a ball. If anything, steering a child toward a particular sport or activity based on genetic data will actually limit their future opportunities, not enhance them. Early specialization may foreclose opportunities. If you give a child access to a general sports program, you are laying the foundation for them to gain abilities that could translate into a whole range of skills. In a paper exploring genetic testing and sports medicine ethics, it was raised several other ethical issues associated with using genetic markers to predict athletic ability [Sports Med 2009; 39: 339-44]. There are confidentiality issues, for instance. What if a test for athletic ability also indicated a predisposition to a particular illness, such as Alzheimer disease? Who has a right to that information? Potential problems could also arise if professional athletes were compelled to undergo genetic testing [11587].
ETHIC ISSUES IN DOPING AND ANTI-DOPING

Since ancient times, competitive athletes have been familiar with the use of ergogenic aids and they will probably continue to use unfair and harmful substances in future, because their inclination to victory, along with the mirage of glory and money, will probably overcome health and legal risks. It was now searched PubMed using the term doping over the period 1990 to the present day. By literature searching, it emerges that the phenomenon of doping is complex and multifaceted. It involves a number of causes and factors that do not originate solely in the athletic field, making universality its main feature. It is in fact observed in all ages and levels of competition, and it concerns all sports, even the most unpredictable. The high number of athletes testing positive for anti-doping controls attests that the current strategy might be analytically adequate to unmask most (but not all) doping practices, but it is probably ineffective to prevent athletes to dope and modify this upsetting trend. As doping parallels the use of medications, food supplements, alcohol and social drugs, a reinforced preventive policy is advisable. The authors concluded that current anti-doping policy should be replaced with a more efficient and practical strategy to identify and monitor abnormal and harmful deviations of the biochemical and haematological profiles [08443].

Assisting sports performance by allowable means is an acceptable behaviour to most athletes who are involved in competitive sport. Physiological, biomechanical, medical, psychological and nutritional supports are routinely provided to high performing athletes and during the natural course of the athlete development, athletes are accustomed to technologies and methods for human performance enhancement. According to the life-cycle model of performance enhancement doping practices likely to grow out of habitual engagement in a range of acceptable performance enhancement practices [08444].

Healthcare professionals working in and outside of sports medicine are bound by a range of codes of professional conduct that inter alia outline their professional obligations. Central to healthcare professionals' obligations is their duty of care of which patient confidentiality is a part. It was presented a range of Codes that direct the healthcare professional to the protection and promotion of patient welfare including the maintaining of confidentiality, which is at the heart of trustworthy relations. In apparent contravention of this duty, the 2009 version of the World Anti Doping Code appears to oblige all healthcare professionals not to assist athletes if they are known to be engaged in doping behaviours under fear of removal from working with athletes from the respective sport. It was shown that, under certain conditions, serving the best interests of their athlete patients may oblige healthcare professionals to give advice and guidance in terms of harm-minimisation. In so far as the professional conduct of a healthcare professional is guided both by professional code and WADC, they are obliged to fall foul of one or the other. It was called for urgent and pressing inter-professional dialogue with WADA to clarify this situation [09383].

Unique among allied health care professions, athletic training is predominately practiced amid competitive intercollegiate sports. Competitive sporting environments have been suggested to adversely impact morality, ethical decision-making, and behavior. The purposes of one study were to (1) investigate the effect of institutional National Collegiate Athletic Association (NCAA) participation level on preferred ethical ideologies and ethical decision-making, (2) determine the relationship between professional status (athletic training student or certified athletic trainer and ethical ideology preferences and ethical decision-making, and (3) examine whether preferred ethical ideology is related to differences in ethical decision-making. A nationally representative sample of 610 athletic training students and certified athletic trainers from 30 athletic training education programs, stratified by NCAA division level, participated in the study. All participants completed a demographic survey, the
Ethics Position Questionnaire, and the Dilemmas in Athletic Training Questionnaire. No significant relationships were noted between NCAA participation level and respondents' ethical ideology preferences. However, athletic training students and certified athletic trainers demonstrated significant preferences for specific ethical ideologies, with students adopting the subjectivist ideology more than expected and the exceptionist ideology less than expected and certified athletic trainers adopting the exceptionist ideology more than expected and the situationist ideology less than expected. In contrast to some previous research, our results suggest that competitive sporting environments do not affect athletic training students' and certified athletic trainers' ethical ideology and ethical decision-making abilities at the collegiate level. These findings serve as a baseline for future research examining the ethical ideologies and ethical decision-making levels of athletic training practitioners and other allied health professionals across clinical settings [09384].

Today's collegiate student athlete is a highly diverse individual and as such is at higher risk for many health problems both on and off the field. To determine if a preparticipation evaluation can be optimized to help the collegiate team physician and athletic trainer assess both current and past health issues of student athletes. Utilizing MEDLINE and other medical literature database search engines, the authors conducted detailed literature searches on this subject. Key words used in these searches included preparticipation physical evaluation, collegiate, athlete, cardiovascular, preventive healthcare, high risk, alcohol, tobacco, sexually transmitted disease, motor vehicle accident, adolescent, and female. Approximately 35 articles were selected for review for this report. Authors reviewed articles within their particular area of content responsibility. Personal communications with several sports medicine experts were also conducted. Twenty-three articles were selected for inclusion, in addition to information obtained from the American College of Sports Medicine and National Collegiate Athletic Association (NCAA) Web sites. Utilizing these sources, as well as guidance and suggestions from other sports medicine physicians, the authors determined that the NCAA-mandated preparticipation physical evaluation should deliver an overview of the athlete's entire health status. As detailed in this report, it is recommended that the NCAA preparticipation physical evaluation serve as a tool in tracking and assessing both current and past health issues of student athletes. These health issues would include (1) on-field health concerns such as cardiac and musculoskeletal conditions, (2) off-field health concerns (that may adversely impact on-field performance) such as sexual activity and substance abuse, and (3) health issues unique to the female student athlete, such as eating habits, nutritional record, and menstrual history. Primary care physicians should be involved in all preparticipation physical evaluations as they have the necessary expertise to recognize potential problems in these areas [09385].

It has been argued that the risk to athletes' welfare provides the only legitimate ground for restricting the use of performance enhancing drugs in sport. In one paper, it was argued that the idea of "sport", properly understood, provides further reason to impose such restrictions. A balance of excellences' argument is proposed whereby doping is considered objectionable on account of its disrupting the relation between the excellences around which sporting competition is organised. There are reason to restrict the use of performance enhancing drugs in sport not only because of the threat they pose to athletes' health but also because of the threat they pose to athletes' displaying the relevant types of sporting excellence [10267].

It has recently been attacked several arguments generally adduced to support anti-doping in sports, which are widely supported by the sports medicine fraternity, international sports federations, and international governments. It was now shown that his attack does not succeed for a variety of reasons. First, it uses an overly inclusive definition of doping at odds with the WADA definition, which has global, if somewhat contentious, currency. Second, it seriously misconstrues the position it attacks, rendering the attack without force against a
more balanced construal of an anti-doping position. Third, it makes unwarranted appeals to matters that is considered morally “clear”, while simultaneously attacking a position many others take to be equally morally “clear”, namely that of anti-doping. Such an inconsistency, attacking and appealing to the moral status quo as befits one’s argument, is not acceptable without further qualification. Fourth, his position suffers from a general methodological flaw of over-reliance upon argumentation by analogy. Moreover, it is argued that the analogies, being poorly selected and developed, fail to justify his conclusion that the anti-doping lobby lacks philosophical and moral authority for its stance. These issues are symptomatic of a more fundamental problem: any attempt at providing a blanket solution to the question of whether doping is morally acceptable or not is bound to run up against problems when applied to highly specific contexts. Thus, rather than reaching any particular conclusion for or against doping products or processes in this article, we conclude that an increased context-sensitivity will result in a more evenhanded appraisal of arguments on the matter [10420].

There is currently much concern over the use of pharmaceuticals and other biomedical techniques to enhance athletic performance – a practice we might refer to as doping. Many justifications of anti-doping efforts claim that doping involves a serious moral transgression. In one article, it was reviewed a number of arguments in support of that claim, but show that they are not conclusive, suggesting that we do not have good reasons for thinking that doping is wrong [10536].

It was thus recently attacked several arguments generally adduced to support anti-doping in sports, which are widely supported by the sports medicine fraternity, international sports federations, and international governments. It was shown that his attack did not succeed for a variety of reasons. First, it uses an overly inclusive definition of doping at odds with the WADA definition, which has global, if somewhat contentious, currency. Second, it seriously misconstrues the position it attacks, rendering the attack without force against a more balanced construal of an anti-doping position. Third, it makes unwarranted appeals to matters Kious considers morally "clear", while simultaneously attacking a position many others take to be equally morally "clear" namely that of anti-doping. Such an inconsistency, attacking and appealing to the moral status quo as befits one’s argument, is not acceptable without further qualification. Fourth, his position suffers from a general methodological flaw of over-reliance upon argumentation by analogy. Moreover, it is argued that the analogies, being poorly selected and developed, fail to justify his conclusion that the anti-doping lobby lacks philosophical and moral authority for its stance. These issues are symptomatic of a more fundamental problem: any attempt at providing a blanket solution to the question of whether doping is morally acceptable or not is bound to run up against problems when applied to highly specific contexts. Thus, rather than reaching any particular conclusion for or against doping products or processes in this article, we conclude that an increased context-sensitivity will result in a more evenhanded appraisal of arguments on the matter [10536].

It is in the nature of medical practice that it is always likely to yield ethical problems because of the role that health, illness and injury play in the lives of patients. Sports physicians can find themselves in particularly difficult (though not unique) contexts because of the role of the body in athletic performance, especially at elite and professional levels. Such difficulties as arise in sports medicine merely reflect the kinds of challenges and dilemmas (e.g. confidentiality, conflicts of interest, consent, disclosure, working with vulnerable populations) as are found in other branches of medicine, though not necessarily in precisely in the same configurations. One common professional response to the recognition of ethical demands and professional ambiguity is to establish codes of conduct such as those published by the American College of Sports Medicine (ACSM), the Australasian College of Sports Physicians (ACSP), the International Federation of Sports Medicine (FIMS) and the Faculty of Sport and Exercise Medicine (FASEM). Within the literature of applied ethics, sports ethics and sports
medicine ethics, the limitations of these codes as instruments of education, guidance and punishment have long been noted. One of the merits of the ACSP code that the article draws attention to is the articulation of standards of expectation, differentiating among other things between those actions that are, on the one hand, compulsory from exhortations to best practice on the other. One of the standard criticisms of any code of professional ethics, which has often been overlooked in discussions of sports medicine ethics, arises from the notion of scope of application. To whom do the rules apply? Sports medicine is not unique in being professionally fractured along disciplinary and geographical lines. The idea of a universal code, an Esperanto of sports medicine ethics, is almost certainly a pipe dream. Nevertheless, what the sport physician can and should do is to achieve the greatest clarity possible about the precise boundaries of their roles before engaging their services in order, first, to identify potential conflict and, second, to agree upon a clear set of priorities with the relevant parties concerned: athlete patient, Club/Team/Federation/ event organiser and so forth. It is now proposed a model of the nature of professional relations between sports physicians, athlete patients and other institutions for whom they offer paid and unpaid services (such as Clubs, Teams and National Governing Bodies) [11550].

Deterring those who in sports would be tempted to cheat and catching those who do are essential components of the current anti-doping strategy. But one should never lose sight of the ultimate goal – to protect honest athletes from losing to less talented and less dedicated competitors who use surreptitious biomedical shortcuts such as testosterone, synthetic anabolic steroids, growth hormone, EPO, stimulants or other means to gain an unfair advantage. The ethical case against drugs in sport is strong and straightforward. As long as drugs are prohibited, athletes who choose to rely on them are seeking an unfair edge over their honorable competitors. If some athletes are using drugs, all those lining up against them either have to accept the disadvantage, drop out of the competition or give in and use whatever they believe their competitors are using. Antidoping is intended to create a superior option – compete drug-free with reasonable confidence that the athletes you face are doing the same. Whenever that goal is reached, athletes are free to develop and display the particular forms of human excellence their sport embodies. Talent and dedication then have their proper place in determining who succeeds on the field of play. Perhaps this is an unconventional way to think about drug testing, but the athlete may rejoice every time an honest athlete receives an “all-clear” from the laboratory. In the end, these are the people whose integrity it is wanted to publicly affirm: the competitors whose natural gifts and hard work we believe should decide who wins and who loses [12482].

Present day elite sport culture is now less about health, courage, community and solidarity, or even character and education, fun or joy. The majority of athletes presumably believes and strive for ethics, fair play, honesty, and most are mindful of respecting themselves, the other participants, and showing teamwork, and will not knowingly break the rules and the law. While few would disagree that elite athletes epitomise the virtues of dedication and commitment, their ultimate and fundamental motivation is ultimately excellence in sporting performance [12015].

With one recently recommended gene therapy in Europe and a number of other gene therapy treatments now proving effective in clinical trials it is feasible that the same technologies will soon be adopted in the world of sport by unscrupulous athletes and their trainers in so called ‘gene doping’. In this article an overview of the successful gene therapy clinical trials is provided and the potential targets for gene doping are highlighted. Depending on whether a doping gene product is secreted from the engineered cells or is retained locally to, or inside engineered cells will, to some extent, determine the likelihood of detection. It is clear that effective gene delivery technologies now exist and it is important that detection and prevention plans are in place [13761].
The use of performance enhancing drugs among elite athletes has been in the headlines recently, particularly with Lance Armstrong’s fall from grace and his admission about widespread doping. Many argue that the use of drugs confers an unfair advantage and is ultimately dangerous to the health of the athletes. Others argue that the use of drugs is no different from other techniques employed by athletes to boost their performance: swimmers shaving their body hair; skiers wearing sleek body armour; archers and shooters having laser eye surgery to improve their accuracy. It has been put forward the provocative argument that since “there is no acceptable proof (that) drugs improve competitive performance and their use is no different from accepted sports practice, banning them is wrong and immoral”. Others argue the other side, that the use of performance enhancing drugs poses a significant risk to the health of athletes and perhaps more importantly, “threatens to undermine the very purpose of sport” by disrupting the “balance of excellences” [13762].

The history of anti-doping ethics

Sport physicians may become invested heavily in the success of their athletes, and even when there is no direct or indirect financial gain, the sport physician may push the boundaries of good medical practice. The ethical dilemmas of the sport physician with regard to return to play and conflicts of interest have been described elsewhere and this commentary will focus on the issue of doping. Over the years, physicians who were involved in the doping of athletes claimed that they were monitoring properly the athletes and simply assisting the body’s efforts at homeostasis. The argument was that if testosterone levels were low from overtraining or heavy competition, it could be boosted back up to “normal” levels. Fatigue in the setting of multiday endurance events could be cured with a small blood transfusion or the judicious use of erythropoiesis stimulating agents. These doctors emphasized that their athletes were monitored strictly, and thus, any medical risks were minimal. “Only with adequate safe controls can anabolic steroids be viewed in the same light as antibiotics, anti-asthmatic and other medications used in daily life,” stated US physician Robert Kerr in 1982, 8 years after androgenic anabolic steroids were banned. He eventually reversed his position upon the realization that athletes often pushed drug use to the extremes. This conclusion should not have come as a surprise as illustrated by the British cyclist Tommy Simpson who died on Mont Ventoux during the 1967 Tour de France while under the influence of amphetamines and alcohol. “If ten pills will kill you, I will take nine,” he reportedly said, not calculating for conditions of extreme heat and dehydration [13763].

The contested opinion of the relative harms of doping substances continued to be expressed. In 1994, Italian physician Dr Michele Ferrari was quoted in L’Equipe, “EPO is not dangerous, it is the abuse that is. It is as dangerous to drink ten litres of orange juice’. Some physicians believed that they were operating in a gray zone of acceptable medical practice and in some historical contexts that may have been an argument. For example, in the early days of blood transfusions, there was little concern over the ethical implications as it was seen to be an extension of a training method rather than ingestion of a substance. At that time, the concept of a prohibited method had not been described in antidoping. Although the first study on blood transfusions was in 1945, this method became more investigated widely in the 1970s at which time athletes began to see the performance-enhancing benefits. A steadying clamor arose to ban this practice for the same reasons as other substances are banned for the health of the athletes and the fairness of the competition. This debate was summarized nicely in 1982 by Dr. N. Gledhill, who stated that although in athletes with low hemoglobin levels the use of blood boosting could be rationalized as being therapeutic, it was nevertheless analogous to the use of physiological substances to gain an artificial and unfair
increase of performance. Therefore he recommended that the IOC prohibit it. Following the use of blood transfusions in the 1984 Los Angeles Olympics, which was publicly acknowledged by the US Cycling team, the IOC Medical Commission made this the first-ever prohibited method. An increasing understanding of this and related prohibited methods developed over the years and is now defined in the World Anti-Doping Agency (WADA) Prohibited List under Section M1; Manipulation of Blood and Blood Components [13763].

**War on doping versus war on drug**

The current anti-doping policy (“war on doping”) resembles the “war on drugs” in several aspects, including a zero-tolerance approach, ideology encroaching on human rights and public health principles, high cost using public money for repression and control, and attempts to shape internationally harmonized legal frameworks to attain its aim. Furthermore, even if for different reasons, both wars seem not to be able to attain their objectives, and possibly lead to more harm to society than they can prevent [12012].

*Doping is here to stay*

There has been a distinct trend of doping in sport for more than the last 50 years, a trend that has culminated in the current status quo that exists between the antidoping authorities and athletes that choose to cheat in this way. Some have described this status quo as a “cat-and-mouse” scenario in which the doping authorities are always one step behind the dopers. No sooner is a detection method proven to be successful than the doping athlete moves on to ever-more sophisticated substances and methods. This may be a rather simplified way of looking at the problem, but it is not wholly inaccurate. What is also clear is that there are no signs of this doping trend coming to an end. Indeed, we are probably now well beyond the stage where we should be referring to doping as a “trend” we now need to completely accept it as another of society’s ills, just as we accept that there will always be those who cheat the system in other aspects of our society [12034].

*“Everybody take doping drugs”*

Indeed, some have argued that we should stop trying to catch the cheaters of doping, and allow athletes to use whatever substances they like. According to their position, the athletes are only harming themselves. However, this view reflects a common misconception that steroid use is restricted to elite athletes. Instead, steroid use has infiltrated high school sports and neighborhood fitness centers. Thus, before it is accepted unregulated steroid use, it is important to acknowledge how widespread the problem is. The argument that “everyone's doing it”, does not take into account who “everyone” is [12100].

**Arguments for doping and their ethical anti-arguments**

The claim that carefully monitoring and modifying an athlete’s physiology with prohibited substances or methods are acceptable professional behavior is a specious one. Although the risk to particular athletes may be low with some doping substances or methods, their use distorts the notion of a level playing field and sense of fairness that reverberates through all ranks of sport. Moreover, developing athletes may come to believe it is acceptable to engage in risky (and often unmonitored) behaviors to emulate the stars and/or attempt to reach the elite level of their sport. The argument that elite sport itself is risky and in some cases far more dangerous than the practice of doping also is found wanting. Danger may be an
inherent element in some sports, and although sport federations often wrestle with the rules to improve safety, there are not many who believe that allowing doping is desirable. Although there are no large-scale social science studies, most athletes accept and encourage the application of antidoping rules. There is certainly a need to continue academic debates on what should or should not be prohibited and how best to apply antidoping rules. There are elaborate processes for stakeholders, as well as individuals, to suggest changes to the WADA Prohibited List as well as periodic revisions of the World Anti-Doping Code and Standards. Nevertheless the point remains; once a substance or method is prohibited, there can be no justification for a physician to assist or administer this to an athlete [13763].

Sport physicians will always be challenged by difficult ethical/medical decisions beyond those related to doping, and not all issues can be resolved by verifying whether the substance is on the Prohibited List. For example, the use of local anesthetics (not prohibited) in different clinical/sporting situations as well as other return-to-play issues may be quite complex. “Do no harm” is still the ultimate maxim to guide physicians, but this principle must apply to the collective health of the athletes as well as to the individual. Physicians and other responsible leaders need to promote respect for the spirit of sport and the integrity of the rules, which exist to promote fair play and the health of the athletes [13763].

The zero tolerance ban on doping has failed. The second fastest runner ever, the American Tyson Gay, tested positive for a banned substance, along with Jamaican sprinters Asafa Powell and Sherone Simpson. There is evidence of widespread doping across many sports including athletics, tennis, and cycling. Evidence from Germany suggests doping is rife in football. Despite apparent advances in the “war on doping,” the success in detecting drug misuse is limited. In 2000, the first tests for erythropoietin were introduced [13764].

Human nature

It appears it was reached the limits of human performance in sprinting about 15 years ago. Starting with Ben Johnson in 1988, only 10 men have ever run under 9.8 sec. Only two (including Usain Bolt) are currently untainted by doping. To keep improving, to keep beating records, to continue to train at the peak of fitness, to recover from the injury that modern training inevitably inflicts, athletes need enhanced physiology. We have exhausted human potential. But, to be human is to be better, and doping is not going to go away [13764].

Regulation could improve safety

The strongest argument against doping is safety. Since there have been no scientific tests of the effects of doping in healthy athletes, there are few good data available. Some have pointed to deaths of athletes as proof, but there is little evidence to attribute many of these to doping. In fact, a recent study shows French Tour de France competitors (1947-2012) had 41 percent lower mortality than the French male population as a whole. Modern doping with anabolic steroids, growth hormone, erythropoietin, and blood can be tightly monitored and, as we put together the evidence, safe limits set. An indication of their likely safety is that most current doping agents are routinely used for patient care under medical supervision. For example, growth hormone is given to children who have normal levels but who are a certain amount below the height expected for their age [13764].

Pushing humans way beyond what is physiological can have ill effects, as the East German experiments with steroids in the 70s showed. On the other hand, extreme exercise itself depletes natural levels of red blood cells, testosterone, and other hormones. But testosterone and growth hormone can all be increased within physiological endpoints (which still
constitute doping) safely under medical supervision with clear, well understood risk-benefit profiles. Anything is dangerous if taken to excess. Caffeine, a legal and popular performance enhancing substance, has been linked to deaths and dangerous overdoses. Yet it is consumed by both athletes and the general population, including children, as a performance enhancer, usually safely. Moreover, there is no such thing as risk-free sport, or life. We need a balance between the values of safety, human contribution and participation, enforceability, and spectacle. Elite sport is itself risky. Around 20 percent of professional riders starting the Tour de France do not complete, many because of crash injuries. Since 1980, at least 21 cyclists have died during competition. We should assess each substance on an individual basis. We should set enforceable, fair, and safe physiological limits. For example, blood doping and the use of erythropoietin could be dealt with at a stroke by allowing doping up to a blood cell count of 50 percent [13764].

*Spirit of sport*

A second objection lies in the nature of the intervention. If a substance came to dominate or corrupt performance, there would be good reason to ban it. For example, if drugs cause boxers to feel no fear or beta-blockers cause archers and pistol shooters to have steady hands, they should be banned because overcoming fear and tremor are integral to these sports. But if a substance allows safer, faster recovery from training or injury then it does not corrupt sport or remove essential human contribution. Indeed, analgesics and anti-inflammatory drugs are already widely used to enhance performance after injury, in competition and out. That is more unnatural and probably more dangerous than physiological doping. Athletes are using many doping products to optimise their physiology, just as they do with diet, fluid, and glucose management. Cyclist Tyler Hamilton claims in *The Secret Race* that he lost a race because he did not take a 100 calorie energy gel at the correct time (despite the fact he was also doping). Will allowing elite athletes to take drugs under medical supervision encourage children and amateurs to imitate their heroes? Again, the current ban fails this test. Amateur doping is already happening in an unsupervised manner. There is doping at college, and it is estimated that 3-5 percent of school athletes use doping. It is better to send the message that you can safely enhance physiology with a doctor when you are an adult. Many practices that have risks if taken to excess, or carried out recklessly, like driving a car or drinking alcohol, are banned for children. Over time the rules of the sport have evolved. They must evolve as humans and their technology evolve and the rules begin to create more problems than they solve. It is time to rethink the absolute ban and instead to pick limits that are safe and enforceable [13764].

*Argument against doping in sports*

The argument against doping in sport is moral, not medical. If performance enhancing drugs were no longer prohibited in sport, then being a talented sportsperson would rapidly become a dangerous occupation. Within weeks of the decision, it would no longer be a choice of whether to take performance enhancing drugs or not. You would either take them and stay competitive, or refuse and retire. Athletes who wanted to live a healthy existence would be pushed out altogether. Soon, the only competition that would matter would be the one to develop the most powerful drugs, and athletic opponents would enter into an exchange of ever escalating doses to stay ahead of each other. In a supposed attempt to level the playing field the exact opposite problem would be created. Only rich and powerful nations would have access to the best technologies, meaning the gap between the privileged and poor would actually widen [13015].

*Escalating problems*
In some nations we might see a return of the state sponsored doping programmes of the 70s and 80s. We still have many countries with totalitarian governments and dictators who show scant compassion for their citizens. Such governments would exploit and abuse their best talent from childhood to create centrally coordinated doping programmes. Politicians would bask in the reflected glory of their gladiator champions, who would later die young. Some from the pro-doping camp argue that the cheats are always one step ahead and therefore the anti-doping programme serves only to give the smartest cheaters an advantage. However the anti-doping programme also serves as a useful lid on the pressure cooker. Without it, the use of performance enhancing drugs would expand exponentially and filter deeper into our society. Some cheats are never caught, but they may still pay a heavy price for their doping. Anabolic androgenic steroids create a deleterious lipid profile (reduced high density lipoprotein cholesterol, raised low density lipoprotein cholesterol), leading to premature atherosclerosis and the risk of premature cardiac death. Much media suspicion surrounded the death of Flo-Jo, sprint queen of the Seoul 1988 Olympics. Provisional reports of a “heart seizure” became epileptic seizure in the final autopsy. Erythropoietin is not risk-free either, with many anecdotal accounts of high blood cell counts leading to sudden cardiac death. Our health systems are already creaking under the pressure of alcohol, smoking, heroin, and cocaine related illnesses. Do we really want to add to this the problems that performance enhancing drugs could create? [13764].

Engineered athletes

Legitimising performance enhancing drugs in elite and professional sport would change the message sport sends to society. A meritocratic society is one in which success is proportional to effort and ability. Sporting success is generally achieved through positive attributes such as diligence in training, effort, and self denial. Athletes inspire us because we appreciate that they got where they are through hard work. Would a bioengineered athlete be able to inspire in the same way? Like the case for a Formula 1 champion (where it is impossible to tell whether success is due to the car or the driver), it would be impossible to separate the effects of the drugs and gene doping from the human element [13764].

Bans can work

Some have argued that because we will never be able to catch every cheat, we should give up trying. The answer to futility is not to give up, however, but to make the anti-doping system more effective. In the words of the Irish political philosopher Edmund Burke, “All that is needed for evil to triumph is that good men do nothing.” So how do we make the system less futile and the deterrent respectable? Recently an area of mathematical study known as game theory has been proposed to have some of the answers. Game theory is the study of how players choose strategies to maximise their own return, in anticipation of what their opponents will do. Currently drug testing covers only a random and incomplete sample of competitors. Therefore inevitably some will elude detection through chance alone. Game theory dictates that the only rational logic is to match your rival’s anticipated strategy – to cheat yourself. However, if testing was ubiquitous, it would be virtually impossible to evade detection, and the equilibrium would be reset in favour of not cheating. Another application lies in relation to the duration of drug bans. Currently a first offence generally only leads to a two year ban, and it is very hard for sponsors and promoters to reclaim their prize money. Many athletes are prepared to take the risk of getting caught, followed by serving a soft sentence. Evidence from a retrospective cohort trial suggests that anabolic steroids continue to enhance performance for years (8 ± 3) after a washout period, so an athlete is able to return to his or her sport legally yet still gain a competitive advantage. In such circumstances, lenient sanctions are not an effective deterrent to doping. Game theory suggests that increasing the risks associated with cheating would greatly reduce its prevalence. To dope is
What justifies anti-doping?

Because of the problems of current anti-doping policy the question arises as to what the reasons for the anti-doping endeavour are. The main justification for anti-doping is formulated as follows in the 'Code', the central document published by WADA outlining anti-doping: “Anti-doping programs seek to preserve what is intrinsically valuable about sport. This intrinsic value is often referred to as “the spirit of sport”, it is the essence of Olympism; it is how we play true. The spirit of sport is the celebration of the human spirit, body and mind, and is characterized by the following values:

- ethics
- fair play and honesty
- health
- excellence in performance
- character and education
- fun and joy
- teamwork
- dedication and commitment
- respect for rules and laws
- respect for self and other participants
- courage
- community and solidarity.

Doping is fundamentally contrary to the spirit of sport.” According to WADA the spirit of sport is thus the celebration of the human spirit, body and mind. The reasons advanced against doping are that it skews a level playing field, can threaten the health of the athlete, is against the spirit of sport, and incompatible with the concept of the athlete as a role model. All these arguments are problematic. Elite sport is by definition a non-level playing field since it is about the celebration of differences. The protection of the health of the athlete argument is paternalistic and neglects the health hazards of sport itself while the distinction of avoidable and unavoidable risk is flawed. Moreover, the spirit of sport argument is fuzzy and fraught with problems, and the role-model argument is out of perspective as compared with any other role model in society. Anti-doping policies in sports have created an image of an idealized 'perfect' human. Obliging athletes to correspond to this ideal appears unfair compared to what is asked of other citizens [12012].

Given the fact that in spite of the increasingly repressive means employed to combat doping, athletes are regularly caught – while others probably get away with it – the question arises: why do athletes continue taking the risk? The underlying question is whether current anti-doping policy in sports, striving for a world free of doping in elite sports, is commensurate with how human (doping-) behaviour is determined. Using a point of view from a sociocultural perspective, but interpreting behaviour as partly determined by our evolutionary past, it may be suggested that doping in athletes is perfectly natural human behaviour. Given what modern sport is today, i.e. an important entertainment enterprise in which large amounts of money go round, strongly biased towards the celebration of winners and applying the general concept of ‘winner takes all’, it is not surprising that athletes can be drawn to whatever
promises an edge in competition. The official credo of the Olympic movement is “Citius, Altius, Fortius”, or faster, higher, stronger. It is often completed by the phrase “The most important thing is not to win but to take part.”. But the latter is not reflected by reality. Today an important tenet of elite sport is the celebration of the winner. This puts a lot of pressure on athletes, who are perpetually seeking the competitive advantage. As Petroczi states: “using doping agents may be more of a rational, outcome optimizing behaviour than deviance”. The ingredients to be a champion are a combination of talent, hard work and some luck. Talent is a licit, albeit unmerited gift and results from the genetic lottery. Hard work is essentially training and using any other means that are allowed to improve performance. Performance enhancement is a logical and essential ingredient of competitive sport. Athletes look for ways to get better, by changing their training paradigm, by eating differently, by taking vitamins, by taking licit medication, by taking supplements. A huge sports supplements industry exists and it is very common for athletes to consume a lot of substances that are not on the list of forbidden substances. Many of the supplements do nothing, a few have an effect, but quite many may pose doping problems because of adulteration, leading to accusation of doping because of positive blood or urine samples or health problems because of excess intake of some compound, erroneously seen as innocuous. Modern sport puts athletes under enormous pressure to win and the use of licit substances and methods to improve performance is explicitly encouraged. The line between licit and illicit fluctuates and has dimensions that can be perceived as arbitrary. Transgression of doping rules is not necessarily accompanied by a fundamentally different mindset as when keeping to the rules. In athletes’ minds, doping may align with illicit behaviour or with functional licit use of chemical or natural preparations [12012].

Alternatives to current anti-doping strategy

Some suggestions for possible strategies have been given. To begin with, the concept of performance enhancement by means of methods or substances, including pharmacology, should be seen as a logical consequence of elite sports endeavour and not be negated by a utopic ideological “spirit of sport” concept. Second, the health of elite athletes should still be protected, but taking into account the specificities of this risky profession (some sports come with a level of risk not acceptable in other professions). This can be done by continuing some form of testing, without going all the way as in today’s testing. For example, a no-starting rule for a haematocrit above a given level, however the way it got to that level, is a pragmatic way to prevent excess use of red cell mass stimulation regimes that lead to a health hazard. Sure enough, athletes will find ways to cheat a bit around such strategies, but that would be part of the game while keeping the problem within acceptable boundaries by associating such a rule with some other rules like the exclusion of plasma expanders, if warranted. The argument that it would change sports into an arena akin to Formula 1 where the best engineering team wins is only partly correct. It will still take talent, a lot of hard work and some luck to become a champion. And then, it is likely that such a scenario is already in place anyway; today well-assisted athletes may engage in complex training regimes and strategic doping while remaining undetected. Third, the list of forbidden substances can be shortened, leaving on the list only those substances with actually proven performance enhancing effects and major health hazards. For example, cannabis derivatives can be taken off the list, allowing athletes to be dealt with in the same way as the general population. The current arguments to keep cannabis on the list are flawed. There are no well-controlled trials that show any performance enhancing effect, while there is evidence for performance decreasing effects [12012].

With regard to the general population, instead of a crackdown on steroid users in gyms and fitness clubs with compulsory testing as in Denmark, a harm reduction approach is probably better. This has already been shown in the UK where so-called steroid clinics, giving out clean syringes and thus lowering the threshold to medical care, have led to the number of
syringes handed out now outnumbering those exchanged for injection for psychotropic drugs. These clinics, offering mostly free and anonymous services, make it possible to reach a previously hidden population. Potential advantages of providing harm reduction measures, besides health benefits, include the personal and direct contact with a hidden population allowing it to be informed of the risks and dangers of doping substances, and helping to take well informed decisions whether to continue use and if so in what way. In Switzerland the federal commission on drug-related affairs developed a conceptual model (‘the cube’) considering that for every substance, with different risk profiles, different levels of use exist (non-problematic, problematic, dependence), needing different levels of intervention at the level of prevention, treatment, harm reduction and regulation. Such a model might help to conceptualise alternative policies on performance enhancing substances respecting public health and ethical principles [12012].

One would have to distinguish between elite athletes, amateur athletes, minors, gym users and the public in general, since it can be expected that the answers to these questions would vary between groups. It is impossible to predict what would happen in these different groups. In elite athletes one might expect limited harm since medical supervision and health oriented testing would constrain the possibilities. In amateur athletes the possibility of refraining from sourcing substances from the black market and having access to general information and proper methods of use, might perhaps have a positive effect. It will of course be difficult to devise an optimal strategy to regulate for children specifically. Since athletic careers often start very early, the protection of young talents would be mandatory. Alternative policies should of course be continuously and extensively evaluated for desired outcomes and unintended negative consequences, carefully balancing the two [12012].

A continuing discussion is needed

Even if sufficiently complete and accurate data on the negative aspects of anti-doping policy are still lacking, it is suggested that the taboo on debate and reform also be broken in this field, now and not in 40 years. It may not be ignored the side effects of current anti-doping policy for society in general. We suggest experimentation with and evaluation of alternative ways of dealing with doping in elite (and amateur) sports, inspired by the experience gained with alternative drug policies, that are scientifically sound, respect human rights and public health, and treat athletes as ordinary humans and not as potential criminals. The modern globalised sport entertainment industry with its almost unlimited financial means should not be allowed to hijack worldwide legal frameworks and orient society towards a zero-tolerance approach to both psychotropic and performance enhancing substances. Those who are advocating harm reduction strategies to help society live with psychotropic drugs at the lowest cost for the individual and the community should therefore be concerned about these developments [12012].

The reasons for anti-doping rules

The considerations of devising bans on drug use in sports have been based on the following Considerations [06006].

- Doping in sports is cheating and unfair. It can be argued that doping should be banned because it is cheating or unfair. The problem with this position is that usually an activity is considered “cheating” or “unfair” only when there is a rule prohibiting it. Without the rules, there may be no issue of cheating and unfairness. Others have suggested that an action can be inherently unfair even if there is no rule prohibiting it,
but a sport is inherently a rule-driven enterprise and without rules, questions of inherent unfairness and cheating would be very difficult to resolve in sports.

- Doping harms the athletes. Here the suggestion is that doping should be banned to protect the athletes who dope. It may be argued that this rationale is paternalistic, inconsistent, and incomplete. It is paternalistic because we do not generally permit intrusion into the lives of competent adults under the guise of protecting them from perceived harms. It is inconsistent because a sport, in particular an elite-level sport, is often a hazardous enterprise. It is not clear why athletes should be protected from the harm that doping may inflict when we do not protect them from possible dangers of the training and performance of these same sports. The argument is incomplete because evidence for harm in doping is mixed. For example, while steroids in high doses may cause adverse side effects, steroids in relatively low doses probably do not. Autologous blood doping has not been shown to have any adverse side effects.

- Doping harms even nondoping athletes. The argument based on the interests of other “clean” (nondoped) athletes is that doping can be coercive. Because doping may improve results, there is coercive pressure “to keep up” placed on those who wish to compete without doping. This argument has some merits, but is incomplete because an elite-level sport is already highly coercive. For example, when full-time training, attitude training, or diet control are shown to produce better results, everyone is forced to adopt these measures to keep up. It is unclear why doping is any more coercive and sufficiently so to warrant being banned than say, training 6–8 hours a day. On the other hand, the coercion argument has merit if it can be shown that doping detracts from what is important to sports. If sports, sporting excellence, and sporting contests are about testing skills, then it can be argued that the improved performance that comes with doping is not about that test of skill. If doping does not enhance the development of sports and sporting excellence, we can choose to reject it as being unnecessarily coercive, as compared to, for example, extended training that may improve one’s skill at the contest at hand.

- Doping harms society. This position is based on the assumption that doping harms others in society, especially children who see athletes as role models. This argument works in two ways. One, if children see athletes having no respect for the rules of the games they play, there will be a tendency to undermine respect for rules, and law, in general. The second version sees doping and athletic drug use as part of a wider social problem of drug use. The argument here is that if children see athletes using doping to attain sporting success, then other drugs may also be seen as a viable means to other ends. The limitations of this argument are that there are many things that we consider appropriate for adults but not for children. Alcohol and cigarettes are obvious examples, as is sex, and although we attempt to control these behaviors, we do not generally ban these substances or activities for adults because of their potential harmful effect on children.

- Bans must be enforceable and therefore require complex infrastructures for fair and just implementation. Many layers of intrusive and expensive testing and monitoring must be established to detect instances of forbidden drug use both in-competition and out-of-competition, to evaluate and judge the results of investigations, to provide mechanisms for appeal and to levy and enforce punishment – fines, suspensions and even at times criminal punishment. It is also necessary to develop and implement programs for responding to proven instances of doping – suspensions from competition, fines, etc. All these are expensive and potentially intrusive into the private lives of the athletes.

- Doping represents a perversion of sports. It converts the beauty of sports, the glory of striving and achieving and outdoing physical limitations into “mere” biotechnology – into rewards for the most clever biological and genetic engineer instead of the swiftest, the strongest. Sport is a social construct that relies on an agreement on the
part of all to abide by sets of rules, no matter how arbitrary, that are the agreed underlying principles. Taking part in organized sport is, contrary to some views, not a human right but rather a voluntary act by athletes who, by the simple act of agreeing to participate, agree to the rules and restrictions of that sport. Of course, one of the human rights that athletes must be allowed to retain is the right to refuse to take part in doping control. No one, athlete or not, should be forced to accede to intrusive monitoring and screening programs without permission and without giving fully informed and voluntary consent. But if an athlete wishes to refuse to accede to doping control measures, he or she relinquishes the privilege of taking part in the sports competition.

- Doping is unnatural and dehumanizing. The unnaturalness argument does not get very far for two reasons. First, we do not have a good account of what would count as "unnatural." Second, we are inconsistent. For any account of "natural" and "unnatural," few things designated "unnatural" would be permitted (e.g. spiked shoes) while other "natural" ones (e.g. testosterone) are on the banned list. The dehumanization argument is interesting but incomplete, since we do not have an agreed-upon conception of what it is to be human. Without this it is difficult to see why some practices should count as dehumanizing. There is also a problem with consistency. Some practices, such as "psychodoping," the mental manipulation of athletes using the techniques of operant conditioning, are not banned, whereas the reinjection of one’s own blood is. There is an attempt later to provide some framework for defining human excellence, thereby allowing us to see how the pursuit of athletic excellence can, and should, be limited in ways that exclude doping from the pursuit of sports.

**Sports as part of the society**

An additional facet is the greater willingness to recognize the broader use of illicit substances in the society. It is noted that many users are not elite athletes at all, but young people who are preoccupied with body image. This point alludes to the relevance of broader cultural studies of body modifications when considering the use of enhancement technologies in sport. While it is tempting to believe that the rationale for any athlete’s use is merely to gain an edge over other competitors, other values are at stake. Yet, related studies of the cultural context of performance enhancement are often overlooked in the debate about the ethics of sporting performance. One could also be skeptical of the claim that society broadly is unhappy about enhanced athletes. Rather, one might more adequately claim that the media discourses surrounding the doped athlete generate a justification for a culture of anti-doping. The discussions on the ethics of hypoxic chambers in elite sport demonstrate how technology gives rise to a mixed reception and that the ethical stance taken by athletes or lay spectators or sports fans is malleable. The suggestion that sport enhancement issues are converging with broader medical enhancement debates is reflected in the activities of key legislative agencies and advisory committees. For example, the Australian Law Reform Commission in 2003 published an extensive document on the use of genetic information within a range of social contexts, one of which includes sport. Also, the UK Government Select Committee for Science and Technology launched a public inquiry into the use of Human Enhancement Technologies in Sport in 2006. To this extent, it is useful to employ a convergent metaphor in the analysis of converging legislation surrounding human enhancement technologies. Nevertheless, of critical value is to understand following section outlines various forms of technology within sport, which establishes a critical response to how technology is framed by sports authorities as a diminishing influence. In short, there is no ethical view “out there” that can justify the current approach to evaluating the role of
technology in sport [06168].

**Doping as a sign of dehumanization**

The are concerns about doping in sport also reveal a rhetoric of “dehumanization” in sport, where technology might reduce the athlete’s role in performance and, in so doing, diminish the value of competition. This view of dehumanization also emerges from a “mechanization” thesis that describes the scientification of sport as bringing about feelings of alienation: the manufacturing of athletes. Such an evaluation of contemporary, elite sports, may describe the athlete as a product of a scientific or technological process, somehow automated in performance. Thus, one might describe a sense of anxiety over tampering with biology on a global scale. Yet, even in these cases, it is unclear why such tampering should matter morally. A further example that raises questions about whether there is a broad social concern about enhancement technologies is *cosmetic surgery* (or more broadly body modification). Very little is known about whether athletes would use elective reconstructive surgery for sports performance. While one might consider aesthetic interventions given the importance of gaining sponsorship within the sports world, one might also envisage other surgical procedures that could enhance the body. Indeed, there is some evidence of athletes undertaking risky, experimental surgical procedures when injured, hoping that their ability will be restored [06168].

It may be put into question whether the human athlete or the technology has achieved the performance. However, to answer such a question requires being able to make clear distinctions between each. This category presumes that something clear can be said about humanness that is lessened or removed by the use of some technology. Nevertheless, the purpose of this categorization can be to demonstrate ideas about the moral implications of technology so as to identify the kinds of argument that are being made about the effects of technology. While various authors discuss how these technologies alter what it means to be human, adding content to such claims is more problematic as identifying the salient characteristics of humanness that are removed or lessened by such technology is not easy [06168].

**Making sport possible**

An initial category of effect for technology involves the constitutive function of technology within sports. The category raises questions about the politics of defining technology, since it reminds us that sports have always been technological and the moral evaluation of this relationship shifts over time. Technology (primitive or sophisticated, premodern or post, recent or ancient) is unequivocally a necessary characteristic of many sports without which they would not be possible. It is thus, no surprise to notice that, as the technology evolves, so too do the sports. In Formula One motor racing, it is possible to see this most clearly where advances in motor engineering vastly affect the outcome and demands upon a driver and race team. In such a performance-driven sport, the technology has often been argued as being the determining factor of success, where the driver plays merely a secondary role. Yet, such a view would be naïve or, at least, incomplete since even those who reject the most recent advances in technology that they consider to have reduced the skills required to be a driver, no such views would argue for a return to cars from the earlier parts of the 20th century. To this extent, one might describe that the relationship between technology and sports has an optimal limit beyond which something critical about the sports particular character is compromised. One important conclusion that must be drawn from this is to
realize that technologies are not antithetical to sports and that it can only be the way in which they develop – rather than their very existence in sport – that raises ethical problems [06168].

**Technology introduced but changing the sport**

One of the central aims of technological change in sport has been to improve safety and reduce the risk of harm. Many rule changes within sports can be viewed as *technologies of knowledge* that aim to restructure the range of technological interactions – such as the foot against the floor or a shoulder’s movement when swinging a racket. Other examples include the redesigning of the javelin in the 1980s, when athletes were throwing dangerously close to the spectators. The only reasonable solution to this impending problem was to change the specifications of the javelin so that the athletes could not throw it as far. This resulted in a change in the kinds of athlete that were successful as javelin throwers, from the strongest to the technically proficient. Other examples include:

- improved floor surfaces within sports halls to reduce shock to athletes when landing or bounding
- introduction of better helmets in American Football and ishockey to reduce head injury
- more sophisticated shoe design for more support to foot during athletic events
- spring board surface in diving to prevent slip and increase resiliency of board tips to reduce injury
- sturdier epee and foil in fencing, as well, Kevlar jackets for more protection but with no loss to movement
- navigational equipment in sailing
- carbon composite poles in pole vaulting and enhanced safety pits, allowed more daring contest and higher vaults

These examples identify the imperative for sports federations or governing bodies of sport to strive for their practices to be less dangerous for the competitors by introducing new technological measures. Their ethical justification derives from an interest in athlete safety and, generally, allowing the athlete to perform at an optimal level without placing undue stress on the body. However, other examples may be more controversial since their implementation can change the kind of test that is constituted by the competition [06168].

Technological innovations can alter the way that sports are played. They can change the conditions of training that are required to be successful at a particular skill, and can even make it easier to perform the required skills. Examples of such technologies include:

- U-groove golf clubs that allowed greater accuracy on stroke
- depth finders in fishing to make it easier to locate large schools of fish to enhance prospects of catching
- superman cycling position that allowed more streamlined position for greater speed
- breathable clothing material used to regulate body temperature in extreme climates

However, innovation considered to have democratized the skills of a sport may devalue or deskilled the activity. While these devices would seem quite useful for a novice who may require assistance to engage in the activity in a meaningful way, their application to competitive sports is implied – yet, it is unclear that such things are beneficial within elite competition [06168].
Doping must be concerned about the human condition and the degree to which enhancement technology can alter it. The ethical debate must take into account the risks to vulnerable groups, such as children or athletes who enhance because they feel coerced and the liberties of adults who make lifestyle decisions about body modification. Yet, it must also consider the limits of ethical policy making within the world of sport and the relationship of this to broader structures of ethical governance within society. When considering what should be the strategy for anti-doping officials in relation to, for example, gene doping, it is necessary to return to fundamental questions about the value of sport, consider how these values might have changed, and recognize the broader bioethical context within which decisions about medical technology are made. This requires that elite sports organizations reevaluate established systems of rewarding excellence, in order to promote a moral climate in sport that takes into account inherent natural and social inequalities, which are constitutive of sports practices. The conceptual framework of technological effects is useful for:

- establishing how ethical issues arise in the context of technological change
- clarifying the interrelatedness of effects arising from any one technology
- revealing that the debate surrounding enhancement as a doping infraction is only one component of a broader relationship between sport and technology

As human enhancements become a constitutive element of broader social circumstances the concept of enhancement and of the natural human will become even more difficult to sustain. In such a future, sports authorities might still attempt to protect a particular way of life for an athlete, though athletes – as humans – might no longer see either the need or the relevance.

**Increased participation and spectatorship**

One of the major interests of a sport governing body is to maximize the breadth of inclusion within the given sport. This ambition often translates into the development of technology that can allow sport to become more accessible to prospective participants. The example is slightly different from developing technologies to make the sport easier, as the main aim here is the maintenance of standards, with the broadening of participation. Alternatively, equipment is often developed that can even exclude particular kinds of individual from participation. For example, the sophistication of technology may demand a level of finance that is beyond many individuals. Examples of such technology include the following:

- artificial turf for field sports
- U-grooved golf clubs
- carbon composite tennis racquets and mass production of other kinds of equipment
- the carving ski (alpine) that makes it easier to learn skiing
- different-sized tennis balls
- varying speeds of squash ball for different levels of competence.

The benefits of such technology are complex. The ability to reach a wider audience can seem a worthwhile ambition. However, the ends of such ambitions can be problematic for the sport. For example, in sports, such as climbing or skiing, there exist limited natural resources, the overuse of which could seriously damage the environment and lessen the aesthetic experience of the performance. These varied examples provide some basis for understanding the complexity and effect of technologies in sport and the range of values that are engaged when considering the ethical implications of any proposed technological
innovation. In addition to these effects, one must also recognize that there are further concerns about the unknown consequences of new technologies. Indeed, it is crucial to recognize how anti-doping authorities develop policy on the basis of lacking scientific evidence that can demonstrate safety [06168].

Alternate conceptualizations

Within the conceptualizations there is the degree of overlap among the different technological effects. For example, the improvement of floor surfaces within sports halls that can significantly reduce injury and which would thus, fit within the safety category, also reskills the activities. As such, the categorization vastly simplifies any single example of technology within sport and, therefore, does not suitably characterize it. Consequently, it is tempting to draw some further categorization about them in an effort to find some conceptual framework that demarcates technologies from nontechologies. Thus, one might separate them into such categories as body, external, internal, environment, or something similar. It is not reasonable to expect that the categorization alone will yield answers to which ones are acceptable or not. Instead, the reason for undertaking this conceptualization is to reveal the range of technological effects that arise within sport and to demonstrate the range of moral narratives that they provoke [06168].

It has been argued that only virtuous nurturing of natural talents is valued in sports. To this extent, they note that an athlete who benefits from the knowledge of an excellent coach, engages with some form of relationship that implies their interacting. Yet, is such a view a reasonable articulation of the athlete–coach relationship? The athlete will not have undertaken any virtuous sacrifice to access such knowledge. The role of virtuous action here is unclear but doubtful. Nevertheless, if virtue were present here, one would not expect the dismissal of a coach merely due to failure to deliver results. Yet, this is the established ethos of sports practices. To this extent, it is false to suggest that the spirit of sport necessitates that only virtuous action is valued. Consequently, one can accept without controversy that nonvirtuous action—actions lacking virtuous content, rather than unvirtuous acts, such as cheating—can also have value in sport. By proposing a virtue theoretical view of ethics, it neglects other ways in which people value sport—for instance the value of witnessing misbehavior on a playing field. While a response to an ethics position does not reject the claim that “the means” are ethically relevant in sport, it does not accept the notion that only virtuous means are valued. Requiring that any enhancement be earned through virtuous action is too great a requirement, which should not be interpreted as too high an ideal. Nonvirtuous action does not mean that it lacks value [06168].

The appeal of making a clear distinction between the athlete and the supportive system through which an athlete journeys to become elite derives from concerns about athletes’ vulnerability to the political will of such systems. It must be remembered the haunted stories of the GDR (East Germany), where the political value of sporting success gave rise to unacceptable exploitation and manipulation of individual athletes. Moreover, it may be expected that any state-funded program to improve athletes will have such a character. This is more broadly contextualized within views about human enhancement more generally. Without a vigilant permissive environment for human enhancements, this will remain a prospect. As such, the burden must be on critically establishing the conditions through which legitimate human enhancements could be permissible [06168].

Ethical aspects of “harm” in sports
Some of the arguments against all forms of doping, including genetic doping in sport revolve around the various forms of harm that would ensue. At least four types of harm or potential harm can generally be envisioned including:

- harm to users
- harm to other athletes
- harm to society
- harm to the sports community

The most obvious kind of harm would result from the known harmful effects of many of the drugs classically used in sport doping in general and from the scientific and medical unknowns that still permeate the field of gene transfer in human subjects and patients in the gene therapy setting [06304].

**Harm to the athlete**

One of the common ethical arguments justifying a ban on doping in sport is the potential for harm to the athlete, that the athlete requires protection, that such protection could be provided by banning the offending substance or procedure. The assertion that the use of gene transfer technology for enhancement will harm the user is not unreasonable. It seems reasonable to suppose that the likelihood of harm likely to befall a healthy athlete or other healthy subject, taking doping substances or taking part in a gene transfer approach to doping without medical indication, is greater than the likelihood of harm that would befall someone suffering from an illness for which the same agent represents a therapy. At least the ratio of harm to benefit would be far greater in normal young subjects than in ill patients [06304].

The desire to act on behalf of others or to protect others from the consequences of their own actions is known as paternalism. Obviously, in the case where potentially injurious actions are being undertaken by minors or people who are otherwise medically and medico-legally incompetent because of illness, age, or injury, paternalism is acceptable, legal, and ethical. Virtually everyone can accept the need for drug and genetic regulation and even banning of doping by minors. But most athletes are not incompetent; most are adults who are fully competent from all ethical and medico-ethical perspectives. Although most elite athletes are competent adult athletes, some argue that banning doping by them could be considered a form of acceptable paternalism — an attempt to protect the health or well-being of a competent adult athlete or to prevent some of the other forms of harm that result from doping. On the other hand, paternalistic interventions in the lives of competent adult athletes can be seen by others to be unwarranted — that others know better than the athletes themselves how to achieve a more general good that could deny for them the very attributes that we so highly value: self-reliance, personal achievement, and autonomy. But of course the flaw in that argument is that sports ethics is not the same as medical ethics. Drug bans are meant not only to protect athletes from harm but also to preserve the concept and ethos of sport [06304].

It can therefore be concluded that the argument in favor of drug bans based largely on the need to protect athletes from harm is an important but an incomplete justification. However, in the case of a technology as immature as gene transfer approaches to sport doping, we find a particularly convincing argument of athlete protection. The state of the technology underlying this approach to doping is extremely immature and full of recognized and even more unrecognized dangers. Autonomous choices are autonomous only if they are enlightened and that a gene-based doping application under the current conditions of
inadequate scientific knowledge and probable secrecy and stealth that would almost certainly be involved, a decision to protect athletes from such inappropriate genetic applications would certainly be an example of acceptable paternalism. Once the tools and methods of gene therapy will have become much more fully established and proven to be safe, the issue of personal safety by itself will become a much less convincing justification for a ban on genetic doping [06304].

Harm to other athletes

The second form of harm from drug- or gene-based doping is not the harm that gene transfer technology could cause to users, but rather the harm that could befall other athletes. The “others” in this argument are usually deemed to be other “clean” (undoped) athletes and the harm comes through “coercion.” If a successful athlete is known to be doping, then others who wish to compete and succeed at that level might feel compelled to resort to the same kind of illicit methods to have a realistic prospect for success. Thus, doping is a harm because it is coercive and puts the well-being and life goals of others in jeopardy. Competitors require protection from the risks of doping and from the damage caused to them and to their careers. In principle such protection might be provided by a ban on the substance or the technique in question [06304].

Harm to society

Any form of doping, whether it be drug- or gene-based, also may harm another group of people, the general public and, in particular, children. People admire prominent successful athletes and view them as role models. We often try to emulate the nobler activities and qualities of great athletes – their struggles against great odds, their tenacity, and their dedication to a goal – and extrapolate their achievements to many nonsports domains of the society. Whether this is a fair or an appropriate burden to put onto athletes rather than other public figures is irrelevant, it is a fact. The trust that we place onto athletes seems to be a societal “good” and anything that brings that trust into question or into disrepute must be a form of harm. From a societal perspective, if the hero or heroine is tainted and becomes morally suspect, the very young may have difficulty distinguishing between the athletic triumphs of their heroes or heroines and the moral or ethical blots on them. The ennobling influence of the hero becomes a crushing negative influence of a fallen idol. Interestingly, the downfall of a hero might not be intrinsic to doping itself but rather could merely be due to the revelation of doping that results from breaking the rules of the sport [06304].

Ethics of the athletes regarding doping

One study presented an opportunistic examination of the theoretical tenets outlined in the Sport Drug Control Model using questionnaire items from a survey of 643 elite Australian athletes. Items in the questionnaire that related to the concepts in the model were identified and structural equation modelling was employed to test the hypothesised model. Morality (cheating), benefit appraisal (performance), and threat appraisal (enforcement) evidenced the strongest relationships with attitude to doping, which in turn was positively associated with doping susceptibility. Self-esteem, perceptions of legitimacy and reference group opinions showed small non-significant associations with attitude to doping. The hypothesised model accounted for 30 and 11 percent of the variance in attitudes to doping and doping susceptibility, respectively. These present findings provide support for the model even though the questionnaire items were not constructed to specifically measure concepts
Sports Federations’ attitudes

It was determined the priorities and activities of International Sport Federations (IFs) with respect to the promotion of health in their sport and for the general population. All 35 IFs participating in Olympic Games in 2014 or in 2016 were asked to rate the importance of 10 indicated topics, and to report their programmes, guidelines or research activities on 16 health-related topics using an online questionnaire (response rate 97%). On average, the “fight against doping” had the highest priority followed by “health of their elite athlete” and “image as a safe sport”. The topics with the lowest importance ratings were “health of their recreational athlete”, “increasing the number of recreational athletes” and “health of the general population”. All except one IF reported to have health-related programmes/guidelines/research activities; most IFs had 7 or 8 of the listed activities. Eight IFs (24%) stated to have activities for “prevention of chronic diseases in the general population” but only FIFA and FINA reported related projects. It was concluded that IFs aimed to protect the health of their elite athletes through a variety of activities, however the health and number of their recreational athletes was of low importance for them. Thus, IFs are missing an important opportunity to increase the popularity of their sport, and to contribute to the health of the general population by encouraging physical activity through their sport. FIFA's “Football for Health” and FINA’s “Swim for All” projects could serve as role models [13765].

Litigation in sports medicine

As with other medical specialties, litigation in sports medicine appears to be on the increase. In most countries, the applicable legal standard is "good medical practice" as identified with reference to the physician's own field of specialisation: what is commonly done by physicians in the same specialty generally serves as the standard by which a physician's conduct is measured. To enhance the quality of sports medicine practice, medical societies have been issuing guidelines for use by sports physicians, and a number of courts have recognised guidelines as evidence of good medical practice. One potential field of malpractice in sports medicine relates to privacy issues: an athlete should be asked to fill in a consent form if the medical information needs to be shared with other parties. Another relevant field is doping: for any act of drug prescription to be legally sound, sports physicians have to be aware of the requirements of the World Anti-Doping Agency Code and its international standards. Ultimately, the best way for sports physicians to avoid sources of liability is for them to keep up to date with the latest research and to act in a careful and diligent manner [13766].

Physician's role in doping

Sports physicians occupy a specific set of roles. For example, they may carry out the role of an athlete patient’s general practitioner; a team doctor for a single club; the sport physician for a national sports federation or an international sports federation; an independent consultant to an employer of the athlete patient; an event physician whose services have been engaged by event organisers; and a specialist consultant in a legal process. Each of these roles brings ethical challenges, but between them there may be conflicts of expectations or duties. Although sports physicians may occupy other medical and healthcare functions beyond their sports medicine practice, the proposals below refer specifically to their
interaction with any athlete patient. Where there is overlap between their role as consulting physician (i.e. general practitioner) and sports physician, it is the duty of the physician clearly to distinguish these and to communicate them both to patients and other interested parties. In all the scenarios that sports medicine presents the sports physician, it is recommended that the sport physician is focused on the care they give to their athlete patients. Best practice may be difficult to determine in a young medical specialisation, where the nature of individual care and the demands upon athlete patients may appear ambiguous. It is not always clear how to interpret the best interests of the patient. Nevertheless, if sports physicians are to give their athlete patients the highest level of care they are able, to it is of the highest importance that they develop trusting relationships. Athlete patients who do not trust their sports physicians to act always in their best interest are unlikely to share with them such information as may help diagnosis, cure and prevention of athletic injuries and other deleterious conditions. In order to provide their athlete patients with the highest level of care, it will be necessary, therefore, for a clear separation of roles where possible between the sports physician, the athlete patient and the relevant third parties such as team coaches or managers; club owners, press officers; and those involved in team or squad selection. While this is not always possible, and while the sports physician may be burdened with a number of potentially conflicting roles, they should always seek to clarify and minimise such risks before the engagement of their services, consulting with colleagues and up-to-date sources of professional guidance [11550].

Athletes may rely on sports medicine physicians for guidance on performance-enhancing substances. The involvement of sports physicians with the “doping” of athletes dates back more than 100 years. Despite the fact that it is unethical to encourage the use of banned substances, there have been well-documented cases of physician involvement with the doping of athletes. Two prominent examples are the Ben Johnson steroid scandal at the 1988 Olympic Games in Seoul and the 1998 Tour de France scandal during which many riders were found guilty of doping with erythropoietin (EPO) and other drugs. Concerning illegal or banned substances, physicians and athletes are bound by the decisions of legislature and sports governing bodies. According to the FIGS, the use of banned substances is unethical and strictly forbidden because they may provide unfair advantages to the athlete and may cause harmful side effects. When counseling an athlete on illegal substances, the physician must respect the law and uphold the dignity and honor of the medical profession; hence, such information should be kept confidential but the physician should denounce usage. The use of legal, approved performance-enhancing substances is not necessarily unethical, but physicians must still proceed with caution. Physicians may counsel athletes about legal performance-enhancing substances provided they are not dangerous, but again this is a gray area. Are analgesics that enable athletes to continue participating while injured considered perform-enhancing substances? Some may say yes. Regardless of whether they are considered performance-enhancing or not, are they dangerous? In the short term, they are probably not, but long-term risks such as the potential acceleration of degenerative joint disease that may occur if the athlete returns to play while still injured are more difficult to quantify. However, physicians are ethically bound to discourage substances that are unfairly available to a limited population, which thereby violates the spirit of the rules of competition. The team physician’s first obligation is protect the player from potential harm, whether short term or long term, associated with performance-enhancing substances, whether illegal or legal. The physician should not terminate the relationship just because an athlete does not heed this warning. The physician should follow the health of the athletes and continue to inform them of the risks based on available scientific evidence, reminding them that it undermines the sport and is potentially unfair to opposing athletes. The physician should not sacrifice his or her own credibility by exaggerating the evidence to dissuade an athlete from using a drug; this would violate the basic tenant of the relationship – trust [07398].

2100
There is little data in India on the use of performance enhancing drugs in sports. But personal and incidental information shows that their use is far more extensive than is believed. This use occurs beyond the arena of high-level competitive sports. Even if the guidelines of the national and world anti-doping agencies were to become effective, they would not impact the larger environment where such drug use appears to be extensive. Which ethical guidelines advise the sports medical practitioner in prescribing medicines and training regimes for the athlete? Of particular concern is the role of paediatricians since training for sports or physical fitness is increasingly a youth phenomenon [13767].

Recent positive doping cases and a series of mistakes of medical doctors of the International Federation of Basketball have reopened the debate about the role of medical doctor in elite sport. This study shows that some sports physicians involved in recent positive doping cases are insufficiently aware of the nuances of doping regulations and, most importantly, of the list of prohibited substances. Moreover, several team doctors are shown to have exercised poor judgement in relation to these matters with the consequence that athletes are punished for doping offences on the basis of doctors' negligence. In such circumstances, athletes' rights are jeopardised by a failure of the duty of care that (sports) physicians owe their athlete patients. It was argued that, with respect to the World Anti Doping Code, antidoping governance fails to define, with sufficient clarity, the role of medical doctors. There is a need for a new approach emphasising urgent educational and training of medical doctors in this domain, which should be considered prior to the revision of the next World Anti Doping Code in 2013 in order to better regulate doctor's conduct especially in relation to professional errors, whether negligent or intentional [13768].

**The challenge of working in sports medicine**

All physicians are faced at some time with fundamental challenges while striving to respect the principle canons which define a physician's ethical code. These canons are:

- primacy of patient interests
- patient confidentiality
- informed consent
- maintenance of a high standard of care

Athletes, because of their focus on performance, often present unique situations which lead to ethical challenges not seen in the general patient population. Adherence to the four principle ethical canons guides physicians to make ethical decisions when dealing with these unique patients. Athletes as patients are a unique group of individuals who test the foundation of medical ethics and deserve special consideration. Olympic gold medalist Vince Matthews offered insight when he proclaimed “Twenty years from now, I can look at this medal and say: “I was the best quarter-miler in the world on that day. If you don’t think that’s important, you don’t know what’s inside an athlete’s soul.” The athlete patient is driven to perform and pursues the extremes of physical capacity to compete. Long term health may take on an importance secondary to achievement. After injury, he often searches for a quick fix to overcome a situation that compromises performance. Speed of recovery becomes a main priority. Extensive training also affords a unique knowledge of his own body, a factor that serves as an asset during medical evaluation and treatment. Respect for this awareness facilitates the decision making process. By nature, the athlete patient is usually very motivated and prepared to commit to a rehabilitation program, eager to push the limits of the healing process. A physician may be drawn into and caught up in this fan mentality or feel a
sense of obligation towards the success of the team that employs him. Ultimately, he must wade through these multiple roles of team employee, businessperson, and fan to focus on the principal role as a physician who is primarily concerned for the health of the patient. The National Collegiate Athletic Association Sports Medicine Handbook declares: “The team physician has the final responsibility to determine when a student-athlete is removed or withheld from participation due to an injury, an illness, or a pregnancy. In addition, clearance for that individual to return to activity is solely the responsibility of the team physician.” Legal precedent has protected this responsibility, citing college sport as a privilege and not a right. The advent of the widespread use and abuse of Performance Enhancement Substances (PES) has resulted in unique ethical challenges for physician and athlete. Advancements in medicine have produced chemical compounds capable of boosting physical performance. When disparities in talent are minimal, an intervention that provides an edge can mean the difference between victory and defeat. Much attention has been paid to drug use by athletes, and the list of banned substances in competitive sport is lengthy and growing. At first glance, the very notion of banning drug use appears to violate respect for autonomy. After all, the risks of using chemical enhancements are assumed entirely by the individual. “Greater risks for greater gains” is a philosophy familiar to competitive sport whether referring to individuals or game management decisions made by a coach. Rules of sport are arbitrary but together combine to define the activity itself. Performance enhancing drugs do not fall within the borders that have been determined as the court of play. While an argument made in this way is sufficient to justify the stance, it is somewhat unsatisfying. Adding substance to the dialogue is a philosophical perspective. The nature of sport is not only in the final performance but in the means to the capacity for such a performance [12486]

Environmental factors

To examine the ethical challenges of upholding patient confidentiality in sports medicine and the practical responses of clinicians to these challenges a questionnaire survey and follow-up semi-structured interviews were performed with the members of the British Olympic Association's Medical Committee and Physiotherapy Forum. Clinicians identified three contextual factors that influenced issues related to patient confidentiality in sports medicine: the use of confidentiality waivers; the facilities available for treatment; and the cultural norms of elite sport. They further identified interpersonal strategies used to lessen or eradicate conflicts, including emphasising the benefits and avoidance of disbenefits for athletes and the potential negative consequences for others. It was concluded that aspects of clinicians' practice environment should be designed to enable compliance with the highest levels of ethical conduct. Professional associations should establish guidelines for clinicians' interpersonal conduct in dealing with confidentiality issues and consider their provision of ethics-based continuous professional development. They should also petition for the establishment of athletes' codes of conduct which identify a context-relevant understanding of “serious harm” and how that might impact on information disclosure [13769].

Case reports of the doctor’s fault but athlete’s verdict

Recent positive doping cases and a series of mistakes of medical doctors of the International Federation of Basketball have reopened the debate about the role of medical doctor in elite sport. This study shows that some sports physicians involved in recent positive doping cases are insufficiently aware of the nuances of doping regulations and, most importantly, of the list of prohibited substances. Moreover, several team doctors are shown to have exercised poor judgement in relation to these matters with the consequence that athletes are punished for doping offences on the basis of doctors' negligence. In such circumstances, athletes' rights
are jeopardised by a failure of the duty of care that (sports) physicians owe their athlete patients. It was argued that, with respect to the World Anti Doping Code, antidoping governance fails to define, with sufficient clarity, the role of medical doctors. There is a need for a new approach emphasising urgent educational and training of medical doctors in this domain, which should be considered prior to the revision of the next World Anti Doping Code in 2013 in order to better regulate doctor's conduct especially in relation to professional errors, whether negligent or intentional [13776].

Rumanian gymnast

Notwithstanding collusion by physicians in systematic doping, the most important case of doctor fault in relation to doping offences was probably that of Andrea Raducan's case in the Olympic Games in Sydney, when she was stripped of her Gold medal after testing positive for pseudoephedrine which was contained in nurofen, a common over-the-counter anti-inflammatory medicine. Andrea Raducan, one of the greatest gymnasts of her generation, was born in 1983. She started to train at the age of 4, while by the time, she was 14, she represented the Senior Romanian team, and made her debut at the Sydney Olympic Games when she was aged only 16. In 1999, she won gold as an individual in the Floor Exercises and gold in the Team Event at the World Gymnastics Championships, and silver on the Beam. At the Sydney Olympics, she won gold in the gymnastics (artistic) women's team finals and won an individual silver medal on the vault. From 1996, she was under the control and direction of, among others, Dr Ioachim Oana, the Romanian gymnastics team doctor. During the competition at the Olympic Games, she reported a headache, a running nose and a feeling of congestion to Dr Oana, who prescribed and issued her a nurofen, an anti-inflammatory drug. He gave her a second nurofen tablet during the warm-up women's individual all-around event. She won gold in the gymnastics (artistic) women's individual all-around event. Subsequently, however, she failed a doping control, testing positive for pseudoephedrine and was stripped of her gold medal by the International Olympic Committee (IOC). Raducan said that she bore no responsibility for the antidoping rule violation (ADRV), since the nurofen pills were given to her by her team doctor with whom she had a relationship of trust, and that the pills had not been performance-enhancing. She competed weighing only 37 kg, a statistic that is important when considering the effects regarding the concentration of the drug. Nevertheless, because of the strict liability condition, the IOC antidoping panel and later the Court of Arbitration for Sport (CAS) were implacable. The World Anti Doping Code (WADC) makes it clear that there is no need to prove the intent to cheat via the use of performance-enhancing substances, but merely the presence of prohibited substances in the athlete's body is enough. Athletes have a duty to avoid the presence of such substances within their person. This is known as “strict liability”. The case generated a significant amount of media attention about the role of team doctor. The team doctor who administered the nurofen was banned for two Olympic cycles. It is noteworthy, however, that the then WADC and antidoping regulations did not precisely define the role of medical doctor. The situation persists today, though greater clarity exists of the role of physicians in relation to the therapeutic use exemption certificate for athletes who have a clinical need for substances that are simultaneously on the prohibited list (PL) because of their (potential) ergogenic or (potential) harmful effects [13776].

Serbian handball player

It was analysed four result management decisions done by the Antidoping Agency of Serbia and the International Basketball Federation (FIBA) in 2010 which involved team doctors: one regarding an international Serbian handball player and three doping cases of FIBA. An international Serbian handball player was tested positive in June 2010 for the substance of hydrochlorothiazide during in-competition testing at the French national championship.
According to his medical history, he had been treated by ACE inhibitors, calcium antagonists and diuretics since 2008 because of hypertension. The therapy is prescribed by a cardiologist from Belgrade, confirmed by the team doctor of the Handball Club Partizan, Belgrade, Serbia, and then by the team doctor of the Dunkerque Handball Club: HB Grand Littoral, Dunkerque, France, and finally by the team doctor of the Handball Club Kolubara, Lazarevac, Serbia. What is important here is that the player has been seen by various medical doctors. What is even more important is the player has informed French doping control officer during doping control about his use of the diuretic, which is on World Anti-Doping Agency’s (WADA) PL. Yet, there are hypertension treatments available that are not on the PL, a point that the physician ought to have been aware of. Nevertheless, the doping control officer did not put it on the list of medications the one the athlete had taken during the week prior to the control. What is clear here, that as an adult, the athlete themselves bears some responsibility (not just liability) to present themselves at competition in such a way that does not fall foul of the WADC’s regulations. Finally, the player was given a 10-month ban, while financial penalties were subjected to the two medical doctors by the civil courts in Serbia [13776].

**Russian basketball player**

Another case can be seen to fall somewhere between the cases of the handball player and Raducan in terms of the role played by sports physicians. A young Russian basketball player, committed an ADRV by the use of nandrolone. He had been treated by a Russian team doctor following two fractures (the instep bone of the right hand, and left fibula). The physician ought to have chosen another medication with similar effects that was not on the PL. In spite of this offence, the player argued that he had no idea that the injections contained substances on the PL, that he had not committed previously any ADRV, and that he was unable to properly give consent as a minor. Nevertheless, under the auspices of strict liability, he received a 1-year punishment while the team doctor received a lifetime ban from FIBA. What is further worthy of note here is the discretion of the FIBA panel. Had the doping offence been detected by, for example, a more lenient antidoping panel, it is not immediately clear that a 1-year sanction would be handed down, given the (fairly clearly) accidental nature of the ADRV from the athlete's perspective [13776].

**French basketball player**

The third case is that of French basketball player, who underwent an in-competition doping test in July 2010 in Zadar, Croatia, on the occasion of the France-Spain semifinal of the FIBA Europe U-20 Men's Championship. Upon being found to have committed a doping offence, he was handed a 1-month ban while there was no punishment for the team doctor concerned. The player had filed a declaration of use for ventolin (salbutamol) with the French National Anti Doping Organization (Agence Française de Lutte Contre le Dopage) (NADO (AFLD)). During the game, however, he suffered an asthma attack and was urgently treated by the team doctor (whom the athlete did not choose, but may reasonably have assumed, was familiar with the PL), with an inhaler containing terbutaline. The team doctor had treated him in the mistaken belief that the declaration of use covered all beta₂-agonists. The physician thus mixed different beta-agonists, and though sabutamol and terbutalin are from the same group, he did not use the drug that had been registered on the therapeutic use-exemption (TUE) certificate. This oversight caused the ADRV to arise in the doping control. Unsurprisingly, it was argued that the player bears neither fault nor negligence for this ADRV, since this was clearly a mistake by the team doctor and that he had committed no previous ADRV. Again, from the athlete's perspective, this is one of the difficulties of the WADC regarding strict liability [13776].
The fourth and final case to be considered is that of a Spanish basketball player, who underwent an in-competition doping test on July 2010 in Toulouse, France, after the end of the USA versus Spain quarter-final of the FIBA U17 Women’s World Championship. The analysis showed the presence of the prohibited substance chlorthalidone in the player's sample. Problems began for the athlete in spring 2010 when, it is alleged, she gained weight as a result of stressful school exams. The Spanish national team coach asked the player's parents to monitor her weight and initiate a diet with the purpose of rapid weight loss. After having unsuccessfully tried to lose weight, she was contacted by the team doctor of the Spanish Basketball Federation responsible for the U-17 Women’s team who recommended a supplement named “Obesity A”. It should be noted that a TUE would not normally be granted for this product. Upon joining the national team's training camp in early July, however, the team doctor asked her to discontinue taking the pills. Despite this, she continued using the drug, so it is clear that her doping offence could at best be considered careless and, at worst, a case of intentional doping. Upon committing the ADRV, she received a ban of 9 months.

**Regulations**

“Athlete-support personnel” (often called the “athlete entourage”) comprises any coach, trainer, manager, agent, team staff, official, medical, paramedical personnel, parent or any other Person working with, treating or assisting an athlete participating in or preparing for sports competition. The WADA code defines, in very general terms, the role and competencies of medical doctors in relation to doping in article 21.2:

- To be knowledgeable of and comply with all antidoping policies and rules adopted pursuant to the code and which are applicable to them or the athletes whom they support (article 21.2.1)
- To cooperate with the Athlete Testing programme. (article 21.2.2)
- To use their influence on athlete values and behaviour to foster antidoping attitudes. (article 21.2.3).

Finally, the code defines punishment of medical doctors in article 10.3.2. For violations of articles 2.7 (trafficking or attempted trafficking) or 2.8 (administration or attempted administration of prohibited substance or prohibited method), the period of ineligibility imposed shall be a minimum of 4 years up to lifetime ineligibility. An ADRV involving a minor is considered especially serious because of the failure of a heightened fiduciary obligation, and, if committed by athlete-support personnel for ADRVs other than specified substances referenced in article 4.2.2, shall result in lifetime ineligibility for athlete-support personnel.

**Aims of the regulations**

The central aim of the WADA code with respect to athlete-support personnel is that those who are involved in “physician-assisted doping” in a strong sense, or assisting athletes in masking doping practices should be subject to sanctions that are more severe than the athletes who test positive. The athlete is always responsible for any prohibited substance in his body (article 2.1) under strict liability, but the period of ineligibility shall be reduced or even eliminated if player can establish that they bear no fault or negligence. In line with strict liability, antidoping panels typically argue that players did not ensure that no prohibited substances enter their body, and because of this, they cannot shift their responsibility under the rules to support personnel. Nevertheless, antidoping tribunals may hold that a player's
negligence is not insignificant and that it is, therefore, appropriate to impose variable sanctions on them [13776].

Athletes are expected to bear most of the responsibility for taking medical drugs and supplements that are potentially performance enhancing, harmful, and/or contrary to the spirit of sport, yet sport physicians are responsible to athletes for questions regarding antidoping and medical care. If athletes cannot rely on the trustworthiness of physicians, particularly in relation to their competence regarding anti-doping regulations, then it would seem that their right to proper healthcare in the contexts of elite sports medicine is jeopardised. On the other hand, one can ask whether the supply of qualified sports physicians may dry up if colleagues are repeatedly found guilty of ADRVs [13776].

From another aspect, medical doctors are often seen to be held responsible by sport administrators, athletes and the general public. The main accusations made are, first, that some are engaged in “physician-assisted doping”, and second, that they supply athletes with doping agents, through carelessness. It has been shown that general practitioners’ (GPs) knowledge of prohibited substances in sport is poor. In one survey it was shown that only 53 percent of GPs were aware of banned drugs, and that 12 percent believed that medical practitioners were allowed to prescribe anabolic steroids for non-medical reasons. A Dutch study of 1000 GPs was even clearer: 85 percent of the respondents admitted that they were not familiar with banned drugs or their side effects. If, as in this study, doctors are the most common source of information for the athletes (61 %) then the situation become more problematic [13776].

What is also clear is that WADA are somewhat impotent in the process of disciplining members of the athletic entourage. National and International Sports Federations can apply sanctions to prevent doctors, physiotherapists and other healthcare professionals from working with individuals or teams. What is more likely to be effective is interagency collaboration between WADA, Institute of National Anti-Doping Organisations (or international antidoping organisations such as Europe’s CAHAMA group) in order to bring pressure to set international antidoping education guidelines. They ought also, however, to bear on individual healthcare professionals via their licensing associations. For the most egregious of infractions, the temporary revoking of licenses might be considered. This would not be without precedent. Indeed, the physician involved in that case, back in 1989 in Canada in the wake of the Ben Johnson incident, was held not to be fit to practice and had his licence revoked [13776].

**Legislative structures on enhancement**

With the development of highly sensitive drug testing technologies that can detect a minute quantity of a prohibited substance in an athlete's body, accidental contamination through contact with publicly circulated materials can more readily result in a "positive" reading. To discharge the burden of a positive finding, the athlete must show the “factual circumstances” in which the prohibited substance entered his/her system. In cases of accidental contamination, the athlete generally cannot even know how it occurred, as there are many known and unknown possible sources of contamination. When an athlete does give an account, it cannot generally be proven or disproven. Outside the realm of sports anti-doping, the use of scientifically established thresholds for drug testing is standard practice. Basic logic dictates that thresholds would enable one to differentiate between relevant and irrelevant amounts in the context of a possible sports doping offence. Such a threshold should be functionally motivated, i.e., enable the differentiation between relevant and irrelevant quantities in the context of a possible doping offence, rather than based on instrument performance limits [12487].
Since the early part of the 20th century, various sports organizations have employed an anti-doping policy, though it was 1967 when the International Olympic Committee first organized a Medical Commission whose primary role was to address the use of doping substances. The main concern of this committee involved the risks to health that doping entailed for athletes, which, expectedly, was also seen to diminish the values of Olympism. In particular, the televised death of Tommie Simpson in the Tour de France in 1967 began a cultural turn in how the doped athlete was represented. His image of a doped athlete has become characteristic of the abjection associated with unnatural enhancements, which sustains part of the political will surrounding anti-doping. In 1998, the Tour de France again was monumental in transforming this political landscape. The images of athletes under siege by police provoked the world of sport to rethink its approach to doping and the World Anti-Doping Agency (WADA) was born soon after. The current international standard for doping technologies is the World Anti-Doping Code, which indicates that two of three conditions must be met in order for a technology to be considered for prohibition from sport. These consist of the following:

- is the technology harmful to health?
- is it performance enhancing?
- is it against the “spirit of sport?”

It is widely recognized that determining whether these conditions are engaged is not simple and requires some form of discursive process to resolve. However, this process does not apply to all forms of enhancement technology, which are not considered in relation to the Code. For instance, when a new design element of a tennis racquet is introduced—such as the use of piezoelectric dampening technology—the anti-doping code is not engaged. Rather, the specific sport’s federation will consult its own guidelines on technical specifications to determine whether the innovation is acceptable.

Since its beginning, WADA’s role has been to harmonize policy and it has gradually worked toward independence from the International Olympic Committee at a time when the International Olympic Committee was under scrutiny for allegations of corruption. During this time, it has succeeded in working with UNESCO to develop a Convention on doping and its relocation to Montreal has been accompanied by renewed efforts from a range of countries whose recent actions suggest greater, rather than less controls over athletes’ actions. In particular, president George W Bush has included references to the “war on drugs” 2004 and 2005. The Hastings Center, has undertaken continual research in this area since the 1980s. In 2006, it was made first substantive intervention by concluding that the use of hypoxic environments (also known as altitude chambers) should be deemed an infraction of the WADA Code because they violate the “spirit of sport.” In 2003, the now infamous Bay Area Laboratory Co-Operative (BALCO) affair reminded anti-doping authorities that designer substances are completely unknown and it will be near impossible developing direct tests for them in advance. Indeed, the challenge of proving positive doping cases has been one of the major obstacles for anti-doping authorities. This challenge has also recently given rise to changes in the law, where the emergence of a nonanalytical positive—a doping infraction without the need for a urine or blood test—means that athletes now face possible disqualification (and sometimes prosecution) based on evidence other than unequivocal facts. These circumstances are also accompanied by an emerging willingness to criminalize doping infractions and to discuss doping as underpinned by an international criminal drug mafia. These terms reshape what is at stake in the issue of doping, transforming a matter related to fairness and ethics in sport to a moral panic over drug use.
Frequent battles occur between drug cheats, antidoping agencies, and the lawyers representing all sides. Elite sport has entered the world of forensics, where winners and losers of competitive events can be declared in the courtroom, not the playing field. Further, legal proceedings take months. Because of forensic procedures, the 2006 Tour de France will not declare a winner until months after the finish of the race [07153].

Evidence-based doping?

Evidence-based medicine makes an important distinction between statistical significance and clinical relevance: real effects may be too small to be therapeutically useful. This is very important in a clinical context; for example, cholinesterase inhibitors have a statistically significant effect on formal measures of cognitive function demonstrable in groups of patients with Alzheimer’s disease, yet individually many such patients and their carers do not notice an improvement, and treatment should continue only if worthwhile improvement is noted on an individual basis. When drugs are used in sport this situation is stood on its head, since a marginal improvement in performance (< 1 %), which would be extremely difficult to detect experimentally even in a randomized controlled trial, could nonetheless make the difference between winning and losing. None of the drugs used in sport (including anabolic agents, erythropoietin, human growth hormone, insulin, beta2-adrenoceptor agonists and others) have been convincingly demonstrated to enhance athletic performance, but this absence of evidence has not impressed contenders in the past half century and is unlikely to do so in future [12003].

Motherhood goes with gold?

Physiological actions of female sex hormones on muscle metabolism, and effects of increased blood volume and oxygen delivery are surely consistent with this possibility – although this is of course a far cry from definitive evidence. Despite this draconian instance, taking advantage of a natural physiological state such as pregnancy could hardly be called into question [12003].

Legitimacy of ban on cannabis and cocaine

The Olympic movement was the driving force behind the anti-doping efforts that led to the establishment of the World Anti-Doping Agency in 1999. A series of widely publicized doping scandals and public outrage in the nineties triggered this increasingly strong movement advocating doping-free sports. WADA aims at eradicating doping by harmonizing anti-doping practices worldwide from a zero-tolerance standpoint. It is backed by the UNESCO anti-doping convention, now signed by a majority of UN member states. These anti-doping efforts were recently dubbed “war on doping”, echoing the declaration of the “war on drugs” by president Nixon in 1971. These wars on drugs and on doping share various features, such as similarities between policies based on zero-tolerance, repression and surveillance, black markets controlled by organized crime, attempts to shape internationally harmonized legal frameworks, ideology and political convenience anchored in media-fuelled moral outrages. Furthermore, when WADA was established and drafted its first list of forbidden substances, the question rose on whether cannabis derivatives should be on the list. They were included largely because of pressure from the ‘war on drugs’ movement, even though there are no known proven performance enhancing effects but rather evidence for the contrary. Even
though only forbidden during competitions, regularly athletes are indicted because of urine traces of cannabis metabolites, which may remain measurable up to weeks after use. Cocaine metabolites are also regularly found and the public announcement of such cases often leads to important media coverage, strongly condemning the athlete, even if the substance was taken in a recreational context and not for sport performance enhancement. Whereas the “war on drugs” proved to be a failure and public debate is now slowly shifting towards finding better policies – based on public health principles – to deal with psychotropic drug use in society, in sport current policy is still essentially based on repression and surveillance from a zero-tolerance viewpoint [12012].

The complexity of anti-doping

Upon first glance the anti-doping rule may seem reasonable and simple, but when looking more closely at the consequences of the rule, questions arise. Some recent examples of indictments for doping, illustrating the complexity of anti-doping policy implementation, are given hereunder. Dwain Chambers, a famous British 100 m runner, tested positive for THG (tetrahydrogestrinone) in 2003 and was banned from competition for 2 years. The British Olympic Association, on the basis of a bylaw, excluded him for life from participation to the Olympics. The Court of Arbitration for Sports (CAS) overruled this decision in April 2012 after an appeal by WADA, opening the way for Chambers to compete in this summer’s Olympics. The case is illustrative for several reasons. First because of the substance THG. It was designed by a clandestine laboratory specifically for doping purposes, and by consequence unknown to the scientific community and anti-doping laboratories in particular. It was discovered when a coach sent a syringe containing traces of the substance to the USA anti-doping agency. Second because there are no published experimental data confirming the alleged performance enhancing effects of the substance. Third, since WADA convinced CAS to overturn the ruling of BOA on the basis of non-compliance to universally applicable WADA rules. And fourth, because of the strong condemning of this CAS ruling by many prominent members of the British sports-establishment which seemed to indicate that a doping offence is not seen in the same way as most other offenses in society; if in general, upon punishment for transgression of a rule, one is offered a second chance, a doping offense is deemed essentially unforgivable and worth exclusion of sports for life. As Sebastian Coe, chairman of the London Organising Committee for the Olympic Games, said: “I am clear cut on the Chambers case – I don’t think there is room for drugs cheats in sport” [12012].

Alberto Contador, a famous Spanish road-cyclist and Tour-de-France winner, was indicted in 2012 for trace levels of clenbuterol in urine samples obtained during the 2010 Tour. Several aspects set this case apart. First because of the long time it took to condemn Contador, contrasting his case with several other similar cases in other, perhaps less famous athletes. Second, the traces found were so low as to exclude any significant physiological performance enhancing effects around the time of sampling. Third, the excuse used by the defence: ingestion of contaminated meat. And fourth, the widely publicized rumours about possible blood doping as the source of the substance, by transfusion of Contador’s own blood, extracted and stored at an earlier time when doping, on the basis of traces of plastic residues in his blood, compatible with the use of blood bags and tubing, even though his blood parameters did not indicate blood doping [12012].

Christine Ohuruogu is a very successful British athletics sprinter who was suspended from competition in 2006, not because of doping, but because she missed three unannounced out-of-competition drug tests. She received a one-year ban for missing these tests, even though she was repeatedly tested at other occasions in the same period as the missed tests, without any adverse findings. This would rather indicate negligence on informing the
authorities on her “whereabouts” and not intentional doping-hiding behaviour. Nevertheless BOA initially imposed a lifetime ban from competing at future Olympics. Her Olympic ban was finally overturned in November 2007. Yanina Wickmayer is a talented young tennis player from Belgium whom end 2009 was initially suspended for a year by the Flemish anti-doping authority. She had failed three times to inform about her whereabouts upon entering the Women’s Tennis Association top-50 and by consequence becoming part of the athlete pool obliged to inform about her whereabouts. She was able to defend herself in a Belgian court, pointing out shortcomings and administrative errors of the official bodies overseeing her introduction to whereabouts obligations and had the ban overturned, allowing her to play again, but her image was probably tainted forever. But her case is not over yet. Even though WADA, claiming a 2-year suspension, has decided to withdraw its appeal against the Flemish anti-doping authority following the decision of a Belgian court to invalidate the authority’s decree, the procedure between the player and the Flemish Tennis Federation has only been suspended for the moment [12012].

Claudia Pechstein, a very successful German speed skater, was frequently tested throughout her long career, but never failed a test. On the basis of fluctuations in the number of young red blood cells in her blood (reticulocytes), she was declared guilty of blood doping in 2009 and banned from competitions for two years. This case is interesting because it was the first time that an athlete was considered guilty of doping on indirect evidence indicating the possible use of a substance that, in itself or its metabolites, were not directly identified. Perhaps the fact that Pechstein’s career started as an East German skater created a climate of suspicion because of state-organized doping practices in former East Germany. But in hindsight it is now highly likely that Pechstein’s higher than “normal” levels of reticulocytes result from a genetic anomaly, and are, in her case, physiological and not the result from doping. In all there remains considerable scientific doubt on the likelihood that Pechstein did indeed use forbidden substances or methods. The case also highlights the difficulties for the CAS to rule in such complicated cases, because of the entangling of scientific, legal, economical, political and personal interests [12012].

It was reviewed a series of cases of doping in tennis. There were 40 cases in the 5-year period 2003-2007, but in only 13 of these a prohibited substance was taken to enhance performance. In the other cases (68 %) it was accepted at independent hearings that there was “no intent to enhance performance” or “no (significant) fault or negligence”. Recreational drugs made up 40 percent of the cases (11 cases of cannabis, 5 of cocaine) [12012].

The ethics of anti-doping

There have been several sceptical challenges to the legitimacy of the antidoping position arising from both philosophers working in the field of medical ethics and sports ethics. They argue, typically, that the bans on certain performance-enhancing processes and substances rest on principles that are inconsistently applied. Sport, it is widely canvassed, is about healthy, natural and ethically regulated activity. The WADA itself employs three criteria, of which at least two must apply in order for a product or process to be proscribed: it should be performance enhancing; it should present an actual or potential health risk and it should violate the spirit of sport (WADC Article 4.3). Performance enhancement per se, is of course the heart of elite sport. The other criteria establish means by which it is unacceptable. In response, sceptics typically argue that:

- doping is no more unnatural than the muscle-bound and technologically saturated athletes and sports equipment, prostheses, and so on
- athletes who train harder do not coerce their opponents to follow likewise and doping should be thought no different
- doping is no more harmful than other legitimated behaviours such as punching in boxing, or brutal tackling in rugby or American football
- doping confers no more of an unfair advantage than is enjoyed by athletes or teams from economically or technologically superior countries/clubs/systems

A more passive case made by the antidoping lobby is that by failing to proscribe socially undesirable behaviours, it would celebrate bad role models and promote undesirable lifestyles. Such a stance is consistent with public health policies against smoking or the use of marihuana (even when the harmed are solely the users themselves). To this, the sceptics may argue that the undesirability of doping would be chimerical if antidoping rules were repealed and not forced upon athletes seeking only to optimise their own athletic potential while only (potentially) harming themselves. Each of these sceptical challenges has some merit. In addition, the ethical and legal legitimacy of doping control may diminish if undetectable gene technology comes into widespread use. Nevertheless, what deflects sceptical challenges is the fact that the sporting practice communities themselves have rejected doping through their engagement in the processes of the formulation of antidoping policies and practices [10270].

It has been argued that the risk to athletes' welfare provides the only legitimate ground for restricting the use of performance enhancing drugs in sport. In one paper, it was argued that the idea of "sport", properly understood, provides further reason to impose such restrictions. A "balance of excellences" argument is proposed whereby doping is considered objectionable on account of its disrupting the relation between the excellences around which sporting competition is organised. There are therefore reason to restrict the use of performance enhancing drugs in sport not only because of the threat they pose to athletes' health but also because of the threat they pose to athletes' displaying the relevant types of sporting excellences [11305].

It was offered criticisms of an earlier article where it was argued that there is no clear reason for thinking that doping in sport is morally wrong. It was sketched a view on which the risk profiles of different sports may make doping permissible in some and impermissible in others. Second, it was suggest that safety-based arguments assume that doping opponents are bent on harm elimination, rather than harm management. Society clearly thinks that some safety measures in sports, such as helmets in cycling, are reasonable. It may therefore be that limiting or eliminating doping is reasonable in the same way. Because sports involve different risks, there may be more reason to limit doping in some sports than in others, so that one cannot say generally that doping is permissible or wrong. There are several responses to make. First, one should note that the argument about safety did not assume that doping's opponents desire harm elimination. The argument was that since many sports tolerate substantial levels of risk related to usual practice and competition, and since many sports – or their characteristics that engender risk – are morally permissible, the fact that doping involves risks does not by itself show that it is morally wrong. Thus it may be happily conceded that harm management in sports is reasonable and even that it could include efforts to curtail doping. One need not regard something as morally wrong in order to restrict it: moral considerations are not the only grounds for rules. It is not, for example, clearly wrong to drink sugary beverages, but it may be reasonable to limit persons' access to them and to create a system of incentives (e.g. taxes) that reduces their consumption. There are a variety of reasons to ask whether doping is morally wrong per se. First, conventional attitudes about doping treat it as something that is deeply wrong rather than as something that is merely unwise. Second, current anti-doping strategies incorporate severe punishments for violators. But if a system involving such punishments is to be justified, a
moral justification should be required. It would be absurd to ruin athletes’ lives to promote their well-being, just as it would be absurd to imprison someone for a year because he failed to wear his seatbelt. A bit more might be said regarding the suggestion that doping may have a different status in different sports, depending on their other risks. First, it is unclear how or why the difference between sports would have this effect. Second, this strategy would represent a weakening of the anti-doping position. Even if we were to tie imprudence to wrongness, to hold that doping should be treated differently in different sports would surely mean that some instances of doping are (considering safety only) morally permissible. Moreover, if the complexity of risk assessments were fully embraced, one would probably find that some types of doping should be broadly permitted. After all, some performance-enhancing drugs are safer than others. Albuterol, for example, is relatively innocuous in comparison to anabolic steroids. And even the most dangerous drugs are probably safe when used in moderation. A touch of EPO – enough to raise one’s hematocrit from 45 to 50 percent, for example – might not be especially dangerous, even though a large amount of EPO is dangerous indeed. And though it would restore consistency for opponents of doping to argue that doping and more conventional risk-laden sporting practices are wrong because they are unsafe, this would be a costly retreat. It would, obviously, imply that society should disband or alter many popular professional sports, which is counterintuitive (indeed, this was the force of my reductio mentioned earlier). It might also have unsavory implications for other realms of conventionally permissible human activity. And it would require opponents of doping to overcome various argumentative hurdles, such as demonstrating that a certain degree of imprudence is morally wrong. Finally, the cost of abandoning a safety-based anti-doping argument is relatively minor: it is to concede not that doping is permissible but only that considerations of safety alone do not show it is wrong [11306].

Education in moral judgment of participants in team sports

The aim of one study was to investigate the effect of age and education on the moral reasoning of the same 535 individuals in sports for whom nature of sport experience was reported. All 535 participants (medium age 25 years3) were involved in sports at the time of the study as athletes (n=342), referees (n=145), or coaches (n=48), and had a wide range of education. Analysis of variance of scores on the Defining Issues Test of Rest showed moral judgment in sports differs significantly amongst different age groups and amounts of education. Generally, with more education, higher moral judgment can be expected. It is apparent that moral development in sport is related to age and education, as also holds for a wider social setting [06303].

Professionalism and the ethics of the sports physician

The physician working on the sidelines of a public sporting event may encounter a unique set of ethical dilemmas. Many of the ethical pitfalls result from the physician’s responsibilities, not only to the patient, but also to teams and events. Standard rules of confidentiality and patient autonomy may not apply. It was proposed using the concept of “professionalism” to fill the gap between the ethical standards of office practice and the modified ethical standards necessary to practice as a sideline physician. Professionalism in medicine demands adherence to principles such as honesty, integrity, reliability, responsibility, respect for others, self-regulation, and, above all, doing what is best for the patient despite outside pressures. A central tenet of most theories of professionalism involves the recognition of responsibilities not only to one’s self and one’s patient, but also to the profession itself. That is, each profession has its own character to maintain and defend. Indeed, much of allopathic
medicine’s power resides with its trusted and respected good name. In general, any factor that moves the physician away from the professional role as an objective medical expert using skills solely for the purpose of evaluation and treatment of the athlete presents a moral pitfall [06302].

Conflicts of interest: the need to minimize

Excessive ties to the success of a team, whether financial or not, may set up a conflict of interest, or at the very least the appearance of a conflict of interest. It is best to keep these to a minimum. For example, although cheering is a natural human reaction toward the team with whom you work, the physician must do everything possible to be and appear as an objective medical expert. When the physician becomes a fan, an investor, or simply rests his or her pride on serving as the team physician, one might begin to question clinical decisions that benefit the home team or burden the opponent [06302].

Need to recognize the limits of athlete’s autonomy

Respect for patient autonomy is a fundamental principle in bioethics that must be balanced against other fundamental principles such as beneficence, justice, and others. Although many bioethical issues arise out of a failure of physicians to respect patient autonomy, there are limits to the scope of choices appropriate to offer patients. Respecting patient autonomy means respecting the choices they make, not necessarily facilitating anything they want to do. The sideline physician may place tighter limits on the choices open to the patient/athlete than the office physician. These limits actually arise from an implicit agreement between the players and the team organization that gives the sideline physician significantly more control than an office physician over the patient/athlete’s actions, at least within the confines of the contest [06302].

The need to maintain informed consent

A number of common dilemmas facing sideline physicians hinge on the potential for outside pressures to compromise the patient/athlete’s right to informed consent. It is imperative that the sideline physician resist any temptation to deviate from the rules of informed consent as it is routinely practiced. The necessary conditions for legitimate, informed consent as it pertains to the sideline physician are that all relevant information that a reasonable person in this player’s position would want to know regarding the proposed treatment, nontreatment, and alternative treatments must be explained to the player in an appropriate fashion and that players must have the capacity to understand this information and appreciate how it applies to them [06302].

Confidentiality in doping

Normally, physicians respect patients’ confidentiality except in rare instances of mandatory reporting laws or imminent danger. However, in the role of the physician covering the team, the scope of confidentiality is not nearly so broad. The dilemma set up by the physician’s competing obligations to both maintain patient confidentiality and serve the team administration can be mitigated. By informing athletes in advance that, unlike a standard
doctor-patient relationship, confidentiality may not be guaranteed, the physician discloses his or her obligations and lessens the dilemma. Critics of this approach might argue that such warnings will ultimately prove dangerous for athletes. That is, informing athletes that information may be forwarded to the coach could result in the dangerous under-disclosure of pertinent medical history. For example, many athletes may be reluctant to reveal a history of concussions – or doping – if aware that it may bar them from competing. Not knowing which facts may be revealed to the coach, athletes might withhold sensitive or embarrassing information that may be very relevant to their general health [06302].

It should come as no surprise that healthcare professionals have been engaged in unethical conduct in sports and notably with respect to doping. This is true in such famous events as the Tour De France and in less elite and sometimes adolescent cases. Although no one suspects that these interventions are the norm, the pressures in elite sports and the money that is often at stake render it likely that the temptation to medically assist doping will always remain. The work of every healthcare professional is guided by ethical considerations over which their relevant professional body has legitimate governance. Doping has itself been the object of discussion over a number of years within sports-related healthcare professions, such as the British Association of Sport and Exercise Medicine, the International Federation of Sports Medicine (FIMS), the Association of Chartered Physiotherapists in Sports Medicine and the International Federation of Sports Physiotherapy (IFSP). Most have publicly declared the unacceptability of doping in position statements. Advice regarding what professionals may and may not do with respect to doping forms a part of their clinical professional guidance. The sources of guidance vary for the healthcare professional working in sports medicine from their profession to the World Anti-Doping Agency (WADA). There may be a potential conflict in the duties of a healthcare professional who cannot simultaneously serve the patient's best interests while disengaging from giving advice regarding doping behaviours. In such a dilemma, the healthcare professional may either find that they are culpable of a doping offence or, in disclosing the confidential information of such, be "struck off" from their respective profession. However, the manner in which healthcare and medical professionals serve their athlete patients is governed by a variety of relevant codes of conduct. A range of codified rules is presented that refer both the welfare of the patient and the maintaining of confidentiality, which is at the heart of trustworthy relations. The 2009 version of the World Anti-Doping Code (WADC), however, appears to oblige all healthcare professionals not to assist athletes if they are known to be engaged in doping behaviours under fear of removal from working with athletes from the respective sports. In contrast, serving the best interests of their athlete patients may oblige healthcare professionals to give advice and guidance, not least in terms of harm minimisation. Although there are nuanced differences among these rules in relation to various aspects of professional performance, there are also easily discerned shared commitments. Among them, the following are of particular significance:

- human dignity
- protection of health and safety
- confidentiality and privacy
- informed consent
- duty of care.

Thus, according to the UK Health Professions Council (2008) – for example, the duties of a registrant demand that

1. you must act in the best interests of service users.
2. you must respect the confidentiality of service users.
3. you must behave with honesty and integrity and make sure that our behaviour does not damage the public's confidence in you or the profession

In a similar vein, the UK General Medical Council advises its members to

- make the care of your patient your first concern
- protect and promote the health of patients and the public
- respect patients’ right to confidentiality

In so far as the professional conduct of a healthcare professional is guided both by professional code and World Anti-Doping Code (WADC), they are obliged to fall foul of one or the other. It was called for urgent and pressing inter-professional dialogue with the World Anti-Doping Agency to clarify this situation [11307].

The 2009 World Anti-Doping Code which is the second version, has been accepted by >100 member states of the UNESCO. Its scope is greater than that possessed by the previous two. Although the processes of its development incorporated widespread consultation, certain aspects of its regulatory framework have brought considerable opposition. One aspect of the WADC not commented upon is the tightening of regulations relating to the medical support team. The rationale for this tightening is well founded. Clearly, where doping was widespread in some sports, it was not the effect of isolated individuals trying to gain illicit advantage. Rather, access, prescription, dosage and removal of doping products/processes were the object of systematic organisation and control by the sports support system that included doctors, physiotherapists, sports massage therapists and coaches/managers. Clearly, then, in attempting both to deter and, where necessary, punish members of the support system, greater attention was required to call to account the full range of individuals contributing to the doping offence. The section of the code stipulating antidoping violations was therefore modified to include the following article, relating specifically to administration of banned substances by a third party. The offence carries with it certain prohibitions that should be put into effect by the relevant sports bodies themselves. WADA has no jurisdiction to punish sports medicine professionals. Two issues arise here. They both relate to the jurisdiction of governance. First, there will be variability as to how any particular professional body will implement the sanction to practitioners in breach of WADC. Other countries will also vary in their interpretation and application of the rule(s). International harmonisation of responses here is unlikely to be achieved, although one might ask whether global bodies such as FIMS or IFSP, or indeed the World Medical Association or the World Congress of Physical Therapy ought to take a policy lead for the sake of consistency. Only bodies who are signatories to the WADC are likely to implement its sanctions. Second, WADA recommends, although it has no power to do more than this, that the offending individual be banned from working with athletes within that body. (It is not, however, possible to ban them from all sports-related assistance. The scope of the ban refers predominantly to the sports governing body in which the offence emerged, though it extends to all signatories). At first sight, this appears to be precisely the kind of regulation required to capture fully the offending parties in the sports medicine community. On closer inspection, however, the list of verbs that enunciate the sanctionable offence may give rise to considerable professional and ethical concern. It is worth citing the list of verbs: “assisting, encouraging, aiding, abetting, covering up or any other type of complicity involving an anti-doping rule violation”. Each of these verbs can be used to capture wrongful conduct for which a sanction may be applied. Take one example from the list to serve as an illustration of the problem faced by the healthcare practitioner: What precisely is “aiding and abetting” to be taken to mean? There is no particular legal or medical meaning of these terms. Courts, no less than healthcare professionals, must use the everyday meanings of these words, which are open to considerable ambiguity and interpretation. A number of everyday scenarios might reasonably
be envisaged, therefore, that would render the healthcare professional vulnerable to a charge of "aiding and abetting" a doping offender. For the purposes of illustration, we shall now discuss one such difficulty in terms of conflicts of duties in a realistic hypothetical scenario. Perhaps the most widely shared healthcare or medical norm is that of serving the patient's best interests while, ceteris paribus, respecting their autonomous decision-making as to the nature and means of any professional intervention. Thus, the healthcare professional, in line with the recent urgings of medical ethicists and sports medics, is deeply concerned with the health of the athlete in their care (understood as their patient). Therefore, in line with general medical norms of respect for autonomy and acting in their best interest, they would consider it important that the athlete comprehends any information and advice imparted. Now imagine further that a patient informs the healthcare professional that they have, for example, been using off-label performance-enhancing products using the information on the web to determine their own dosages and cycles. Then they approach the healthcare professional, concerned with what they perceive to be a moderately serious condition. Let us imagine that our hypothetical healthcare professional is committed to doping-free sport. Nevertheless, healthcare professionals also feel compelled to make their patient aware of the potential consequences of inter alia continued use, lowering doses, arranging cycles in the least harmful way and doing their best to persuade the athlete to cease their doping practices. All to no avail, the apparently well-informed patient argues that it is their body and no one else's that is at risk, and that they deem continued doping a risk worth taking. To cap it all, they state in no uncertain terms, that the professional may, under no circumstances, inform third parties regarding the consultation. They threaten legal action if this route is taken by the healthcare professional. Every healthcare professional is thus charged with serving the best interests of their patient. Part of this will entail respecting patient autonomy by imparting the relevant information and advice. An hypothetical colleague appears to have met this obligation by informing the patient of dangers and recommending the cessation of doping. However, there is an obvious difficulty in what to do with this information, or "guilty knowledge." Are the best interests of the patient served by releasing this information into the public domain? The client has forbidden this possibility and the healthcare professional is properly aware of their duty to respect the patient's autonomous decision-making. It is at this point, mindful of their differing frameworks for professional governance, that the relevant codes are consulted [11307].

Patient confidentiality

A variety of ethical questions may have arisen in the mind of the committed antidoping healthcare professional. Should they deliberately overestimate risks in their communication to their patient? With whom, if anyone, should they share the information? Should they write up the notes in full or fail to document incriminating data? Should they leave such notes, full or otherwise, in the purview of others who might discover them? It might be concluded that the duties of care and of confidentiality appear to have priority. The sources of the duty of care, of which confidentiality is a key part, are threefold. They may arise individually or jointly from contract, law or professional codes. Recall that the second duty of a healthcare professional as stated by the UK Health Professions Council is to the confidentiality of patient data, i.e. you must treat information about service users as confidential and use it only for the purposes they have provided it for. You must not knowingly release any personal or confidential information to anyone who is not entitled to it, and you should check that people who ask for information are entitled to it. You must only use information about a service user to continue to care for that person, or for purposes where that person has given you specific permission to use the information. Patients also have a right to expect that information about them will be held in confidence by their doctors. Confidentiality is central to trust between doctors and patients. Without assurances about confidentiality, patients may be reluctant to
give doctors the information they need in order to provide good care. If you are asked to provide information about patients you must:

- inform patients about the disclosure, or check that they have already received information about it
- anonymise data where unidentifiable data will serve the purpose
- be satisfied that patients know about disclosures necessary to provide their care, or for local clinical audit of that care, that they can object to these disclosures but have not done so
- seek patients’ express consent to disclosure of information, where identifiable data are needed for any purpose other than the provision of care or for clinical audit – save in the exceptional circumstances described in this booklet
- keep disclosures to the minimum necessary
- keep up to date with and observe the requirements of statute and common law, including data protection legislation

Patients should be able to expect that information about their health, which they give in confidence, will be kept confidential unless there is a compelling reason why it should not. There is also a strong public interest in maintaining confidentiality so that individuals will be encouraged to seek appropriate treatment and share information relevant to it. Therefore, in the prosecution of this advice, everything is going to hang on whether there is a “compelling reason” to disclose the doping athlete patient. Yet it is far from clear what might constitute this. On the one hand, it might be said that preventing a star athlete from gaining gold, thus depriving other athletes (presumably clean) of their right to a fair contest, was compelling. However, the contrary position could also be argued. Thus, it might be argued that the dishonour brought upon relevant others by a potential doping scandal was compelling, although again a counterargument is also fairly obvious. Writing a professional code of conduct is not a task for the faint-hearted because it requires the author(s) to envisage the unlimited variety of contexts that may standardly apply in the patient–professional encounter.

Thus, rules for disclosure against the general prohibition have been made. It is a widely accepted norm that information may be shared within a healthcare team to provide treatment that is in the best interest of the patient. However, the information shared should proceed on the authorisation of the patient, in line with their conception of best interests. Against the general norm for confidentiality, disclosure may be justified:

- where to withhold it would be to put others at “risk of death or serious harm”
- that those who receive disclosure – subject to the above condition – are bound by confidentiality to
- that it may be justified in a medical emergency.

It is thus argued that there is a tension with regards to a variety of professional codes and the new WADC. Insofar as healthcare professionals must serve the best interests of their patients, they must inter alia give advice and information as to the health choices of the doping athlete as well as respecting the privacy of the clinical encounter where doping choices may be revealed. Although it may be difficult to harmonise codes that cater for a variety of professional labour, dialogue between WADA, (inter)national sports governing bodies and relevant (inter)national healthcare professions is urgently needed to protect healthcare professionals who are committed both to their clients, their own and their employers’ requirements for doping-free sport. Clearly, more precise guidelines on how such conflicts will be interpreted by both WADA and the relevant bodies should be made public to prevent professional dilemmas such as this from occurring [11307].
Fairness in sports

What does it take to keep sports fair? And what does fairness require? It’s easier to see what’s unfair in sports. Among the countless differences between competitors, from eye color to favorite food, only certain differences are meant to be highlighted in each particular sport. Successful short-track speed skaters possess explosive strength and finely honed technique. Each sport calls upon its particular mix of physical talents. Every sport requires the commitment to perfect those talents and to learn how to employ them skillfully and strategically. It may not be easy to say exactly what fairness means, but the ease with which we can call out unfairness suggests that the task is worthwhile and far from hopeless. Talent and dedication determine the winner. The varieties and degrees of impairment among Paralympians in no way detract from the talents and dedication that competitors bring to the games. But the variety also requires that the playing field be made level so that every athlete is competing against people with similar levels of impairment. In that way, talent and the many things we admire about dedicated athletes are on display and shape each athlete’s performance [10268].

The first thing to note is that a fair sports competition does not require that athletes be equal in every imaginable respect. Some basketball players are taller, stronger, quicker, or more agile than others. No one regards such differences in natural talents as unjust or unfair. Some have better coaches or more favorable training environments. At what point such differences cross the line from inevitable and acceptable to iniquitous and deplorable is something to be debated and settled by the people who participate in, understand, and love that sport – not by distant and disinterested philosophers. After initial dithering, the Fédération Internationale de Natation (FINA) – the international governing body for swimming – last year banned many suits on the grounds that they changed the nature of the sport by allowing bulky athletes to float on top of the water rather than having to push through it. Whatever one thinks of FINA’s ruling, it was right to focus on the meaning of the sport and on what characteristics lead to excellence and success. Then again, the most gifted, hardest-working athlete or team does not always win. A random bounce, a slip, a hesitation can give victory to the side that might lose nine of ten matches. That’s why we play the game [10268].

When it comes to performance-enhancing drugs, gene doping, and the panoply of manipulations banned widely in sports, the challenge is less about fairness than about meaning. If the rules ban performance-enhancing drugs, then using those drugs to gain an advantage over athletes who refuse to cheat is unfair. Antidoping skeptics, however, often proclaim that the problem isn’t with the drugs, but with the ban on drugs. It would be fairer, they argue, to give all competitors access to the same drugs. If everyone had ample supplies of anabolic steroids, erythropoietin, growth hormone, or whatever drugs boosted performance in their sport, then – they claim – unfairness would be eliminated along with the nuisances of drug testing, adjudication, and enforcement. One response to the skeptics is to ask a different question: Is it not unfair to put the athletes who want to compete without drugs or gene doping at a competitive disadvantage by permitting everything – to tilt the playing field in favor of the drug users [10268]? Any serious ethical commentary on the uses of performance-enhancing technology in sports must confront two compelling realities. First, sports science has provided a great deal of information about how to optimize training and performance. It has also led to a plethora of technologies and methods to enhance performance, from altitude chambers that allow athletes to gain the benefits of “training low, living high,” to ice-filled vests runners can wear before a long race to cool their core temperatures, to esoteric measurements of muscle and organ function. Why, the skeptics ask, should we distinguish between these technologies of
performance enhancement on the one hand and drugs like steroids on the other? Part of the answer to this challenge is to recognize that sports are about what can be accomplished under specific limitations. Soccer players, other than goal tenders, may not use their hands or arms to direct the ball, even when that would be far more convenient and accurate than one’s foot or head. Golf imposes strict limits on balls and clubs. Marathon runners may not use wheels, whether attached to their shoes. The other piece of the answer requires an understanding of what that particular sport values. What makes a great weightlifter does not make a great distance runner. Bodies that possess massive explosive strength are rarely the lithe, sinewy bodies best suited to run great distances. The limitations each sport chooses for itself reflect a shared understanding of what that sport is meant to display and reward. The rules of sports are arbitrary in the sense that they could be otherwise, and, in practice, sports modify their rules in response to changes in equipment, tactics, and athletes’ abilities. But in another sense, the rules and the changes wrought in them are far from arbitrary: they must pass muster with the community of those who play and love that sport. The community must be satisfied that the new rules keep alive what it values, what natural talents enable athletes to excel, and what, in the end, is meaningful about participating and winning [10268].

One proposed solution is to continue to ban some drugs – those deemed to be particularly harmful – but allow athletes free reign to use all others. Consider what is likely to happen. We’ll continue to need drug testing and enforcement to deter athletes from using the substances on the banned list, so all the complaints about the inconvenience and intrusiveness of testing will remain. And now athletes will feel pressured to take ever more drugs, often at higher dosages, in untested and possibly dangerous combinations. It’s hard to see that scenario as progress. Whether performance-enhancing drugs or gene doping should be permitted in sports is, in the end, a matter to be decided by the communities of athletes and those who understand and love each sport [10268].

**Criticism of the fairness of the testing procedure**

The stated mission of the World Anti-Doping Agency (WADA) is “to lead a collaborative worldwide campaign for doping-free sport”. This is (arguably) a laudable goal but a vociferous group of analytical scientists is far from satisfied with efforts to achieve it, questioning the effectiveness of anti-doping programs, the injustices created in their implementation, and the non-collaborative approach that WADA has taken. Current practice is not state-of-the-art; sub-optimal methods are being used for data analysis. Instrumentation is not the problem. What is being done with (or to) the measurement results, that’s the problem. The criteria for a positive result, that is, the decision limits, are not statistically underpinned. There is not a single test for which the risk of false-positives and false-negatives is known. The whole system is closed, which is maintained under the auspices of independent accreditation bodies. There is a lot of confirmation bias (the tendency to favor information that confirms your beliefs), under the guise that “every athlete is guilty, we just can’t prove it.” Evidence-based studies in respect to sport performance-enhancing effects and health effects are often missing. They may be under- or overestimated, but because of a lack of adequate studies, this can’t be confirmed or denied.” Unfortunately, anti-doping control does not solve the problem of doping, but rather shifts it to the abuse of sometimes more dangerous substances. Pharmacological substances still under investigation, not yet approved for clinical use, or even disapproved for clinical use, are becoming of interest. Given that the latest doping drugs are designed to be undetectable, the historical (and very long) list of prohibited substance must be constantly updated and evaluated. The list of prohibited substances and methods originated in the 1960s, without clear objective reasoning. Only afterwards was the reasoning objectified, with criteria written to extend the
list. In the early days, the list was evaluated and extended by the International Olympic Committee (IOC) mainly based on scientific grounds, but WADA’s current list is also influenced by political arguments. Politicians have more influence on WADA than they ever had within the IOC, because they finance 50 percent of WADA’s budget. For that reason, a relatively low threshold for a cannabinoid was maintained on the prohibited list longer than could be justified scientifically. Only very recently, was its low threshold upgraded from 15 ng to 150 ng/ml of urine. Analytical science is not infallible, indeed margins of error must be tolerated in all scientific data. For athletes, false positive tests for doping are a huge concern (while the “victims” of false negative results are unlikely to complain). Our panel holds that athletes who challenge a test result are treated unrighteously. Anti-doping regulation is such that the laboratory result is assumed to be reliable. One can only appeal on procedural grounds, that is, was the test properly carried out? As long as the principle of strict liability is applied to athletes (meaning that they are responsible regardless of whether they were aware or not), “fair” has a very special meaning. This is especially so if one measurement and, in theory, even one molecule, is enough to sanction an athlete. Anti-doping authorities reveal as little detail about their analytical strategies as is legally required because of potential abuse of those details by dishonest athletes. Thus, the anti-doping authorities hide themselves behind regulations, which they themselves set up and write. Thus there are several part of the procedure worth criticism:

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Criminal Trials</th>
<th>Sports Doping Hearings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burden of proof?</td>
<td>On the prosecution</td>
<td>Defence must prove innocence</td>
</tr>
<tr>
<td>Right to an attorney?</td>
<td>Yes</td>
<td>No, if cannot afford</td>
</tr>
<tr>
<td>Right to a jury trial?</td>
<td>Yes</td>
<td>No (three-member panel from WADA list)</td>
</tr>
<tr>
<td>Proof of chain of custody?</td>
<td>Yes</td>
<td>No (panel decides on admission)</td>
</tr>
<tr>
<td>Hearsay testimony?</td>
<td>No</td>
<td>Yes (decision by panel)</td>
</tr>
<tr>
<td>Reanalysis by another lab?</td>
<td>Option for defence</td>
<td>No (only the same WADA-certified labs)</td>
</tr>
<tr>
<td>Rebuttal testimony?</td>
<td>Yes - after passing voir dire</td>
<td>Not permitted by WADA lab analysts</td>
</tr>
<tr>
<td>Lab’s proficiency tests?</td>
<td>May question past performance</td>
<td>WADA will not divulge</td>
</tr>
</tbody>
</table>

The WADA mission

Before changes can occur at the laboratory and analyst level, there must be changes at WADA. This can begin with the mission statement: WADA and its accredited laboratories must only be concerned with monitoring those substances that can have a positive effect on athletic performance. For example, can it be clearly shown that marijuana use positively affects athletic performance? Also, there must be established cut-off levels for every banned substance. Below these levels, any indications of their presence should be considered neither capable of improving performance nor an indication of an athlete’s intention to cheat. For example, traces of clenbuterol were found in a urine sample from the cyclist, Alberto Contador. Whether these traces were or were not the result of eating meat obtained from Spain should not be the question. More importantly, about 50 percent of the convictions are questionable because thresholds are not being applied. The use of party drugs outside competition is expressly allowed in anti-doping rules; it is the negligible trace found in-competition that leads to a conviction: “Drink a few alcoholic beverages on Friday and played a match on Saturday? Not a problem, because there’s a threshold for alcohol. Smoked cannabis on Wednesday, and played a match the following weekend? Positive test and a sanction!” In theory, athletes are stakeholders of WADA and therefore have influence on the development of regulations. In practice, they are not well organized or well represented.
Analytical scientists within anti-doping laboratories also limit themselves to regulations and claim that it is not their task or responsibility to sanction. However, if analytical progress is pushing identification limits lower and lower, the science will play an increasingly important role. Analytical scientists are also stakeholders in WADA and can exert their influence and provide proper advice – while doing so, they should keep the dilemma of fair chance in their minds. This is a social responsibility, rather than their analytical responsibility [13778].

Sport and the history of ethics in sports medicine

Sport has always played an integral role in society and naturally serves as a vehicle for education, health, leadership and fair play. Fairness is one of the core principles of sport and can be a metaphor for everyday behaviour in life and communities. Whether the principle is adhered to depends on how the sport is managed, taught and practised. Sport has clearly become a global enterprise as well as a recreation for billions. In the early Olympic Games, victors were crowned with wreaths from a sacred olive tree and marched around the grove to the accompaniment of a flute in the admiration of the crowd. Nowadays, athletes can demand lucrative sponsorship contracts and appearance fees, and within moments of their triumph, their faces are found plastered on billboards and advertisements around the world endorsing some product or other. International sport dates back to the 19th century, and the commercial exploitation of sport is even older than that. What is new is the degree of commercialisation and its spread to the emerging markets. As a result, the value of victory in monetary terms has never been greater. The pressure on athletes to win has increased considerably and so too have the demands on sports-medicine doctors to facilitate these victories through fair means or foul [10269].

The adversarial relationship between sporting performance and patient welfare is not a new phenomenon and stretches back to early Greek and Roman civilisation. Aelius Galenus, one of the forefathers of sports medicine, served as a physician to the gladiators in Pergamum in AD 157. In this capacity, he learnt the importance of diet, fitness, hygiene and preventive measures, as well as living anatomy and the treatment of fractures and severe trauma. His fastidious attention to gladiators' wounds resulted in a significant reduction in the mortality, when compared with his predecessor. In spite of this, he argued vehemently against the immoderate lifestyle of athletes and their obsession with victory, which he believed was unhealthy and potentially dangerous behaviour. He wrote a small work called 'That the Best Physician is also a Philosopher,' and he saw himself as being both. His theories dominated and influenced Western medical science for well over a millennium [10269].

When there are conflicting principles it is not always easy to decide which principle should dominate. The principles framework does not take into account the emotional element of human experience. Another approach to bear in mind is the concept of virtue ethics, which emphasises the character of the practitioner, or moral agent, as the key element of ethical thinking. This approach holds that morality stems from the identity and/or character of the individual, rather than being a reflection of the actions of the individual. Once again, much of the teaching on virtue ethics is derived from the ancient Greek philosophers. Aristotle believed that a virtue lay at the centre point between two divergent vices and referred to it as "the mean by reference to two vices: the one of excess and the other of deficiency." Courage, for example, lies between foolhardiness and cowardice. Compassion lies between callousness and indulgence. Plato believed in the Four Cardinal Virtues; wisdom, justice, fortitude and temperance. Beauchamp and Childress considered there to be five virtues which were applicable to the medical practitioner: trustworthiness, integrity, discernment, compassion and conscientiousness. Yet, there is no comprehensive list of virtues. The
Scottish philosopher, MacIntyre, believed that any account of the virtues must indeed be generated out of the community in which those virtues are to be practised. His approach also seeks to demonstrate that good judgement emanates from good character. The application of virtue ethics to the sports medicine field may have some advantages over the principles approach. It considers the motivation of the team doctor (agent) to be of crucial importance. Ethical decision-making hinges on the characteristic virtuous disposition of the team doctor who typically wants to behave well and in the best interest of the player. As there are no strict rules to be obeyed, it permits the adaptation of choices to the particulars of a situation and the people involved. This flexibility promotes creative thinking and problem solving to deal with complex dilemmas. In applying virtue ethics, it is important to be aware that tragic dilemmas can rarely be resolved to the complete satisfaction of all parties and that any conclusion is likely to leave some remainder of pain and regret [10269].

Multiple obligations

Multiple obligations are most obvious when sports medical practitioners have an employment relationship that places demands on them while traditional obligations to their patients remain. For example, the desire of the coach to succeed can result in pressure on a sports medical practitioner to return an athlete to the field of play before medically indicated. Here the medical practitioner has an obligation to his employer, but also to the athlete. However, individual sports physicians varied in their perceptions and management of the complexity of their environment and their multiple obligations [09386].

As an employee, a sports doctor has obligations to their employer, but also professional and widely accepted obligations of a doctor to the patient (in this case the individual team member). The conflict is evident when sports doctors are asked by an athlete to keep personal health information confidential from the coach and team management, and yet both doctor and athlete have employment contracts specifying that such information shall be shared. Recent research shows that despite the presence of an employment contract, there appears to be a wide range of behaviours among sports doctors when an athlete requests that information about them be kept from team management. Many seem willing to honour requests to keep health information about the athlete confidential, thereby being in breach of the employment contract, while others insist on informing team management against the wishes of the athlete. There are a number of potential solutions to this dilemma from forcing doctors to meet their contractual obligations, to limiting the expectations of the employment contract. One paper suggests that at times it may be appropriate to do both, making the position of the doctor clearer and supporting the ability of this group to resist pressure by coaches and management through having a robust code of ethics [08445].

Attitudes on anti-doping

The aim of one study was to determine if there is a relationship between attitudes toward doping and achievement goal orientations of athletes. Questionnaires from 830 athletes (including 263 females) were obtained. Athletes declared moderately positive attitudes, with attitude toward anti-doping controls as the strongest and attitude toward sanctions as the weakest. Females declared significantly more favourable attitudes than males. With respect to the relationship between goal orientations and attitudes toward doping, it was found that athletes who were high task/low ego oriented declared the most favourable attitudes, while athletes who were low task/high ego oriented declared the least favourable attitudes. Multiple regression analyses confirmed that ego orientation was significantly negatively related to,
and task orientation was significantly positively related to attitudes toward doping. It means 
that with the increase in task orientation, attitudes toward doping became more favourable. 
The opposite can be said about the increase in ego orientation. It could be argued then, that 
creating a motivational climate which promotes task orientation (mastery climate) may aid 
anti-doping efforts [08446].

**Hypotheses on background of doping**

There is no reason to expect fundamental differences in the cognitive or motivational process 
involved in the athlete’s decision cycle whether it deals with acceptable performance 
enhancing or doping methods. Both need a sustained, motivated, goal-directed action. The 
difference is brought about by the current convention of the sport, in particular whether or not 
the method is deemed to be acceptable or unacceptable in it [08447].

Strelan and Boeckmann’s model of hypothetical doping use posits that personal moral beliefs 
and health concerns act as preventing factors, whereas drug testing and sanctioning have 
little deterrent effect [08448]. Interestingly, in a search for predicting factors of steroid use, no 
significant difference was found in the characteristics of the steroid users and those who 
were offered but reportedly declined the drug [08005]. Availability or access to performance 
enhancing drugs is perceived by athletes as a barrier they must overcome if they are 
determined to use such means [08449].

The degree of rationality in doping decision making is highly debated. Economic models of 
doping mainly assume that athletes act according to economic rationality. The literature in 
this area [08442-08446] considers doping as a special case of a “prisoners’ dilemma”, where 
one actor’s action has consequence for both actors and the best collective strategy is difficult 
to reach due to lack of information on, and trust for, the other actor’s decision. To translate 
the dilemma into sports, athletes’ best case would be to compete at doping-free events. 
However, the widespread suspicions and speculations about other athletes’ possible actions, 
coupled with the lack of information about the others’ doping behaviours have the potential to 
bias most athletes in favour of doping: game-theoretic modelling suggests that the majority of 
competitors are likely to see doping as their best option and, under certain circumstances, 
the only feasible strategy to ensure winning [08450].

Whilst economic models of doping have ignored individual dispositions toward doping when it 
comes to decision making, they emphasise the importance of a broader situational context, 
within which decisions are not only made on individual preferences but in consideration of 
others’ actions. Existing behavioural doping models have made attempts to incorporate 
personality, decision making rationality and situational context, including peer group and 
subculture influences [08451-08457].

The widespread medicalisation of life creates an environment in which using medical or 
chemical assistance to life is widely accepted and normal. Many athletes believe that they 
need assistance in order to cope with the physical demand of training, workload, injury and 
recovery. Under the current anti-doping regulation, athletes may turn to performance 
enhancing methods and substances that are not prohibited in order to eliminate the risk of 
being caught, play by the rules and conform to the ethical or moral principles imposed on 
them by their social environment [08458].

One of the more sensational and oft-cited studies in the sports medicine literature and 
popular media on doping in sport is the Faustian bargain offered by Goldman’s dilemma. In
Goldman’s dilemma, elite athletes are asked if they would take a drug that guaranteed sporting success but would result in their death in 5 years time. The first iteration of Goldman’s dilemma was posed to 198 world class athletes in 1982 in which 52 percent (103/198) answered in the affirmative. Goldman continued to pose evolving and improved variants of the initial dilemma and expanded the subject pool with bi-annual surveys from 1982 to 1995. Goldman notes the results have been the same each time the study has been run; approximately half of the athletes accept the deal to take the drug and win, but die within 5 years. It was now again tested the Goldman’s dilemma on a general population sample by asking whether they would take the Faustian bargain of a drug that guaranteed sporting success but would result in their death in 5 years’ time. Between 1982 and 1995 a bi-annual survey using this dilemma suggested half of all elite athletes would take the drug. Respondents were presented with one of two differently worded Goldman dilemmas, counterbalanced for presentation of success and death outcome: “Would you take an illegal performance enhancing drug that was undetectable:

- condition 1: “and guaranteed you would win an Olympic gold medal, if it would kill you in five years?” (n=125, 65 % male, mean age 34 years)
- condition 2: “but going to kill you in five years, if it guaranteed you won an Olympic gold medal?” (n=125, 59 % male, mean age 33.4 years)

In both condition 1 and condition 2 only one out of the 125 respondents gave a positive response (0.8 %). The size of the discrepancy between 50 percent and 0.8 percent negated the need for statistical testing. Analysis by demographic or sports engagement would be meaningless given the low rate of positives. The results provide compelling evidence that athlete responses to the Goldman dilemma vary markedly from the general population. The results raise serious concerns about the reliability of official prevalence rates. The consequences of these concerns become even more profound when considered in the context of the 2009 revision of the World Anti-Doping Code (WADC), in which the culpability of support personnel (such as sports medical practitioners) is placed as equal to and sometimes above that of the athlete. Testing the Goldman dilemma on a control group raises a problem for prevalence rates. The rate of temptation among athletes suggests that if any non-trivial proportion (say, 10 %) succumbs the incidence of performance-enhancing drug use in sport may be well above that reported by anti-doping agencies around the world (typically <2 %). However, in the absence of a reliable epidemiology of sport estimates of performance-enhancing drug usage remain educated guesses. The epidemiological ignorance raises serious concern. First, the testing regimes of the World Anti-Doping Agency and the National Anti-Doping Organisations must be questioned as the anecdotal evidence, including the Goldman dilemma, indicates a much higher use rate. Therefore, the tests are either flawed in administration (athletes can avoid tests or manage their drug use) or accuracy (laboratories cannot detect substances or the limits are too high). The strongest evidence of the problems with testing comes from high-profile doping cases in Europe (Madrid Cycling) and the USA (BALCO). Marion Jones was the highest profile athlete caught as a result of the BALCO investigation and has been struck from the Olympic record books. Importantly, it was investigation rather than testing that caught Jones – who had returned negative results for banned substances at the Sydney Olympics. This raises a profound challenge to anti-doping education and enforcement as the official prevalence rates appear to reflect the unlucky or pharmacologically unsophisticated and fail to reflect actual use. A complementary explanation is that the elite athlete subpopulation is a discrete group with a set of norms, values and beliefs that are quite different to those of the wider population. This is a compelling argument given anecdotal evidence of the obsessive and perverse nature of elite athletes. This body of evidence indicates that athletes, to reach the elite level, must display a singular focus and desire often to the exclusion of other life-affirming activity. Furthermore, the intense desire to win, fuelled by this commitment may make it more likely
that they would accept such a bargain. We do know that some athletes will accept such a deal without the guarantee of success, as shown by those who are caught. It is important for sports medicine practitioners and sports psychologists to recognise that athletes demonstrate this alarming flaw in health decision-making when winning is given precedence over survival. Part of the explanation for this alarming flaw in health decision-making comes from a growing literature that recognises the peculiar social circumstance of the athlete [09387].

Youth and adolescents

Until recently, much of the extant literature of anabolic steroids use in youth and adolescents focused on participation in organised sport as the primary risk factor, based on the historical relationship between sports and AAS use and the face validity of the AAS use and “win at all costs” relationship of elite sports. However, emerging evidence indicates that this model may be outdated, prejudicial to young athletes and, ultimately, detrimental to adequately addressing the use of AAS in the children and teenagers because it fails to recognise AAS use in this population as a public health issue with more complex mechanisms driving it. It has been found health-, social- and personality-related factors including average/low self-esteem and school achievement, and high immigrant status to be “significantly and independently associated with lifetime use of AAS in male adolescents.” Expanding this approach, it has been presented three theoretical models:

- the sports perspective (motivation for use is related to desire to win)
- the “ideal body” perspective (to increase self-concept and/or be more attractive to potential partners)
- “problem behavior” theory (anabolic steroid use is part of a larger syndrome of problem behaviours).

Results indicated that “AAS use was first and foremost associated with types of problem behaviours, that is, drug involvement and aggressive-type conduct problems” and there “was no difference in AAS use among sports competitors and noncompetitors,” although involvement in power sports was associated with increased lifetime prevalence. In another study it was found that “use of anabolic steroids was associated with the use of other forms of drugs, which indicates that use of anabolic steroids may be part of a general pattern of drug use and risk-taking behavior” and not driven by the pursuit of athletic success. It has also been reported significant associations between AAS use and other illicit drug use as well as a variety of deviant behaviours, in line with problem behaviour theory, that were consistent across six European countries. They also examined the association between lifetime AAS use and sports participation. However, this variable was confounded by the inclusion of exercising in the participation question. Although a significant association between AAS use and daily physical exercising was identified (odds ratio 1.4, 95 % confidence interval 1.06 to 1.85), it is not possible to discern the independent effect of organised sports participation on AAS use as exercising to improve appearance occurs in non-sports playing AAS users. Previous studies that examined the relationship between sports participation and AAS use may also be compromised by confounding sports participation with non-specific exercising or physical appearance criteria. It has been concluded that high school athletes were significantly less likely to use AAS than those not participating in sports, and males who used AAS were significantly more likely to use other drugs. Improvement in appearance was cited as the main motivation for AAS use [09024].

Environmental factors
Environmental factors include the socio-cultural, political milieu, the legislative system, as well as the availability of drugs, new drug discoveries and permitted alternatives, such as nutritional supplements, minerals, herbs and non-herb non-mineral substances. The importance placed on sporting success in a society and the direct and indirect reward for such success influence the motivational climate in which athletes prepare and compete. Criminalisation or decriminalisation of the performance enhancing drugs may only have an effect on the level of use via the distribution mechanism. Studies show that decriminalisation of social drugs do not have the effect on the prevalence rate [08459-08462].

**Anti-doping programs**

Factors that may avert individuals from using performance enhancing drugs are related to the current punitive-sanctioning anti-doping system, cultural – religious norms, moral values, social pressure from close relatives and friends and health concerns. Some personality traits can act as inhibitors of doping engagement (e.g. positive and stable self-esteem, conscientiousness and low risk-taking propensity). Intervention as a preventive factor has been the most extensively researched aspect. Gender specific, team centred education has been shown to result in self-reported behaviour change in the Adolescents Training and Learning to Avoid Steroids (ATLAS) [08462, 08463], Athletes Targeting Healthy Exercise and Nutrition Alternatives (ATHENA) [08464, 08465] but effectiveness of such education programmes should be validated beyond self-reports.

**The life-cycle model of performance enhancement**

The current anti-doping policy has received criticism for its elite focus, sanction-based approach and associated costs [08466-08470]. However, the growing number of educational programmes are designed and implemented by the sport governing bodies to target varying groups from top performing athletes to young talents focus on the principles of performance enhancement and fair play. A holistic approach with the emphasis on choices, health issues and broader life goals to the individual and the systemic factors is needed in order for athletes to make informed decisions about their performance enhancement, which may lead – at the population level – to a sustainable change in doping behaviour. Overall, anti-doping interventions are likely to benefit from a dual focus on the risk factors and doping expectancies, as well as from targeting the athlete population from preadolescents to adults at all stages of their athletic life-cycle [08446].

The use of performance enhancing methods is unlikely to be an accident. It requires sustained, self-regulated, goal-directed effort [08471]. Doping use is assumed to exhibit similar characteristics to “functional drug use” which has been recognised as a specific from of drug use [08472, 08473]. Functional drug use is distinct from experimental, recreational or dependent use (abuse/addiction) and it refers to a strategic use of substance to achieve a set goal (i.e. improve a function or skill). Examples for functional use include but are not limited to using stimulants to increase alertness or balance long working hours; or taking hypnosedatives to help coping with anxiety, stress or depression. Functional drug use is not necessarily problematic in the sense of addiction although physical and psychological dependence may develop from functional use. Athletes’ reasons for taking performance enhancing substances are in keeping with the definition of functional drug use. One of the main advantages of the life-cycle model over the traditionally used traitor situational models is that the developmental nature of the proposed model offers various intervention points and suggests a varying set of methods to match those points [08446].

Chronologically, the first intervention points focus on the risk factors by preventing the onset of risks or transforming risks that are already present. Whilst these options are considered
less effective in substance abuse, the unique nature of performance enhancing substance use offers scope for intervention at this stage by changing not the athlete but the environment that influences the onset of doping use. With that in view, the responsibility for deterrence is broadened from individual athletes to the inter-related system of rules, regulations, and expectations by coaches, support personnel and policy makers. An intervention approach that aims to alter expectancy trajectories or modify current maladaptive expectancies (e.g. in relation to steroid use which is prone to developing physiological and psychological dependence) is likely to produce more lasting effects. Based on research on substance abuse [08474], it is envisaged that preventing the development of positive doping expectancies before the onset of a doping-related event may work well with pre-adolescents, whereas athletes with doping experience or in positions to seriously contemplate the use of prohibited methods may respond better to modifications of expectancy pathways, especially if comparable and acceptable alternatives are offered [08432].

A gold medal but also death in 5 years’ time?

The “Goldman dilemma” is one of the most cited results in the antidoping literature. The dilemma presents a Faustian bargain to athletes, asking if they would trade longevity for Olympic success by taking a drug that not only guaranteed a Gold Medal but also their death in 5 years’ time. Goldman is reported to have presented this dilemma to world-class athletes biannually between 1982 and 1995. He reported a remarkably stable set of results with about half accepting the gold for death deal. There has been little in the way of replication of the Goldman dilemma since 1995, with sporadic adaptations for different contexts showing athletes of various levels to be less likely to take the bargain. Despite the extensive reporting of Goldman’s results and the adaptations, questions remain around the validity and reliability of the dilemma to accurately capture an athlete’s willingness to trade longevity for Olympic success. The first weakness of Goldman’s work is that no comparable measure of acceptance exists among the general population. That is, there are no data to suggest whether the athletes are responding in the same manner or differently to members of the general population. Contemporary data suggest that the general population take a very conservative approach to the dilemma; out of a representative sample of n=250 Australians, only two respondents accepted the bargain. That is, if athletes respond to the dilemma in the same way as the general population, approximately 1 percent of athletes would take the Faustian bargain. The second weakness in Goldman’s work is found in the wording of the questions. The question presented the outcome (Olympic gold) followed by the consequence (death). The dilemma responses may thus represent a positive response bias as a function of wording, necessitating replication using the counterbalanced presentation. There is a growing literature on the social circumstance of sport and the drivers of behaviours defined as “illegal”, which indicates that athletes, to reach the elite level, must display a singular focus and desire often to the exclusion of other life-affirming activity. Further, the intense desire to win, fuelled by this commitment, may make it more likely that they would accept such a bargain. It is clear that some athletes will accept such a deal without the guarantee of success, as shown by those who are caught doping. The current study provides data on the proportion of athletes willing to accept the Faustian bargain offered by misuse and abuse of performance-enhancing drugs. This research evaluated the dilemma with contemporary elite athletes. Participants at an elite-level track and field meet in North America were segregated into an interview or online response. After basic demographics, participants were presented with three variant “Goldman” dilemmas counter-balanced for presentation order. Only 2 out of 212 samples (119 men, 93 women, mean age 20.89) reported that they would take the Faustian bargain offered by the original Goldman dilemma. However, if there were no consequences to the (illegal) drug use, then 25/212 indicated that they would take the substance (no death condition). Legality also changes the acceptance rate to 13/212 even
Regression modelling showed that no other variable was significant (gender, competitive level, type of sport) and there was no statistical difference between the interview and online collection method. The results show that the proportion of athletes willing to take the Faustian bargain offered by the Goldman dilemma has changed significantly, approximating the proportion observed in the only sample of general population responses. This indicates that responses to the dilemma from 1982 to 1995 should no longer be taken to reflect the approach taken to the use of drugs in sport by contemporary athletes. The counterbalancing of question design had no effect on acceptance of the bargain, and neither did the response format. Future research on the dilemma can use any version of the wording in an online survey. Athletes were sensitive to the consequences of performance-enhancing drug use (death vs non-death) and the legality of substances. This was consistent with other work showing that athletes are sensitive to the health and legal implications of using performance-enhancing drugs. The radical change in proportion of athletes willing to accept the dilemma is explained in two ways, although others are equally plausible alternative explanations. The problem with defining an explanation in this context lies in the significant changes in the social and sportive contexts. Attempting to explain them fully is to attempt to describe the evolution of drugs in sport since 1982. The first explanation flows from the significant impact of the consequences and legality on acceptance. The social context of the original Goldman results may have had a very different understanding of the consequences of using performance-enhancing drugs. There is also the possibility that the subset of athletes (power-sports) that Goldman approached is not representative. Doping knowledge was usually obtained informally via word-of-mouth or underground handbooks rather than medical advice, and was often erroneous. As a result, the consequences of doping were uncertain. Unsupervised experimentation with erythropoietin among endurance athletes revealed the danger of misuse and abuse. In terms of legality, the epoch around the original Goldman results was one of uncertainty arising from imprecise testing technology. The second explanation flows from the social marketing of antidoping. The original studies were conducted during the debate around the role of drugs in sport, which led to the current legalistic prohibitionist model. The moral stance on the role of drugs in sport was being thrashed out and was therefore ambiguous. Contemporary athletes now contend with an ethically unambiguous (although arguably morally ambiguous) statement that “doping is fundamentally contrary to the spirit of sport”. The current study shows that the results collected in 1982–1995 no longer have relevance in the contemporary context. The wisdom that athletes are willing to die to win applies to only a handful of athletes. Put simply, the sensational reporting of the 1982 to 1995 responses to the Goldman dilemma is no longer relevant to the contemporary debate around the role of drugs in sport [13770].

Prevention of athletes from harm

The prevention of harm to the athlete, the guarantee of fair play, and a level playing field for all competitors are the basis of the current anti-doping strategies. As healthcare systems are forced to allocate increasing resources to prevent and treat the prevailing pathologies worldwide, funding for anti-doping campaigns will necessarily be restricted. Ideally, additional resources should be devoted to increasing the number of athletes tested, the panels of tests used, and the frequency of out-of-competition controls. Since doping prevention cannot be considered a priority for most healthcare systems, such an approach is unaffordable and an alternative framework should be devised, focused primarily on harm reduction rather than fair play. The identification of abnormal deviations from reference values, regardless of pathological or artificial causes, would allow the athlete to be followed and tested using conventional laboratory tests, which are affordable to governments and healthcare systems and available to clinical laboratories. Although this strategy would not detect cheating, it would safeguard athletes' health [08475].
It was discussed the assumptions underpinning the current drugs-in-sport policy arrangements by examine the assumptions and contradictions inherent in the policy approach, paying particular attention to the evidence that supports different policy arrangements. It was found that the current anti-doping policy of the World Anti-Doping Agency (WADA) contains inconsistencies and ambiguities. WADA's policy position is predicated upon four fundamental principles; first, the need for sport to set a good example; secondly, the necessity of ensuring a level playing field; thirdly, the responsibility to protect the health of athletes; and fourthly, the importance of preserving the integrity of sport. A review of the evidence, however, suggests that sport is a problematic institution when it comes to setting a good example for the rest of society. Neither is it clear that sport has an inherent or essential integrity that can only be sustained through regulation. Furthermore, it is doubtful that WADA's anti-doping policy is effective in maintaining a level playing field, or is the best means of protecting the health of athletes. This means that the WADA anti-doping policy is based too heavily on principals of minimising drug use, and gives insufficient weight to the minimisation of drug-related harms. As a result drug-related harms are being poorly managed in sport. It was argued that anti-doping policy in sport would benefit from placing greater emphasis on a harm minimisation model [08476].

**On cheating**

One frequently used argument in the discussion on human enhancement is that enhancement is a form of cheating. This argument is well-known in relation to doping in sports, but recently it has also been used with regard to cognitive enhancement in the context of education and exams. One paper analysed the enhancement-is-cheating argument by comparing sports and education, and by evaluating how the argument can be interpreted in both contexts. If cheating is understood as breaking the rules in order to gain an unfair advantage over others, it can be argued that some enhancements are a form of cheating. This problem of cheating is, however, relatively easy to remedy by either changing the rules, or by instituting controls and sanctions. This does not, therefore, constitute a categorical objection to enhancement. A further analysis of the intuitions behind the enhancement-is-cheating argument, however, shows that if sports and education are understood as "practices", with their own internal goods and standards of excellence, some potential problems of enhancement can be articulated. These concern the internal goods and standards of excellence that are characteristic of specific practices. Seen from this perspective, the important question is how enhancement technologies might be embedded in specific practices – or how they might corrode them [08477].

**Different of shades of blood doping**

Blood doping (reputedly favoured by cyclists) exemplifies the shades of grey, as well as some of the problems of detection: transfusion of heterologous packed blood cells with intent to boost athletic performance rather than to treat intercurrent illness seems eminently unfair (and easy to detect), autologous transfusion of one’s own harvested and stored blood seems no better (though harder to detect), injecting oneself with epoietin is cheating (and can be detected through differences in the exogenous and endogenous human hormone), training at high altitude when one’s home is say in Ethiopia is hard to find fault with, but how about sleeping in a low oxygen tent at night – and how could this be detected after the event? This raises the semantic question of whether oxygen is a drug, and if so whether lack of oxygen – and hence an inert displacing gas such as nitrogen – is also a drug. If a bedroom is shared
with the “wrong end” of an oxygen concentrator which delivers oxygen to a patient with chronic obstructive pulmonary disease in the adjacent room would she be receiving therapy but I am guilty of a lifestyle offence by virtue of the erythropoietin I secrete in response to the partly oxygen-depleted nitrogen that is breathed overnight? [12003].

The grey zone of undiscovered doped

An unknown number of athletes likely remain undiscovered and get away with some forms of doping. This is because of the limits to surveillance and laboratory testing technology. The ideal situation would be black and white: the forbidden substance is present or is not present in a urine or blood sample. Those two extreme cases exist, but there is, depending on the substance, often a large area of uncertainty. A test can be positive (showing the presence of a substance) when it is indeed present (true positive) or not present (false positive); conversely, a test can be negative when there is indeed no substance present (true negative) or when in fact the substance is present (false negative). Anti-doping policy enforcers need to keep false positives as low as possible, while striving for the highest sensitivity possible. The probability for false positives rises with the number of tests performed, as well as with a drop in prevalence of actual doping. Furthermore, for some forms of doping practices there exist no laboratory tests. WADA does not want to publish WADA-accredited laboratories’ test performance, saying that this would permit athletes tailoring doping practice to current testing technology. At first sight, this seems reasonable, but, at the same time, it leaves room for doubt about the impartial nature of anti-doping testing. The absence of transparency is not a good gatekeeper for quality assurance [12012].

Perversely, anti-doping is thus limited in its scope since, in fine, a “clean” sample will never allow to fully exclude doping, while occasional sacrifice of innocent athletes from false positives appears inevitable. Even if anti-doping efforts certainly have changed current doping practices – certain types of doping cannot be used anymore because too easily discovered – the purpose of anti-doping, the celebration of clean athletes with a strong degree of confidence, or even certainty, thus remains an unattainable objective. One is forced then to question whether the champions are clean; a question that unfortunately remains unanswered. So, if the principle of the anti-doping rule may initially seem simple, one can see that its implementation is complicated, very technical, highly costly, only partially successful and condemns athletes who did not dope or had no intent to dope [12012].

Enforcement of anti-doping policy today

Anti-doping is enforced by a combination of repression and surveillance. The latter includes the so-called “whereabouts” rule, or the obligation for a selected pool of elite athletes to inform the anti-doping authorities where they will be each day of the year, to allow unannounced out-of (and in)-competition testing, with the obligation to be present at the announced site for one specific hour per day. The athletes have to provide this information to the authorities in advance, four times a year for three months periods at a time, using electronic and paper-based means and informing in time of any changes. This rule aims at preventing out-of-competition doping in preparation for competition. To force athletes to comply, three missed tests within an 18-months period constitute a doping offence, as happened in the Ohuruogu and Wickmayer cases mentioned, and regularly occurs for other athletes. The actual testing involves providing urine samples (produced in full view by an anti-doping officer), consenting to blood sampling, and in some instances also providing hair samples for doping history and tissue for gene profiling for forensic practices. Longitudinal
testing, looking for fluctuations in certain blood parameters compatible with doping, is now also being introduced. This practice, known as the athlete biological passport (ABP), has recently led to the first indictments of athletes, based on indirect indices of presumed doping rather than laboratory tests directly showing the presence of the forbidden substances or their metabolites in urine or blood. The authorities see the ABP as an improvement of anti-doping. But the ABP may produce false-positive results due to analytical variability and outlying individual patterns resulting from the effects of behaviour (training, altitude exposure) and genetics [12012].

Mostly related to its enforcement strategies, anti-doping has a non-negligible cost. The IOC finances half the budget of the WADA, while the other half comes from national governments. National anti-doping agencies are mostly co-financed by national sports federations and governments. Overall the tendency is towards increasing costs with a new costly anti-doping industry steadily asking for more. The application of new national anti-doping legislations also comes with an increase in cost.Taken together, all of these costly surveillance practices seriously impinge upon the privacy of athletes and set them apart from the general population, for whom the protection of the private sphere and autonomy are generally respected in democratic societies [12012].

**Smart drugs (“brain doping”)**

Debates about the feasibility of cognitive enhancement rarely begin with what seems to be a pertinent question: How effective are cortical networks in performing the complex steps underlying serial thought, planning, memory retrieval, and other operations that go into cognition? If the substrates are not particularly efficient, then there should be numerous opportunities for improvement. Conversely, networks that are finely tuned with regard to cognition would presumably not be amenable to selective enhancement, at least with current technologies. Another natural question is whether improvements in one dimension of performance (e.g. speed, or accuracy) will necessarily lead to improvements in others (e.g. creativity, or judgment). The reason that these points are not generally discussed is, of course, that, despite enormous advances in neuroscience over the past few years, we still know very little about the neurobiology and operating characteristics of cognition-related networks. But perhaps the room for improvement issue can be recast in terms of brain evolution by asking whether comparative anatomical evidence points to strong adaptive pressures for designs that are logically related to improved cognitive performance [11434].

Whether drugs that enhance cognition in healthy individuals will appear in the near future has become a topic of considerable interest. It was addressed this possibility using a three variable system (psychological effect, neurobiological mechanism, and efficiency vs capabilities) for classifying candidates. Ritalin and modafinil, two currently available compounds, operate on primary psychological states that in turn affect cognitive operations (attention and memory), but there is little evidence that these effects translate into improvements in complex cognitive processing. A second category of potential enhancers includes agents that improve memory encoding, generally without large changes in primary psychological states. Unfortunately, there is little information on how these compounds affect cognitive performance in standard psychological tests. Recent experiments have identified a number of sites at which memory drugs could, in principle, manipulate the cell biological systems underlying the learning-related long-term potentiation (LTP) effect; this may explain the remarkable diversity of memory promoting compounds. Indeed, many of these agents are known to have positive effects on LTP. A possible third category of enhancement drugs directed specifically at integrated cognitive operations is nearly empty. From a neurobiological perspective, two plausible candidate classes have emerged that both target
the fast excitatory transmission responsible for communication within cortical networks. One acts on nicotinic receptors (alpha7 and alpha4) that regulate release of the neurotransmitter glutamate while the other ("ampakines") allosterically modulates the glutamate receptors mediating the post-synaptic response (EPSCs). Brain imaging in primates has shown that ampakines expand cortical networks engaged by a complex task; coupled with behavioral data, these findings provide evidence for the possibility of generating new cognitive capabilities. Finally, it was suggested that continuing advances in behavioral sciences provide new opportunities for translational work, and that discussions of the social impact of cognitive enhancers have failed to consider the distinction between effects on efficiency versus new capabilities [11434].

Anatomists often resort to allometry when dealing with questions of selective pressures on brain regions. Applied to brain proportions, this involves collecting measurements for the region of interest – e.g. frontal cortex – for a series of animals within a given taxonomic group and then relating it to the volume or weight of the brains of those animals. This can establish with a relatively small degree of error whether a brain component in a particular species is larger than would be predicted from that species’ brain size. While there is not a great deal of evidence, studies of this type point to the conclusion that cortical subdivisions in humans, including association regions, are about as large as expected for an anthropoid primate with a 1350 cm³ brain. The volume of area 10 of human frontal cortex, for example, fits on the regression line (area 10 vs whole brain) calculated from published data for a series composed of gibbons, apes and humans. Given that this region is widely assumed to play a central role in executive functions and working memory, these observations do not encourage the idea that selective pressures for cognition have differentially shaped the proportions of human cortex. Importantly, this does not mean that those proportions are in any sense typical. The allometric equations involve different exponents for different regions, meaning that absolute proportions (e.g. primary sensory cortex vs association cortex) change as brains grow larger. The balance of parts in the cortex of the enormous human brain is dramatically different than found in the much smaller monkey brain: area 10, for instance, occupies a much greater percentage of the cortex in man. But these effects seem to reflect expansion according to rules embedded in a conserved brain plan rather than selection for the specific pattern found in humans [11434].

What constitutes a cognitive enhancer? Would this include agents that only secondarily affect cognition via actions on broader psychological variables? Should distinctions be made between drugs influencing psychological processes (e.g. short-term memory) that feed into cognition versus those acting on higher, integrative activities? Rather than trying to reach agreement on such questions, it may be more useful to classify potential enhancers according to multiple dimensions of action. A critical first dimension then involves the issue of “how”, in psychological terms, the treatment acts to change cognition (dimension I). There can be little question that fundamental states such as arousal and alertness affect complex cognitive operations; similarly, it seems only reasonable to assume that drugs with positive effects on psychological operations subsidiary to cognition, such as attention and the encoding of memory would have. A provisional classification scheme for candidate cognitive enhancers with an “x” axis (dimension “I”) could list possible psychological processes affected by the compound. The other two axes indicate neurobiological mechanisms (y; dimension II), and the question of whether the compound affects the efficiency of cognition or allows the subject to exceed normal boundaries (z; dimension III). The axes are collections of associated variables that have no quantitative relationship with each other. Finally, enhancement could, in principle at least, be achieved by actions on the integrated mental activities incorporating planning, cataloguing, memory retrieval, etc. that underlie seconds-long cognitive episodes ("integration"). Neurobiological mechanisms provide a second dimension for defining enhancers (dimension II) and one that helps deal with dimension I
problems that have long plagued preclinical attempts to develop such drugs. Specifically, how can one be confident that behaviors used to assess cognition in animals engage the same psychological processes employed by humans? Most learning tests involve pre-determined (by the experimenter) optimal behavior; similarities between species could result from forcing of very different brain processes to reach the same computational end points. Finally, there is the question of whether a proposed treatment affects the efficiency versus the capabilities of cognition (dimension III). Consider, for example, a complex problem that an alert, healthy individual solves with a given accuracy and with expected improvements over successive trials. An effective enhancer in this instance could reduce errors during early testing and/or the amount of sampling needed to reach asymptotic performance –in essence an increase in efficiency. A somewhat different case concerns the effects of the treatment on asymptotic scores that hold for a large population of over-trained subjects, values that might be thought of as a species limit. Such a limit could also be described as an empirically defined level of problem difficulty at which no member of a large population achieves more than a minimal level of performance [11434].

The introduction of cognitive enhancers would have profound and unpredictable consequences for society. Some authors take a generally positive stance towards the potential for such drugs to enhance human life, arguing they should be generally classed with “education, good health habits, and information technology” as means of cognitive enhancement, but warn that their risks must be identified and managed (neither simply left to the mercies of market forces nor addressed primarily through legislation). It has been discussed potential problems including questions of fairness (whether to those unenhanced in competitive situations, or cost and availability considerations), of safety (both for healthy and non-healthy individuals over time, and particularly for developing brains of children), and of personal freedom: whether the freedom to choose to use cognitive enhancing drugs on the one hand, or the potential for explicit or implicit coercion to improve performance (e.g. by employers, in the military, or in the classroom) on the other. It has been recommended a four-fold approach to managing the risks and promoting the benefits of cognitive enhancement, including increased research into their effects; collaboration among doctors, educators, and regulators to develop appropriate policies; public dissemination of their risks, benefits, and alternatives; and “careful and limited legislative action. Drugs that act on ascending biogenic amine projections and thereby change psychological state will be limited in their use because these state variables are, for reasons discussed, likely to be greater than their actions on cognitive efficiency. The state effects in other words may be the larger, and more familiar, issue for society. But compounds such as modafinil that couple moderate effects on catecholamines with actions on hypothalamic systems and thereby improve attention and working memory without pronounced arousal effects are good fits for the issues raised in the above discussions of social issues. It is suggested that the advantages associated with such drugs are somewhat limited, mainly involving conditions in which sleepiness is a factor or artificial testing circumstances that involve heavy loads on operations that feed into cognition. But there is little reason to think that such drugs will in any sense constitute “smart pills” – something that will give healthy, alert individuals any intellectual advantages in real world circumstances. Agents that selectively enhance components of cognition, and in particular memory encoding, also raise regulatory questions that are described in the above noted reviews. But one would suggest that computational neuroscience points to additional, even more difficult to evaluate societal issues. Specifically, modeling work using biologically realistic networks and empirically derived synaptic learning rules based on LTP strongly suggest that accelerated learning will change the manner in which newly acquired information is organized. The simulated networks not only store memory but also place it into hierarchical categories, without outside supervision. Changing the synaptic rules, the functional effect of selective memory enhancing drugs, not only increases the speed of acquisition in the models but also alters the size of categories (does
this item belong or not) and the number of exemplars needed to form them. These observations lead to the idea that the use of selective memory enhancing drugs could cause people to create cognitive structures of a type that do not occur within the range of normal human experience. Regulatory considerations in this case would not be restricted to acute advantages (e.g., learning specific material for a test) gained by using memory enhancers but would also require weighing the consequences to society of individuals who gradually develop a world view that may differ in unique ways from that shared by the general population. There is no way, given our current very limited understanding of how networks generate cognition, of predicting whether these internal constructions would be more or less effective than the baseline human condition. If benign or positive, then there would have been agents that both accelerate learning and have the potential for producing what would literally be a new way of seeing things. Why would society prevent the introduction of such drugs? Perhaps the most obvious reason goes to the predictability problem noted earlier: would the steady accumulation of novel cognitive architecture eventually affect inter-personal relations, a person's integration into society, emotional life, and so forth? Since cognitive neuroscience can't begin to answer such questions, it will not be possible to develop a risk-benefit analysis for drug effects on normal subjects. But the point can be accommodated empirically by running long-term tests on a population of carefully monitored individuals. Furthermore, devices with cognition enhancing properties have been introduced into the public at least since paleolithic times (e.g. painting) and never more so than today. There can be no question that such inventions have altered the human experience in ways that were unforeseen at their introduction. In the end, only further research and long-term trials can provide the material needed for an evaluation of the opportunities and problems that will accompany the arrival of memory enhancers. Others have considered the potential impact of a "smart pill" with regard to fairness -- who will get the pill? -- and related issues. Humans have mental abilities such as language that are barely detectable in closely related primates and, again, most researchers assume that this reflects the physical expansion of networks in large areas of cortex. The sudden appearance of new abilities would likely have profound and quite possibly irreversible effects on society. They could, for example, lead to concepts and arguments that would be difficult to translate back into the world of normal cognition. And once having experienced new abilities, wouldn't a person be reluctant to give these up and return to what might be perceived as a more limited form of mental life? Questions of this kind could prove to be the most difficult issues in any debate about the regulation of cognitive enhancers [11434].

One article challenged recent assumptions that physicians may ethically and legally prescribe psychopharmacological enhancement drugs to patients and the counterintuitive notion that in some cases ingesting an enhancement drug constitutes the more ethical choice than foregoing this option. Although our society seems to be engaged in a pharmacological arms race of efficiency, competition, and self "betterment," the FDA has not approved stimulant drugs such as modafinil for PESA (performance enhancement and sleep avoidance). The drive to seek out stimulants including modafinil is strongly linked to cultural perceptions related to achievement, accomplishment, and efficiency. Use of modafinil to improve concentration, alertness, or forgo sleep would be classified as an enhancement because it constitutes "an intervention designed to improve human form or functioning beyond what is necessary to sustain or restore good health." Our culture fundamentally values the capacity for autonomy, self-improvement, and contributions to the world. An individual at rest is inherently unconnected to society and viewed through this standard as idle and unproductive. The pressure for enhancement is ingrained in both social and workplace environments, where individuals feel a duty to improve one's self and one's performance capacity and output. As a result, the off-label use of modafinil for PESA means these individuals are part of a large-scale experiment that poses serious immediate and long-term side effects to the individual user as well as society. More importantly, a physician's
practice of prescribing a controlled substance such as modafinil for PESA runs contrary to a physician’s ethical duty to the patient and the standard of practice set forth in legal requirements governing the prescription of controlled substances. Exploiting and ignoring these legal standards of practice will put the patient, the physician, and society at risk for dangerous health and social consequences. Enhancement proponents have touted modafinil as an ideal mechanism to improve concentration, alertness, and forego sleep and keep pace with our society's demands. However, patients who use modafinil for these reasons risk potentially severe side effects and addiction, and face unintended consequences related to their cognitive, emotive, and physiological functioning. Importantly, prescribing a controlled substance such as modafinil for performance enhancement and sleep avoidance runs contrary to a physician’s ethical duty to the patient and the standard of practice set forth in legal requirements governing the prescription of controlled substances. Obtaining a prescription for modafinil serves the purpose of utilizing technology in such a manner to accomplish personal and professional outcomes that we would normally attain through slower and less efficient methods. If this technology will be embraced and continue on the path of the “psychopharmacological arms race” marked by eagerly accepting, and even demanding, drugs to facilitate our progress, then the future population may be “stuck in overdrive, searching out the last bits of competitive advantage, business insight, and radical innovation” [11412].

Currently, few sectors of society openly endorse the use of stimulants for PESA purposes. The military is one arena that directly promotes the use of stimulants (and sedatives) for use in combat or in circumstances of “operational necessity.” If soldiers encounter fatigue during continuous or sustained operations, the military classifies this fatigue as a commodity to be managed. Counter-fatigue training declares that all commanding officers and squadrons should adopt this philosophy by utilizing a combination of stimulants and sedatives to achieve the performance goals of optimal productivity with minimal adverse impact on safety, health, and well-being. Under the US Uniform Code of Military Justice, soldiers are mandated to accept medical interventions that make them fit for duty, which suggests that utilization of PESA drugs is not merely optional, but a job requirement in these circumstances. The military sets forth use indication for situations when a soldier will be placed in an unpredictable setting when attention to detail and sustained vigilance is required for perceived job performance success. Scholars have recently suggested that some proponents of psychopharmacological enhancement will use similar rationale to justify expanded usage of PESA drugs such as modafinil into the civilian sector. Numerous publications connect the professional demands of select military operations to physician residents and surgeons, who may experience fatigue as a barrier to mental acuity and alertness during sustained continuous work. One study examining health care workers who experience fatigue concluded that taking modafinil would improve their professional judgment and performance compared to health care workers who would not take the drug [11412].

Pharmacological neuroenhancement refers to the use of psychoactive substances by healthy subjects with the purpose of cognitive enhancement, e.g. vigilance, concentration, memory, or mood. "Brain doping", however, refers to the illicit use of a subcategory of these substances such as prescription drugs. This subcategory includes psychostimulants (e.g. amphetamines, methylphenidate), modafinil, antidepressant drugs (acetylcholine-esterase inhibitors, memantine), and antidepressants (selective serotonin reuptake inhibitors) which are being prescribed for the treatment of ADHD (attention deficit/hyperactivity disorder), Alzheimer's disease, and depression. Only psychostimulants and modafinil show significant effects on concentration, attentiveness, and vigilance in healthy subjects. However, a general use by healthy persons can not be justified because of relevant side effects and safety risks. Caffeine for pharmacological neuroenhancement can be seen as an equally effective alternative. "Brain doping" raises numerous ethical and social concerns that require a
continued discussion. Demands of liberalization should be critically questioned [10311].

The term neuroenhancement refers to improvement in the cognitive, emotional and motivational functions of healthy individuals through, inter alia, the use of drugs. Of known interventions, psychopharmacology provides readily available options, such as methylphenidate and modafinil. Both drugs are presumed to be in widespread use as cognitive enhancers for non-medical reasons. Based on a systematic review and meta-analysis we show that expectations regarding the effectiveness of these drugs exceed their actual effects, as has been demonstrated in single- or double-blind randomised controlled trials. Only studies with sufficient extractable data were included in the statistical analyses. For methylphenidate an improvement of memory was found, but no consistent evidence for other enhancing effects was uncovered. Modafinil on the other hand, was found to improve attention for well-rested individuals, while maintaining wakefulness, memory and executive functions to a significantly higher degree in sleep deprived individuals than did a placebo. However, repeated doses of modafinil were unable to prevent deterioration of cognitive performance over a longer period of sleep deprivation though maintaining wakefulness and possibly even inducing overconfidence in a person's own cognitive performance [10421].

Memory, attention and creativity represent three different cognitive domains, which are interconnected and contribute the "mental performance" of an individual. Modern neuroscience has investigated some of the neuronal circuits and of the neurotransmitters and molecular events underlying the above-mentioned cognitive functions. Within this renewed reference context, some of the properties of the components of the remedies to increase mental performance have been studied and validated in experimental models and, to date, these substances are named "smart drugs", "memory enhancing drugs" or "nootropic drugs" (from the Greek root noos for mind and tropein for toward). Recently pharmaceutical industries are increasingly focusing on the research for potential substances in this field: several "smart drugs" are in clinical trials and could be on the market in few years. Furthermore, a quick survey from Internet highlights the presence of a great variety of both approved and non-approved drugs, with some of them addressing to only medical and others to performance-oriented use, opening room to some reflections or speculations from scientific and ethical points of view. In order to point out the effect of nootropic drugs on cognition of healthy people, it was reviewed the literature on drug enhancement of various cognitive functions, including memory, attention and creativity. As their simplest, memory is regarded as the ability to remember events or learned material, attention is the cognitive process of selectively concentrating on one aspect while ignoring distracters and creativity could be described as the ability to create products or ideas which are original and which possess a social usefulness. Reports from literature reveal that some medications currently available to patients with memory disorders may also increase performances in healthy people and that drugs designed for psychiatric disorders can also be used to enhance certain mental functions. However, the long-term effects of these drugs are unknown, but their apparent effectiveness allows room to their use and misuse. At variance with these literature data showing scientific, even if poor, evidence of the effect of smart drugs in the field of memory and attention, only indirect information on creativity can be obtained by studies of the effects of diseases and drugs on the artistic productivity of classic painters and famous authors, offering a link to understand the neuronal basis of this cognitive function and a cue to understand how drugs (used to correct the illness) may affect the function [08478].

The subject of cognitive enhancement is always topical and has been considered from numerous different angles. The issues addressed cover concerns over unfair advantage and cheating, indirect coercion, safety, and the practical difficulties involved in regulation. It should be noted that the level of risk in the use of cognitive enhancers will be a key factor in determining the appropriateness and necessity of regulation, and calls, as others have done,
for further investigation into safety and efficacy aspects of cognitive-enhancing drugs in the healthy [09388].

The use of medications to enhance performance in healthy individuals is increasingly being propagated even by neuroscientists under such colorful terms as "neuro-enhancement". A large number of medications, including psychostimulants have been advocated in this context. Recent data from the German health insurance company DAK indicate that a number of employees already take medications to fulfill their professional needs and enhance performance. The use of new drugs in the healthy has even been advocated by prominent neurobiologists in a commentary in a recent edition of Nature. There are a number of ethical objections to this use and there is an apparent risk of addiction. To date German psychiatrists have surprisingly not been outspoken on this issue. The author would like to make an emphatic plea against "brain doping" in healthy individuals [09407].

In ancient Greece, it is said that students would entwine rosemary sprigs into their hair in the belief that it would improve their memory. Although the desire to enhance one's cognitive abilities has not abated since then, modern advances in psychopharmacology now offer the possibility of one day realising this ancient dream. Cognitive enhancing drugs, smart drugs or "nootropics" (from the Greek roots noo-, mind and -tropo, turn, change), not only represent important pharmacotherapies for neurocognitive disorders such as dementia, attention deficit disorder and schizophrenia, but might also augment the minds of the healthy. The possibility of purchasing "smartness in a bottle" is likely to have broad appeal to students with normal or above average cognitive functioning to begin with. Reports in the popular press suggest that smart drugs or "nootropics" such as methylphenidate, modafinil and piracetam are increasingly being used by the healthy to augment cognitive ability. Although current nootropics offer only modest improvements in cognitive performance, it appears likely that more effective compounds will be developed in the future and that their off-label use will increase. One sphere in which the use of these drugs may be commonplace is by healthy students within academia. However, although several authors have considered the issue of "academic doping", none have examined the main ethical issues to any large extent. This is despite the widespread non-medical use of psychostimulants such as methylphenidate across universities for the purposes of enhancing concentration. It seems apparent that cognitive enhancing drugs would be highly attractive to high school and university students, and the largest non-therapeutic market for future nootropics could very well be this demographic. As a corollary, the ethical and pragmatic issues that will emerge from the use of nootropics by students warrants earnest consideration. In the absence of any existing ethical framework with which to view this issue, it may be relevant to examine the one paradigmatic human endeavour that has already wrested with the problem of performance-enhancing drugs for several decades: competitive sport. One article reviewed the ethical and pragmatic implications of nootropic use in academia by drawing parallels with issues relevant to the drugs in sport debate. It is often argued that performance-enhancing drugs should be prohibited because they create an uneven playing field. However, this appears dubious given that "unfair" advantages are already ubiquitous and generally tolerated by society. There are concerns that widespread use will indirectly coerce non-users also to employ nootropics in order to remain competitive. However, to restrict the autonomy of all people for fear that it may influence the actions of some is untenable. The use of potentially harmful drugs for the purposes of enhancement rather than treatment is often seen as unjustified, and libertarian approaches generally champion the rights of the individual in deciding if these risks are acceptable. Finally, whether the prohibition of nootropics can be effectively enforced is doubtful. As nootropics use becomes widespread among students in the future, discussion of this issue will become more pressing in the years to come [09389].

Whether drugs that enhance cognition in healthy individuals will appear in the near future has
become a topic of considerable interest. It was address this possibility using a three variable system (psychological effect, neurobiological mechanism, and efficiency vs capabilities) for classifying candidates. Ritalin and modafinil, two currently available compounds, operate on primary psychological states that in turn affect cognitive operations (attention and memory), but there is little evidence that these effects translate into improvements in complex cognitive processing. A second category of potential enhancers includes agents that improve memory encoding, generally without large changes in primary psychological states. Unfortunately, there is little information on how these compounds affect cognitive performance in standard psychological tests. Recent experiments have identified a number of sites at which memory drugs could, in principle, manipulate the cell biological systems underlying the learning-related long-term potentiation (LTP) effect; this may explain the remarkable diversity of memory promoting compounds. Indeed, many of these agents are known to have positive effects on LTP. A possible third category of enhancement drugs directed specifically at integrated cognitive operations is nearly empty. From a neurobiological perspective, two plausible candidate classes have emerged that both target the fast excitatory transmission responsible for communication within cortical networks. One acts on nicotinic receptors (alpha7 and alpha4) that regulate release of the neurotransmitter glutamate while the other (‘ampakines’) allosterically modulates the glutamate receptors mediating the post-synaptic response. Brain imaging in primates has shown that ampakines expand cortical networks engaged by a complex task; coupled with behavioral data, these findings provide evidence for the possibility of generating new cognitive capabilities. Finally, it was suggest that continuing advances in behavioral sciences provide new opportunities for translational work, and that discussions of the social impact of cognitive enhancers have failed to consider the distinction between effects on efficiency versus new capabilities [11309].

Drugs developed to treat cognitive impairments are proving popular with healthy college students seeking to boost their focus and productivity. Concerned observers have called these practices a form of cheating akin to athletes’ use of steroids, with some proposing testing students’ urine to deter “academic doping.” The ease with which critics analogize the academic enterprise to competitive sport, and the impulse to crack down on students using study drugs, reflect the same social influences and trends that spur demand for these interventions—our hyper-competitive culture, the commodification of education, and our attraction to technological quick-fixes. Rather than focusing on the technologies that are being put to troubling uses, it would be better served reforming the culture that makes these practices attractive [12433].

One study tested the hypothesis that college students’ substance use problems would predict increases in skipping classes and declining academic performance, and that nonmedical use of prescription stimulants (NPS) for studying would occur in association with this decline. A cohort of 984 students in the College Life Study at a large public university in the US participated in a longitudinal prospective study. Interviewers assessed NPS; Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) cannabis and alcohol use disorders; and frequency of skipping class. Semester grade point average (GPA) was obtained from the university. Control variables were race, sex, family income, high school GPA, and self-reported attention deficit hyperactivity disorder diagnosis. Longitudinal growth curve modeling of four annual data waves estimated the associations among the rates of change of cannabis use disorder, percentage of classes skipped, and semester GPA. The associations between these trajectories and NPS for studying were then evaluated. A second structural model substituted alcohol use disorder for cannabis use disorder. More than one-third (38%) reported NPS for studying at least once by Year 4. Increases in skipping class were associated with both alcohol and cannabis use disorder, which were associated with declining GPA. The hypothesized relationships between these trajectories and NPS for studying were confirmed. These longitudinal findings suggest that escalation of substance
use problems during college is related to increases in skipping class and to declining academic performance. NPS for studying is associated with academic difficulties. Although additional research is needed to investigate causal pathways, these results suggest that nonmedical users of prescription stimulants could benefit from a comprehensive drug and alcohol assessment to possibly mitigate future academic declines [12484].

**Performance-enhancing drugs create an uneven playing field**

One of the most frequently invoked arguments against the use of performance-enhancing drugs in sport is that they confer an unfair advantage to those who use them. If the difference between winning and losing were determined not on the running track but in the chemical laboratory, it would result in an “uneven playing field” because athletes would not be competing on equal grounds. It is often concluded, therefore, that doping in sports is a form of cheating, because it provides doping athletes an unfair advantage over their clean competitors. Unsurprisingly, cognitive ability is a significant predictor of academic performance and twin studies indicate that IQ has a heritability of approximately 50 percent. That is, a sizeable proportion of one’s academic successes are due to the genes with which one has been naturally endowed. To be sure, nootropics would probably make an already uneven playing field more unfair, and one that is likely only to favour the wealthy who can afford to purchase them. Not only do the rich get richer, but in the future it seems that they might also get smarter. However, using unequal distribution to justify the prohibition of nootropics is akin to prohibiting private tuition, which also increases academic performance while exacerbating educational inequalities between social classes. If socioeconomic inequalities in education are readily tolerated by society, then it would be hypocritical to apply this criterion selectively to nootropics and not to other performance-enhancing strategies. If nootropics represented the most cost-effective means of enhancing academic performance, social programmes might seek to make them accessible to the underprivileged. Moreover, it is entirely possible that some nootropics would primarily benefit those in whom cognitive deficits are present, with little, no, or perhaps even deleterious effects upon the healthy. This appears to be the case with modafinil, in which the greatest improvements in cognitive performance are seen in those with lower IQs [09389].

**Everybody else is taking them**

Some athletes are motivated to use performance-enhancing drugs because they believe their competitors are doing so. Although it is difficult to determine the prevalence of drug use in sports, anecdotal reports few successful Olympians do not dope. Therefore, in order to compensate for what might be considered an unfair advantage against them, an athlete who would otherwise not be compelled to dope may decide that this is the only avenue through which they can remain competitive against those who do. One concern surrounding the widespread use of nootropics is that it may indirectly coerce other students into taking them in order to keep up with their peers. For example, if the majority of students were to use modafinil and their doing so vastly improved their academic performance, then the remaining non-users would feel a certain amount of pressure to follow suit in order to remain competitive. The Red Queen Principle applies here, in which an individual must continue developing in order to maintain their fitness relative to others with whom they are competing. In the absence of empirical data, one can only speculate if any of these factors presently hold true in academia. For example, caffeine is a widely used nootropic that can reduce fatigue and promote alertness and vigilance, but it is unlikely that its use is necessary for academic success and that many feel coerced into consuming it. It has been reported that in the USA the non-medical use of methylphenidate and amphetamine in the previous year is as high as 25 percent in some college campuses. The most commonly cited reason for using these stimulants is to enhance concentration (58 %) and increase alertness (43 %),
indicating that they are being used for their performance-enhancing properties. However, difficulties in this reasoning arise when considering indirect coercion with respect to dangerous or toxic nootropics. Should individuals be protected from the pressure of taking potentially dangerous drugs such as methylphenidate in order to succeed academically? What if the nootropic were innocuous? Would this relegate indirect coercion regarding caffeine use as they would towards methylphenidate. It is apparent then, that the issue of indirect coercion to use performance enhancers hinges upon the safety profile of the drug in question and that this may be a major determinant of future policy towards nootropics use [09389].

**Performance-enhancing drugs are dangerous**

According to the World Anti-Doping Agency (WADA) one criterion for prohibiting a drug in sport is whether or not it poses an actual or potential risk to an athlete’s health. Although the dangers of drugs are often overstated, these dangers seemingly justify their prohibition because legalisation may be perceived as tacit endorsement of their use. Indeed, the safety profile of a performance-enhancing drug appears to be a large determinant of whether or not it is prohibited. Caffeine, for example, reliably increases performance in a range of sports including swimming, cycling and running at doses allowed by WADA. Yet despite being a form of “cheating” in the same vein as anabolic steroids, caffeine’s use in sport is permitted because it is relatively harmless. For nootropic psychostimulants such as methylphenidate, the dangers are real and relatively well known. Aside from its abuse potential, methylphenidate may aggravate mental illness, produce sleep disturbances and is associated with cerebrovascular complications. It is clear that there are risks inherent in the use of any drug, and given that the use of nootropics by the healthy would be for the purposes of enhancement rather than treatment, some clinicians would deem any risk unacceptable. On the other hand, the general libertarian perspective argues that provided the individual is cognisant of the potential side effects, they are free to make their own decision to take nootropics. It would appear that both extremes are untenable – on one hand people should have a right to decide whether or not the risks of nootropics are acceptable, but civil liberties must also be balanced by the need to safeguard the public good [09389].

**Drug use would be almost impossible to control**

The widespread access to and use of performance-enhancing drugs in sport despite their prohibition suggests that current anti-doping measures are inadequate. The competitive advantages derived from their use, the low likelihood of drug testing and the relatively minor punishment for getting caught make them attractive to many athletes. This is not influenced by ethical considerations concerning such use, but rather the belief that any attempt at prohibition is not pragmatic and possibly more harmful than regulation. As in the case of controlled substances such as methylphenidate and amphetamine, the high rates of non-medical use and the ease with which they can still be obtained demonstrate the inability of prohibition to control their illicit supply effectively. As nootropics would probably have legitimate therapeutic applications in the treatment of neurocognitive disorders such as dementia, diversion from legitimate sources would be highly likely. Considerations of supply notwithstanding, just how the prohibition of nootropics in academic contexts could be enforced remains unclear. One conjures to mind the scenario of students taken to one side, cup in hand, and asked to provide a urine sample to test officials. Scandal would erupt and rumours abound when the magna cum laude is stripped of his title for testing positive for modafinil – a drug that gave him near-superhuman levels of mental endurance. As laughable as it may seem, it is possible that scenarios such as this could very well come to fruition in the future. However, given that the benefits of nootropics can also be derived from periods of
study at any time leading up to examinations, this would also require drug testing during non-exam periods. If the current situation in competitive sport is anything to go by, any attempt to prohibit the use of nootropics will probably be difficult or inordinately expensive to police effectively [09389].

**Danish ban on gyms not adhering to doping-tests**

If anti-doping would only concern elite sport, one might accept the arguments in favour of the exceptions made in sports, for the sake of what sports aspires to be. But anti-doping has unintended side effects outside elite sports. One illustrative example of how anti-doping policies directly influence society outside the scope of competitive sport is the 2005 extension of Danish national anti-doping policy to commercial fitness clubs (gyms) in which clients engage into weight lifting and other types of exercise for health and appearance purposes, but not necessarily for sports competition. Danish gyms have the obligation to put either a happy green smiley on the entrance, indicating adherence to Anti-Doping Denmark, which includes surprise urine testing of clients, or an unhappy red smiley with the explicit message that the club does not adhere to anti-doping Denmark. About 20-25 percent of the samples are found to contain (forbidden) anabolic steroids. As a consequence these clients are excluded from the gym in question for 2 years, and from all other gyms that adhere to the rule. According to the law the client should also be excluded from all sports in Denmark. And, as in elite sport, a refusal to be tested is counted as a positive test. This example illustrates the potential for generalization of anti-doping surveillance practices in society in general. This practice is not far from the introduction of testing of students for cognitive performance enhancing substances and other drugs, and possibly other groups, like teachers, trainers, coaches, sports referees, police personnel, amateur athletes, etc. Such increased surveillance and testing would lead to increased numbers of convictions, with an important burden imposed on the judicial system and the families of the convicted. For simple reasons of stochastic and procedural error frequency, a greater number of tests would lead to a greater number of false positives, wrongly accusing innocent citizens. The prospect of such a development has worrying characteristics of a dystopia of Orwellian kind. It appears paradoxical that gym users, generally conscious about their health and complying with general preventive principles like regular exercise and a healthy diet, making a balanced decision on steroid use to aid them in attaining their aspired body form, are punished for anabolic steroid use, while the general population can freely engage in dangerous behaviour combining bad nutrition, lack of exercise, tobacco and alcohol use without much of a constraint [12012].

**Cannabis**

Contrary to health risk and performance enhancement, the spirit of sport criterion does not rely on established scientific facts; rather, it relies more on ethical and societal considerations encompassing a wider view of sport beyond physical achievements and health. Therefore, the fundamental rationale for this aspect of the Code does not include a strict definition of the spirit of sport, but instead provides a collection of essential values to be shared in sport. The values included are ethics, fair play and honesty, health, excellence in performance, character and education, fun and joy, teamwork, dedication and commitment, respect for rules and laws, respect for self and other participants, courage, community and solidarity. These values are in essence contrary to doping. Such essential principles guide WADA scientists and ethicists when determining whether a substance or a method violates values embedded in the spirit of sport. Cannabis is classified as an illegal substance in most of the
world, with penalties ranging from no action to long-term incarceration. The consumption of cannabis and other illegal drugs contradicts fundamental aspects of the spirit of sport criterion. The international anti-doping community believes that the role model of athletes in modern society is intrinsically incompatible with use or abuse of cannabis. Although some anti-doping officials proposed also banning cannabis for out-of-competition testing, this appeared beyond the anti-doping mandate and it was believed to violate athletes’ privacy. For these reasons, cannabis use is prohibited only in competition. Use of illicit drugs that are harmful to health and that may have performance enhancing properties is not consistent with the athlete as a role model for young people around the world. Banning a substance only in competition creates challenges in differentiating new cannabis smoking during competition from evidence of prior out-of-competition cannabis use [11584].

Gender as more than a binary quantity

The case of Caster Semenya provides a vivid illustration of the ways in which natural genetic variation can generate large differences in athletic performance. But since we normally segregate athletic sports along the lines of this particular variation-gender, her case also highlights problems with the current approach to justice in sporting competition. Female athletes seem to have a valid complaint when they are made to compete against athletes who are, in one sense or another, male. But once we recognise that gender is not a binary quantity, sex segregation in competitive sport must be seen as an inconsistent and unjust policy, no matter what stance we take on the goals of sport or on the regulation of doping [10422].

On how to make illegal drugs less dangerous

Substances, such as alcohol, opiates and cannabis, have been used by humans for millennia. Today, a much wider range of substances are used for a range of purposes, including the enhancement of performance during university studies, sexual experiences, sports, exercise, at celebrations, socializing and the experience of art and music. Substance use is also associated with a range of harmful effects to the individual and society as a whole. Prohibitions, regulation, prevention and treatment have all been used to protect against this harm. In this commentary, it is argued that public health interventions should target relevant harms and not to evaluate which aspects of human endeavors and experiences should be enhanced and which should not. It is argued that interventions should directly target the harmful effects, using the best available evidence. Two examples are given of substances that may be altered to prevent serious harm – one for alcohol and one for cannabis. In the case of alcohol, the addition of dissolved oxygen could reduce both the risk of accidents and the risk of liver damage associated with alcohol consumption. In the case of cannabis, there is strong indication that the reduction of content Delta-tetrahydrocannabinol and the increase of cannabidiol could reduce the risk of psychoses and the addiction associated with its use. The aim of one article was to show that responsible regulation should not necessarily be restricted to preventing the use and/or (in the case of alcohol) a reduction in the amounts and frequency of its use, but should also aim to include a range of other strategies that could reduce the burden of illness associated with illicit substance use [10423].

Hidden assumptions and inherent contradictions in anti-doping policy

2142
There is little disagreement that drug use in sport is problematic, but the rationale for and mechanisms of drug control are a subject of debate. On one hand, powerful global sport authorities such as the International Olympic Committee (IOC) the World Anti-Doping Agency (WADA) and international sport federations claim that the use of drugs in sport is cheating and should be eliminated through the imposition of punitive measures. An alternative approach is one that is more concerned with the protection of athlete health and societal impact of drug use. In short, there is an ongoing tension between the benefits of a deterrence-only model of drug control (as enacted by WADA) versus a multi-level approach of harm minimisation (as adopted by many drug education and treatment support agencies). One paper therefore considered the assumptions underpinning the current drugs-in-sport policy arrangements. It was examined the assumptions and contradictions inherent in the policy approach, paying particular attention to the evidence that supports different policy arrangements. It could be found that the current anti-doping policy of the World Anti-Doping Agency (WADA) contains inconsistencies and ambiguities. WADA’s policy position is predicated upon four fundamental principles; first, the need for sport to set a good example; secondly, the necessity of ensuring a level playing field; thirdly, the responsibility to protect the health of athletes; and fourthly, the importance of preserving the integrity of sport. A review of the evidence, however, suggests that sport is a problematic institution when it comes to setting a good example for the rest of society. Neither is it clear that sport has an inherent or essential integrity that can only be sustained through regulation. Furthermore, it is doubtful that WADA’s anti-doping policy is effective in maintaining a level playing field, or is the best means of protecting the health of athletes. This, it was concluded that the WADA anti-doping policy is based too heavily on principals of minimising drug use, and gives insufficient weight to the minimisation of drug-related harms. As a result drug-related harms are being poorly managed in sport [10312].

Setting a good example

One of the first claims made by sport policy makers is that athletes in particular, and sport in general, have an obligation to set a good example, as many young people are influenced by sporting heroes and use them as role models. Under these conditions it might make sense to implement punitive policies that discourages drug use. However, this policy position rests upon two key propositions: first, that sport can positively shape the moral behaviour of its participants and followers, and secondly, that heavy punishment for drug use in sport will lead ultimately to abstinence. While these propositions are intuitively appealing, there is little evidence to support them. The idea that sport should set good examples for impressionable children and provide them with a reliable moral compass is widely held, and is mirrored in the long list of personal and social benefits ascribed to sport participation. It is linked to improvements to mental health and self-esteem, mental toughness, the control of stress, anxiety and depression, better physical development, community-building and diminished health spending. However, the way in which sport influences moral development remains unclear. Indeed, there is evidence that sport can actually increase the risk of injury, encourage binge drinking, undermine an athlete's long-term health prospects and facilitate cheating. In other words, sport can just as easily act as a catalyst for socially dysfunctional behaviour. This consequently weakens the argument that drug-free sport will ensure its integrity and set clear moral guidelines for participation. The concept of sport as an example-setter must also be balanced against the fact that sport holds winning as sovereign, which in turn produces a demand for anything that gives athletes a competitive edge. The hyper-competitive nature of sport and its emphasis on achievement and rewards encourages drug taking, and in some cases the combination of self-gratification and public approval may be a kind of addiction. The incentives for drug use are therefore substantial. In addition, the combination of immense pressure for success and severe punishment for failure teaches
young athletes another important lesson, which is to avoid getting caught [10312].

**A level playing field**

A second assumption made by WADA and the IOC is that their drug code is essential to the maintenance of a level playing field where no athletes are disadvantaged unfairly. On the surface this is a reasonable argument, as sport should be about an equal chance of success for all competitors. However, this level playing-field argument is fraught with inconsistencies. In the first instance, the scientisation and medicalisation of sport means that only certain privileged athletes have access to the latest training advantages that will give them a competitive edge. While erythropoietin (EPO) (a drug which increases the oxygen-carrying capacity of blood) may be a banned substance, those who can afford to train at high altitude or sleep in an altitude chamber can obtain legally a lesser but similar benefit. Furthermore, all athletes respond differently to training and nutritional regimens, while others bring unique genetic advantages, such as the naturally occurring gene mutation which helped Eero Mäntyranta secure two gold medals at the 1964 Winter Olympics. In fact, there is a multitude of non-drug-related factors that can provide a competitive edge and tilt the playing field in favour of better-endowed and better-resourced athletes. This begs the question as to whether it would be appropriate to handicap athletes with extraordinary natural abilities. This is effectively the intention behind the use of weight-classes in boxing, wrestling and rowing. In some professional sports, leagues go to great lengths to regulate the competition in order to achieve competitive balance. Indeed, by radically amending the rules of a game and restricting the movements of able-bodied players, it may be possible to allow disabled athletes to compete with a chance of winning. Similarly, it may be possible to regulate sport activities in such a way as to allow mixed-gender participation. At one level this provides equity for everyone, but at another level becomes a bizarre exercise in equalisation that can never lead to 'true' equality. WADA is not concerned with inequality as such; it accepts, for example, that the naturally occurring ratio of testosterone to epitestosterone can vary between individuals and confers a relative advantage to some. What WADA does not accept is the boosting of individual advantage through the use of certain designated drugs. In general, sporting competitions are inherently unbalanced owing to such factors as genetic advantage, gender bias and differing levels of socio-economic access to training technology. A second ambiguity can be found in the fact that not all performance-boosting drugs and substances are universally banned, caffeine being a prime example. In addition, some drugs that clearly reduce performance are also forbidden. The inconsistency is compounded when we find that alcohol and tobacco, two of society’s most destructive drugs, are tacitly accepted. It therefore comes as no surprise that WADA’s policy has similar punishments for performance-reducing and performance-enhancing drugs. This confusion over which drugs are problematic, and the penalties for using banned drugs, is partly a symptom of the method used to determine which substances are prohibited. To be included on the WADA prohibited list, a substance must either be a potential masking agent or must meet two of the following three criteria set out by WADA:

- the substance is performance-enhancing
- the use of the substance poses health risks to the athlete
- the use of the substance violates the spirit of sport

Marijuana, for instance, meets criteria 2 and 3 and is therefore banned even though it actually reduces performance. In contrast, over-the-counter substances such as bicarbonate/citrate, creatine and caffeine are performance-enhancing but, because they only meet criteria 1, are not banned. The social acceptance of different drugs therefore plays a significant role in determining whether the use of a substance violates the ‘spirit’ of sport. WADA’s criteria for determining which substances are prohibited therefore leads to the
banning of some drugs that do not enhance performance, while allowing others that do. It is therefore unclear as to how elements of WADA policy work to maintain a level playing field. A third contradiction in WADA’s claim for a level playing field is that in some sports where performance-enhancing drugs offer a substantial advantage and are used commonly by elite performers, parity is more likely to come from complete de-regulation rather than further regulation and testing. Allied to this problem are the mixed messages that bombard the sport-watching market-place. On one hand, the general public condemns athletes for using drugs, but on the other hand they laud their record-breaking performances [10312].

**Protecting the health of athletes**

The dangers of unregulated drug use in sport, both of the performance-enhancing and performance-reducing variety, have been clearly established. There is also agreement that sport and its regulating agencies must take some responsibility for the health of athletes. Risk is an inherent part of sport, and governing bodies attempt to mitigate it wherever possible. However, sports such as American football, mountaineering, base-jumping and motor racing continue to be played despite warnings of the risks of serious injury. In fact, to remove all the risk is to remove an intrinsic part of sport itself. Given that athletes are free to engage in sports with substantial risks, why are they not also free to utilise performance enhancements that are, in some cases, less risky than the sports in which they engage? A punitive drug use policy that is defended on the grounds that it protects the health of players sits uncomfortably with a tacit acceptance of sports such as boxing, where the intent of the participants is to inflict serious harm. It also sits uncomfortably with a sporting tradition that embraced a close association with tobacco products for so long, and supports continuing association with alcohol-based products, both of which come with a serious community health risk. Moreover, the policy of banning drugs has made it more difficult for athletes to obtain medical advice that might reduce the health damage of the drugs they are using. It has been shown that athletes who self-medicate tend to use substantially more than necessary, thereby amplifying their risk of illness and injury. The issue here is to strike an appropriate balance between widespread drug use under a legalised system and less prevalent but higher-risk drug use patterns under an anti-doping regimen. There is also the problem of the market-place reaction to a culture of legalised drug use among athletes. Would fans exert pressure on athletes to abstain, and unintentionally promote masking and experimental drug intake, or would they concede that bolstering testosterone levels in a medically safe manner is as socially acceptable as undergoing a breast augmentation? The fact remains that in both scenarios drug use to enhance performance will be an ongoing feature of sport, and the critical issue is to identify the approach that best protects the health of athletes and minimises the cost to society. Most sporting activities, especially at elite level, require athletes to perform at the outer limit of their physical capacity and therefore demand risk-taking and pain tolerance. A masculine ethos holds risk-taking at its core, and the combination of illegality, risk of exclusion and potential for physical damage can be part of the attraction of taking drugs. A punitive anti-doping policy may therefore have the unintended consequence of making drug use even more attractive to some hyper-masculine athletes because of its association with deviant and high-risk behaviour. On the other hand, a policy that acknowledges the logic of using drugs to enhance performance might normalise its consumption, and provide space for a more open public debate on drug use in sport. By focusing upon the importance of performance and winning, sport also provides favourable conditions for its scientisation and medicalisation. Even the use of approved drugs for rehabilitation encourages risky behaviours, such as the use of pain-killers to allow players to re-take the field after injury. Athletes now operate in a sporting culture which supports the use of medical treatments and substances to boost and sustain performance, and managers of professional sport teams have a vested interest in getting injured players back onto the field of play in the shortest possible time, using pain-killing and anti-inflammatory drugs to
speed up the process. However, in so doing they put the long-term health of players at risk by increasing their likelihood of sustaining chronic injury problems [10312].

Preserving the integrity of sport

The final assumption underpinning WADA policy reflects the imperative to protect sports' public image and reputation. In a sport official's ideal world, players will volunteer their free time to assist disadvantaged groups, treat women and people of other races respectfully, obey all traffic laws, drink alcohol within legal limits and in general be model citizens. The thing they fear most is an allegation that one of their players has taken either a performance-enhancing or illicit drug. In this context drug use is particularly vexing because, like match-fixing, it goes against the fundamental ethic of sport, which is all about adhering to a set of intrinsic rules and the values they mirror. Performance-enhancing drugs are seen to threaten sports' integrity by removing any sense of fair play, while the illicit (mainly performance reduction) drugs threaten sports' integrity by tarnishing its public image. However, all this strong rhetoric begs the question as to just how effective a punitive policy will be in eliminating drug use and shoring up sports' public appeal and good standing. The evidence is ambiguous in this regard. Whereas a lack of vigilance in testing may lead to more drug use, the punishments handed out to the few who are caught using anabolic steroids are not effective in discouraging use. While heavy sanctions and punishments may play a role in discouraging drug use in sport, these types of regulations are just some of many of factors that impact on players' decision to use drugs. WADA also claims that taking drugs to enhance sport performance is inappropriate because it compromises the ethical foundations of sport and reflects poorly on its organisation and management. In other words, doping practices should be punished because they undermine the social value of sport and its fundamental authenticity. However, the idea that all sport is bound by the same values and customs is a romantic one, and ignores the peculiar cultural histories and evolution of different sports and the impact of science, technology and commercialisation on their structure and operation [10312].

Policy implications

The realities of sport are that, even in drug-free situations, athletes do not set particularly good examples, sport is not a level playing field, attempts to protect athlete's health are often no more than token gestures and the integrity of sport is determined just as much by its structures, management systems and culture as it is by the behaviour of its players. As a result it is fanciful to think that a selective and punitive anti-doping policy will, of itself, ensure the social and moral progress of sport. Indeed, draconian polices that are embedded with heavy penalties can just as easily force players to take even greater risks in the quest for sporting stardom. For these reasons we argue that anti-doping policy in sport could learn from the harm minimisation principles advocated by agencies managing illicit drug use in the broader community. Policies that consider only the reduction of drug use (such as the number of people using drugs or the amount of drugs being used) are not concerned predominantly with the relative danger of the different types of drugs being used or whether they are used in a high-risk or low-risk manner. They also have a limited capacity to inform the differing domains of education, law, rehabilitation and public health. Policies that aim to reduce drug use can also promote "collateral harms". For example, intensive policing and punishments have been shown to increase the risk of harms associated with illicit drug use. Furthermore, evidence suggests that not only will prohibition fail to reduce drug use, but the cost of enforcement may also lead to an increase in the street-price of drugs, thereby making their trafficking more appealing. In contrast, harm minimisation, which covers policies that aim to reduce drug-related harm, is concerned primarily with addressing the negative

2146
consequences of use, rather than the act of use itself. The harms associated with drug use can include health-related dangers such as risk of death and serious illness, as well as social stigmatism and loss of personal dignity. While harm minimisation policy may incorporate strategies to promote the reduction of drug use, it does so in a harm-sensitive manner so as to avoid unwanted collateral problems. There are three elements in a harm minimisation model which deserve special attention in the sporting context, and they include, first, the importance of context in determining a harm minimisation policy, secondly the development of strategies to reduce demand, and thirdly an emphasis on prevention and early intervention. However, WADA’s anti-doping policy aims to reduce drug use, not harm, and therefore overlooks many of these domains. In addition, drug use in elite sport occurs in an environment where there is significant emphasis on winning, with associated social and economic benefits. Furthermore, social values in the West condone and promote the use of technologies to gain a personal advantage, such as increased physical attractiveness or improved physical capacity. It is consequently unrealistic to expect that performance-enhancing drugs can be eliminated from the sporting milieu, given the situational incentives for their use. Another element of harm minimisation models which deserves consideration in sport is its emphasis on prevention and early intervention. Importantly, this includes the prevention of escalating patterns of drug use. Progression of this type is not being addressed by WADA, which relies on its zero-tolerance, sanction-only approach. While the anti-doping policy of WADA may appear to be promoting harm minimisation by deterring the use of drugs in the first place it may actually encourage high-risk drug use, as the negative outcomes of prohibition include increased risk from unsafe use. Therefore, if prohibition is to be used, it needs to be managed in a harm-sensitive manner. In the context of sport, harm minimisation reflects three empirically tested principles. First, drug use is not simply a sporting matter, nor is it a criminal or legal matter. Instead, drug use in sport is a serious societal issue. Secondly, harm minimisation obviates the need for any form of moral certitude. Instead, it accepts that drug use exists in sport, and will never be completely eliminated. Thirdly, although harm minimisation does not condone the use of drugs in sport, it acknowledges that when it does occur policy makers have an obligation to develop public-health measures that reduce drug-related harm to athletes at all levels, irrespective of whether they compete or qualify for testing. From a harm minimisation perspective, the key question is whether it is preferable to be interested in the short-term brand equity and credibility of elite sport, or in the long-term best interests of athletes. Rather than eliminating the use of drugs, draconian rules and sanctions will only send it further underground as players search for more exotic and less detectable options. The need for a broad-based drug management policy in sport is therefore paramount. Moreover, any drug management policy should include not only performance-enhancing drugs, but all other drugs, illicit or not, that may undermine the long-term health prospects of players and the sustainability of their sport. This means that alcohol and tobacco should fall within the purview of a balanced drugs-in-sport policy. Given the complexities that characterise the drugs-in-sport landscape, it is not surprising that polices designed to punish athletes for taking drugs have not been successful in removing drugs from sport. In addition, there is little evidence that indicates any significant improvement in the health and well-being of players resulting from the current anti-doping policy arrangements. The alternative model is a harm minimisation policy that allows athletes to manage their usage in a safe environment free from ill-informed advice, contaminated supply and the threat of severe shame and punishment. Although controversial in a sporting context, it accepts that drugs will always be part of a risky and tilted playing field full of moral ambiguity. It was argued that a socially responsible philosophy that focuses on the reduction of collateral harm, and seeks out a sound evidence base, should be sovereign in determining future drugs-in-sport policy [10312].
WADA provides the following information: WADA is extremely careful with investigators conducting research activities on its behalf to ensure they work with occasional or recreational athletes to circumvent the risk of exposing elite athletes to prohibited drugs or methods. Even when working with occasional or recreational athletes, WADA ensures that sufficiently long wash-out periods are applied to guarantee that no unfair advantage is provided to volunteers participating in research studies [13774].

The WADA response to research on doping is thus very clear, especially point 8, which states their position in no uncertain manner: “8. WADA considers that conducting research studies with elite athletes should not expose them to prohibited substances and methods unless specific dispositions are made such as retiring the athletes as active athletes for a considerable period” [13775].

Ethical codes

A code of ethics for sports physicians needs to be clear, appropriate and practically useful to clinicians in everyday clinical circumstances and for situations that may be difficult or contentious. The code of Australasian College of Sports Physicians (ACSP) was recently rewritten based on research into the ethical concerns of sports doctors and physicians in New Zealand. Further appraisal of the existing code in the light of these findings led to concerns about its adequacy. The previous ACSP code had not been updated in 25 years, and had gaps in topic areas and errors within it that made it potentially confusing. Any code written for a specialist group must acknowledge the context of its governance. By virtue of their medical registration, sports medical practitioners are required to comply with the code written for all registered medical practitioners in their local jurisdiction. They may also be subject to specialty codes written specifically for physicians who have received further training in sports medicine, and there may be consensus guidelines written on various topics [09386].

A code of ethics with a foundation in evidence

To ensure that a new code meet the needs of physicians and covered appropriate areas requires that it be informed by evidence. It had previously been carried out qualitative research with 16 sports medical practitioners working with elite athletes and teams in New Zealand. Sports medical practitioners were asked to identify and discuss a range of ethical concerns they experienced in their work. There were three key findings from this research [09386]:

- sports medical practitioners work within a complex and pressured environment that has the potential to compromise patient welfare and limit a doctor’s ability to provide quality care or demand better conditions for their patients
- sports medical practitioners may have multiple obligations to others including individual patients and employers. At times these responsibilities may conflict when meeting one obligation will result in neglecting others
- sports medical practitioners vary widely in their perceptions in two particular aspects of practice

The complex environment of elite sport

Sports medicine at the elite level occurs within a complex setting that may include the pressure of large amounts of money riding on results, together with a high level of media
attention into the wellbeing of athletes. Those two elements are further intertwined as media attention can attract sponsorship and advertising and even greater medical services if the athlete/team is successful. Coaches, team management and sponsors can seek to transfer this financial pressure to sports doctors. Although some coaches respect the doctor’s knowledge and experience, this was not the case among all medical practitioners interviewed. Some sports medical practitioners have fought difficult battles with coaches, sponsors and governing bodies, sometimes dealing with entrenched views about the nature of injury and rehabilitation. Some participants described situations in which their decisions were overridden, disrespected and in which the athlete’s wellbeing was overlooked by others with divergent aims. In this kind of environment sports medical practitioners may lack the independence and support required to attend to the needs of their patient group [09386].

Sharing personal information about athletes with others

Employment contracts commonly oblige medical practitioners to share health information with coaches and team management. Correspondingly, employment contracts between athletes and team management make similar demands to share health information. Despite this, athletes may request sports medical practitioners to withhold health information from the coach and management [09386].

Risk taking by athletes

When an athlete wished to take a course of action with a significant risk to health, all sports medical practitioners took an educational role seriously, but there is a lack of consistency about whether to support or limit an athlete’s ability to assume risk. For example, one doctor said he would do whatever the patient requested so long as it was not illegal, whereas another has it written into the athlete’s contract that the sports medical practitioner has the final say on who can play. Although these responses may be acceptable depending on the individual clinical situation faced, ambiguity exists about how medical practitioners should respond [09386].

Aims for a new code of ethics

A code of ethics serves a number of purposes. These include articulating professional standards for its members, ensuring the protection of the public and to enhance the trust society has in this group. Codes provide an opportunity to express the shared values of the group and the profession. On a practical level, codes can be used to support members with ethical decision making, and can act as a shield to protect practitioners from unacceptable demands and external pressures. Codes of ethics also set out standards of behaviour to be used as a yardstick against which to measure the actions of its members. For example, as a more organised group with clear expectations and limits sports physicians could respond more effectively to new developments in sport that have the potential to affect athlete wellbeing negatively, including new forms of high-risk sport, new cell technology and genetic enhancement [09386]:

- *Comprehensible* (accessible). A code of ethics is, in part, a form of communication between the general public and the profession, and as such it needs to be accessible to both patients and clinicians. The document must therefore be written without jargon and in plain language
- *Unambiguous*. A code of ethics must avoid confusion so it must be plainly expressed, clear in its requirements and not contain unintended double meanings
- *Compatible with existing codes and laws*. A code of ethics should seek to be compatible with legislation governing the practice of medicine in the relevant country
or state. Although medical codes should always reserve the right not to comply with any law considered unjust, in general codes should comply with legislation. A code of ethics written for a specialist medical group should also be compatible with relevant codes written for all registered doctors in that jurisdiction.

- **Must/should.** The must/should structure allows the document to set minimum standards expected of all physicians expressed through the term "must", whereas the term "should" was used to convey aspirational aims of the college. The use of "should" statements allows for guidance in situations in which no strict rule would be appropriate or possible. Problems exist in deciding where to place the division between “must” and “should”. Setting expectations for behaviour too high will result in “must” statements that are unachievable. The use of the word “should” in the wrong place would result in a failure to impose minimal standards.

- **General/specific.** Excessive detail renders the code long and unwieldy and threatens its usefulness. It is impossible to imagine every clinical scenario. Any attempt to do so within a code will inevitably lead to some unconsidered possibility being overlooked, leaving the clinician unsure how the code applies. If the code is too general we also run into problems. A code that has grand statements such as “always act for the good of the patient” or “always act with the highest integrity” without further detail can be banal and of little practical use. It is hard to know how such general statements apply in specific situations. Such injunctions also fail to provide standards to measure a physician against.

- **Reflecting patient needs.** Physicians as stakeholders in the new code will bear the greatest burden from any obligations set out therein, but patients are also stakeholders as they clearly have an interest in the behaviour and obligations of their physicians. No input can be sought from patient groups in the development of the code, partly due to limited time, but also because it was considered that an improved code could only be an advance for athletes. It was thought that if physicians were strengthened in their ability to resist demands that eroded their commitment to patients then patients would ultimately benefit.

- **Implementation.** To implement the new code effectively will require member support of the code. To achieve this, members need to be informed about the existence of the new code and how their obligations may have changed. An education campaign of members must be undertaken. Because it is not possible to police every action taken by a physician we must rely on the commitment of members, so each individual member must feel that the code is appropriate and fits with the ethos of the group.

- **Administration of the code.** Members will quickly lose respect for a code that is not applied appropriately, in a timely fashion, or fairly. Therefore any complaints must be dealt with in a procedurally just and appropriate manner and disciplinary action must be consistent, fair and in proportion with the breach by the member. Obviously, a good organisational structure is required to be able act on complaints about members. On occasions breaches may be dealt with by governmental bodies set up to deal with complaints regarding patient care and so the organisation needs to be aware of any legal obligations to direct complaints elsewhere.

**Practicalities and negotiating the politics**

Banned performance enhancing drugs in sport is one area where the author wished to clarify expectations but was unaware of the politics involved. This should not be interpreted to mean that the code development group did not want the area to be covered, but there were concerns about where such duties should originate from. Another matter that generates discussion is the inclusion of mental health and eating disorders. The reasons for including these two topics were based on the research. Some sports medical practitioners had expressed concern over dealing with them, particularly mental health issues. For example,
some created fictitious physical injuries for their patients with mental health problems so that coaches would understand the patient needed to take time off. Also, as medical practice evolves and new developments occur in sports science, sporting practices, or law that affects medical practice, such change will need to be reflected in the code [09386].

Scientific cooperation

There are many areas of common interest between anti-doping laboratories and those working in the clinical, legal and forensic fields. In addition to methodological similarities, there are aspects of the findings in sport drug testing that overlap with other fields in such a way that sport drug testing and clinical, legal or forensic work may benefit from mutual interaction. Three recent examples are presented from the author’s experience. Case report 1 concerns the clinical relevance of hCG findings in sport drug testing as potential indicators of the presence of a (testicular) tumour in athletes. Case report 2 refers to difficulties that accredited laboratories can encounter due to differences between national legal systems and the administrative regulation systems of sport authorities. The example involves a network of blood collection for further autologous transfusion. Case report 3 relates to additional forensic-type investigations needed to interpret a situation where intoxication of a whole delegation was responsible for apparent doping cases. Clinical, legal and forensic fields must recognize the added value that some results and developments coming from anti-doping laboratories may have. At the same time anti-doping analysts should be aware of new issues, methodologies and problems appearing in related fields [09390].

The formal ethics of research with human subjects

All gene transfer studies in human subjects or patients for the purpose of preventing or treating even the most fearful and dire disease are highly experimental. As we have seen in the cases of immune deficiency diseases, there are many ways in which such procedures can go seriously wrong, but we all – patients, doctors, and society – all accept such methods in the cause of easing suffering. All progresses in medicine require multiple layers of research and testing; some in the laboratory in model systems, some with test animals, and some necessarily with human subjects. Each layer of research and testing requires a set of appropriate ethical standards for how such research should be conducted. Research, at all of these levels, is appropriate and necessary, and there is nothing intrinsically wrong or unethical in experimentation with human beings, as long as such studies conform to socially approved and agreed-upon standards. At this stage of development of gene transfer technology in human beings, human gene transfer and gene therapy studies are highly experimental and, therefore, must be subject to the accepted norms and standards that pertain to all human experimentation. As is true in all other areas of human clinical research and experimentation, failure to comply with these, or other comparable universally accepted standards, must rightly be considered medical malpractice and professional misconduct when carried out by licensed professionals or even criminal in cases in which the use of drugs and other materials or devices is not in accordance with governmental licensing and commercial requirements [06310].

Following World War II, the most influential of these codes was the set of principles that were enunciated as part of the Nürnberg War Crimes Doctors’ trial in 1947. This code identified the following 10 conditions that must be satisfied in order for any experiment with human subjects to be ethically acceptable [06310]:

2151
- The voluntary consent of the human subject is absolutely essential. This means that the person involved should have legal capacity to give consent; should be so situated as to be able to exercise free power of choice, without the intervention of any element of force, fraud, deceit, duress, overreaching, or other ulterior form of constraint or coercion; and should have sufficient knowledge and comprehension of the elements of the subject matter involved as to enable him to make an understanding and enlightened decision. The duty and responsibility for ascertaining the quality of the consent rests upon each individual who initiates, directs, or engages in the experiment. It is a personal duty and responsibility which may not be delegated to another with impunity.
- The experiment should be such as to yield fruitful results for the good of society, unprocurable by other methods or means of study, and not random and unnecessary in nature.
- The experiment should be so designed and based on the results of animal experimentation and a knowledge of the natural history of the disease or other problem under study that the anticipated results justify the performance of the experiment.
- The experiment should be so conducted as to avoid all unnecessary physical and mental suffering and injury.
- No experiment should be conducted where there is an a priori reason to believe that death or disabling injury will occur; except, perhaps, in those experiments where the experimental physicians also serve as subjects.
- The degree of risk to be taken should never exceed that determined by the humanitarian importance of the problem to be solved by the experiment.
- Proper preparations should be made and adequate facilities provided to protect the experimental subject against even remote possibilities of injury, disability or death. 8. The experiment should be conducted only by scientifically qualified persons. The highest degree of skill and care should be required through all stages of the experiment of those who conduct or engage in the experiment.
- During the course of the experiment the human subject should be at liberty to bring the experiment to an end if he has reached the physical or mental state where continuation of the experiment seems to him to be impossible.
- During the course of the experiment the scientist in charge must be prepared to terminate the experiment at any stage, if he has probable cause to believe, in the exercise of the good faith, superior skill and careful judgment required of him, that a continuation of the experiment is likely to result in injury, disability, or death to the experimental subject.

At the heart of the code are the principles that participation in experimental studies must be voluntary based on informed consent and justified by a potential for benefit that outweighs the potential for harm. Being “informed” requires that the study participants understand what will be done and agree to bear the expected risks and reap the anticipated benefits. These principles have served as the basis for many variations and subsequent modifications by worldwide medical and scientific bodies, including the influential codes developed by the World Medical Association in the form of its Declarations of Helsinki; first stated in 1964, and amended and extended many times, most recently in 2004. Parts of the Helsinki Declaration as revised in 2004 that are relevant to the potential inclusion of healthy, young subjects in nontherapeutic research state that it is the duty of the physician in medical research to protect the life, health, privacy, and dignity of the human subject [06310]:

- Medical research involving human subjects must conform to generally accepted scientific principles, be based on a thorough knowledge of the scientific literature,
other relevant sources of information, and on adequate laboratory and, where appropriate, animal experimentation.

- Appropriate caution must be exercised in the conduct of research which may affect the environment, and the welfare of animals used for research must be respected.
- The design and performance of each experimental procedure involving human subjects should be clearly formulated in an experimental protocol. This protocol should be submitted for consideration, comment, guidance, and where appropriate, approval to a specially appointed ethical review committee, which must be independent of the investigator, the sponsor or any other kind of undue influence. This independent committee should be in conformity with the laws and regulations of the country in which the research experiment is performed. The committee has the right to monitor ongoing trials. The researcher has the obligation to provide monitoring information to the committee, especially any serious adverse events. The researcher should also submit to the committee, for review, information regarding funding, sponsors, institutional affiliations, other potential conflicts of interest and incentives for subjects.
- The research protocol should always contain a statement of the ethical considerations involved and should indicate that there is compliance with the principles enunciated in this declaration.
- Medical research involving human subjects should be conducted only by scientifically qualified persons and under the supervision of a clinically competent medical person. The responsibility for the human subject must always rest with a medically qualified person and never rest on the subject of the research, even though the subject has given consent.
- Every medical research project involving human subjects should be preceded by careful assessment of predictable risks and burdens in comparison with foreseeable benefits to the subject or to others. This does not preclude the participation of healthy volunteers in medical research. The design of all studies should be publicly available.
- Physicians should abstain from engaging in research projects involving human subjects unless they are confident that the risks involved have been adequately assessed and can be satisfactorily managed. Physicians should cease any investigation if the risks are found to outweigh the potential benefits or if there is conclusive proof of positive and beneficial results.
- Medical research involving human subjects should only be conducted if the importance of the objective outweighs the inherent risks and burdens to the subject. This is especially important when the human subjects are healthy volunteers.
- Medical research is only justified if there is a reasonable likelihood that the populations in which the research is carried out stand to benefit from the results of the research.
- The subjects must be volunteers and informed participants in the research project.
- In any research on human beings, each potential subject must be adequately informed of the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study, and the discomfort it may entail. The subject should be informed of the right to abstain from participation in the study or to withdraw consent to participate at any time without reprisal. After ensuring that the subject has understood the information, the physician should then obtain the subject’s freely given informed consent, preferably in writing. If the consent cannot be obtained in writing, the nonwritten consent must be formally documented and witnessed.

In summary, all experimental procedures in human patients or normal volunteers must conform to these standards or to one of many other codes that have been derived from these original sets of principles [06310].
The ethical principles used by review bodies

Given the understanding that some experimental studies in human subjects and patients are an acceptable part of acquiring knowledge of normal human biology, all the review and regulatory bodies involved in gene transfer studies are charged with two principle tasks common to all human experimentation. The first is to minimize risks of harm to research subjects and to assure that the risks are reasonable in relation to the expected benefits to the subject or in relation to the importance of the knowledge likely to be derived. Harm could include physical harm such as pain and serious injury, psychological damage such as stress or guilt, invasion of privacy, and breach of confidentiality. Risk varies from minimal to severe, with minimal risk generally taken to mean a risk of harm no greater than a subject would encounter in daily life or during routine performance of physical or psychological testing. An undeliverable or excessive promise of reward or benefit can be considered another form of harm; a particularly appropriate issue in the case of healthy young athletes in the context of genetic enhancement applications. Identification of benefits or potential benefits is also a vital part of the decision process in these panels. Benefits include potential increased well-being of the subjects, but since most gene therapy studies to date have been phase I studies in which the goal is merely to establish safety and not efficacy, research participants cannot expect to derive any direct benefit [06310]

The decision to enter into an experimental procedure requires full disclosure by the investigator, informed and voluntary consent by the subject, with the word informed underscored in the context of potential genetic doping. There are some basic requirements that must be satisfied when seeking informed consent from research subjects. It should be clearly stated that the subject is being invited to participate in a research project. There should be a comprehensible description of reasonably foreseeable harms (physical, psychological, social) and benefits that may arise from participation. Assurance that prospective subjects are free to decline their participation must be provided and that subjects have the right to withdraw at any time without prejudice. The possibility of commercialization of research findings and the presence of any actual or potential conflicts of interest on the part of researchers, their institutions, or sponsors [06310].

Further amendments

It has also been agreed on a series of conclusions on gene technology, some general and other specific to sports [06308]:

General principles
A. Gene transfer technology, which is still at the investigational stage, is nevertheless already beginning to demonstrate clinical efficacy
B. While genetic technologies hold immense therapeutic promise, there is potential for their misuse, including attempts at the enhancement of athletic performance
C. The collective efforts of scientists, ethicists, athletes, sports authorities, medical practitioners, professional societies, pharmaceutical and biotech industries, and public authorities (including governments) will be required to avert such misuse
D. The compliance with established international standards pertaining to genetic experimentation involving human subjects, such as the Helsinki, Geneva, and Lnuyma Declarations that prevent unethical research is essential. The application of genetic transfer technologies should be consistent with established standards of professional behavior
E. The pace of research in the field of genetic transfer technology is such that governmental
and other regulatory agencies must work with a continued sense of urgency to establish a social and policy framework to guide this research and its applications and sanction breaches of the framework.

F. Broad public discussion and the development of social and policy frameworks must surround the distinction between genetic therapy and genetic enhancement. The time for the social framework to be established is before abuses occur, not after-the-fact.

**Sports specific**

A. Athletes, in common with other people in society, are entitled to the benefits of genuine therapeutic applications to treat injuries and other medical conditions.

B. There are evident risks that genetic transfer technologies might be used in a manner that would be contrary to the spirit of sports or potentially dangerous to the health of athletes. Akin to doping in the present generation, genetic transfer technology that is nontherapeutic and merely performance enhancing should be prohibited.

C. The definition of doping used by WADA, the IOC, international sports federations (IFs), and national authorities should be expanded to include the unapproved use of genetic transfer technologies.

D. One of the benefits of genetic technology is its potential use in the detection of prohibited substances and methods.

E. The scientific community has recognized the need for the continued development and refinement of methods that will permit the detection of the misuse of genetic transfer technologies in sports. It has been noted there are a number of approaches that currently exist, or are in development, that will permit such detection.

F. The present focus of WADA’s research grants toward the study of the detection methods for the misuse of oxygen-carrying agents and growth factors should be extended to include the detection of genetic transfer technologies and their effects.

G. The World Anti-Doping Code, which is planned for implementation by 2004, should include language prohibiting the use of genetic transfer technologies to enhance athletic performance.

H. WADA calls upon its government members, in particular, to expedite the development of a global social framework for the application of genetic transfer technologies that address the potential misuse of these technologies in sports and a publicly stated deadline for the adoption of that framework.

I. WADA calls upon governments to consider the following recommendations for inclusion in the regulatory framework pertaining to genetic transfer technologies and related research:

- Address breaches of the social framework within the criminal or penal realm.
- Extend corporate liability to directors, officers, and senior employees.
- Extend civil and criminal. There are evident risks that genetic transfer technologies might be used in a manner that would be contrary to the spirit of sports or potentially dangerous to the health of athletes. Akin to doping in the present generation, genetic transfer technology that is nontherapeutic and merely performance enhancing should be prohibited.
- Require detailed record-keeping in respect of all applications of gene transfer technologies with independent audit requirements.
- Expand standards of medical and professional behavior to prohibit the improper use of genetic transfer technologies and that such rules be actively enforced.

J. WADA calls upon governments and the sports movement to establish and fund educational and ethics programs designed to prevent the possible misuses of genetic transfer technologies in sport. WADA is willing to coordinate the design and dissemination of such programs.

K. WADA and the scientific community will establish a mechanism for continuing dialogue and consultation around the subject of genetic transfer technologies.
Sport as an occupation

Occupational medicine deals with all work related aspects of health that affect the employee's ability to function effectively: the workplace itself, the type of work, the state of health of the employee. In addition to purely physical aspects, social and psychological influences must also be considered. It is easy to see that construction workers who are paid according to how much work they complete will be subject to greater stress than, say, a gardener or office worker employed on standard terms. Moreover, within any occupation there are those – often a considerable percentage – who will regularly need medicines to function properly—for example, those with diabetes, high blood pressure, allergies, rheumatic disorders. In such cases, any extra stress in the work environment can easily lead to a situation where the ability of the person to function is close to the borderline of what can be physically expected. These people can often become incapable of continuing in the job or of only doing so under medical supervision and with the prescription of suitable medicines.

There are definite limits to the level of stress under which such people can function and it is the concern of occupational medicine to recognise and deal with these limits. Occupational medicine aims to point out to both employer and employee that only under certain specified conditions will optimum performance be possible. The conditions that could be recommended in such cases might include changes in the workplace, in working hours, in the pressures of the job, or might specify regular medical treatment for the employee—for example, prescription medicines to protect the employee from the effects of workday stress, such as beta-blockers. If we now consider a sport such as football, a number of examples can be identified. The proportion of players who have allergies is similar to that in the general population, and the treatment will be the same—that is, appropriate therapy often involving the taking of medicines, especially during those times of the year when the allergen count is high. But when we are dealing with open air sports, the treatment prescribed could lead to problems since many of the drugs usually prescribed are on the list of banned substances (such as corticosteroids) even though their prescription is medically justified. An example of an American professional international woman player makes the situation clear. She has a relatively rare disease that makes her blood pressure and fluid balance subject to extreme variations; this in turn makes it impossible for her, without medical help, to pursue her profession at the required level. She needs ongoing treatment with a mineralocorticoid (fludrocortisone). However, in contrast with those mentioned above, this medicine has neither an anabolic nor an anti-inflammatory effect and is thus not technically a doping substance in the true sense of the term. This raises the question whether it really constitutes doping if a player can perform at the expected level only after taking such a medicine. It is suggested that this is a problem that falls within the scope of occupational medicine. If such treatment is prescribed for genuine medical reasons and involves taking a drug that in itself has no doping effect, then we cannot be talking about a case of doping, rather merely of enabling a professional player to exercise his or her normal profession. Occasional treatment with banned substances for "bona fide" medical reasons should be permitted if the facts of the case are presented openly to the doctors in charge of the doping control. A quite different question is whether the ever increasing demands faced by professional footballers, in terms of the number of matches and tournaments in which they are expected to play, can be compensated for by taking medicines so that the required level of performance can be achieved over and over again. Playing so frequently, in football as in other sports, under circumstances necessitating more or less continual treatment with painkillers and anti-inflammatory agents, can have serious long-term consequences that really cannot be justified on the basis of occupational medicine or medical ethics. In this case, the limits of doping are recognisable [06001].
Admittedly there is no doping in the first two examples in terms of performance enhancing drugs being taken. However, in the sense of medical treatment being used to suppress the symptoms of injuries and overexertion, clearly there is an aspect of doping involved. The workplace pressures on players in the short term lead to long term effects being ignored. As long as the players in question and their associations all have the same approach, only a firm stand by sports and occupational medicine will have the effect of providing the players with at least partial protection from such long term damage. This is further reason why the campaign against real doping must be actively pursued [06001].

**Legislation of drugs aimed for doping**

The arguments for and against the legalization of performance-enhancing drugs in sport operate on two different levels. On one level, there are pragmatic arguments concerned with the effort required to establish and enforce controls, the quality and quantity of these controls, and the responsibility for and costs of regulations. Also on this level are arguments concerning the need to preserve the audience’s trust in sport, freedom of choice for athletes, the justification for introducing additional risks and the need to avoid risk, especially in children and adolescents. On another level, there are also arguments that touch on the “spirit of sport” and the “naturalness” of performance in sport. Whether this “spirit of sport” has a “central constitutional quality” which one may not change under any circumstances, or whether in fact it can be modified, remain as controversial as the question of whether doping is consistent with the true “spirit of sport” or not. Even if it were thought acceptable to abolish normative behaviour consistent with “the spirit of sport” and that it would be then still possible to perform with a degree of “naturalness”, this step should be taken only if advantages could be expected to ensue. Doping also entails avoidable risks that are not necessary to increase the attractiveness of the sport. Furthermore, many risks, particularly over the long term, are difficult to anticipate. Legalization would not reduce the restrictions on athletes’ freedom; the control effort would remain the same, if not increased. Extremely complicated international regulations would have to be adopted. Above all, the function of sport as a role model would clearly be damaged [11308].

The doping problem in sport has yet to be solved. One must continue to assume that controls do not identify all athletes who use performance enhancing drugs. As a result, a general suspicion has arisen that some of the most outstanding achievements in sport may have been achieved by doping. For this reason, several authors have claimed that controlled use of performance-enhancing drugs in sport should be permitted. If that were to happen, the central argument against doping – i.e. the fairness argument – would immediately be rendered irrelevant (assuming that all athletes used performance-enhancing drugs within the permitted boundaries), and a ban on doping would be more difficult to justify. However, any legalization of performance-enhancing drugs in sport, if it were to occur, would need to be subject to limitations. This is not disputed by either opponents or supporters, for complete legalization would also sanction actions that introduced risks and possibly irreversible damage to an athlete’s health. That would certainly be unacceptable, even if the athlete were willing to accept the risks and damaging effects, because it is inconsistent with the favourable health requirements of sport. In extreme cases, the medically induced, transient success achieved by doping would be paid for with definite and permanent damage to health or even loss of life. This is considered to be too high a price to pay in sport, even in a society that permits self-destructive behaviour. One review therefore examined whether performance-enhancing drugs should be permitted in sport under the control of physicians, and evaluates the expected outcomes of such a scenario. Such a change in regulation would need to be tightly controlled because of the risks involved. The results of legalizing
performance-enhancing drugs in competitive sport would be either unhelpful or negative, and the unwanted aspects of doping control would not disappear. Athletes, including children and adolescents who wanted to pursue competitive sports, would be forced to take additional, avoidable health risks. How the appropriate limits of the use of performance-enhancing drugs can be determined is by no means self-evident and requires further investigation. The current lack of knowledge means deciding which doping activities lead to which unwanted effects is often difficult to determine, particularly with regard to the long-term perspective. This lack of knowledge is particularly problematic in relation to new substances. Also, no biological agent powerful enough to achieve major changes in body or mind is likely to be entirely safe or without side effects. Moreover, if a legalization of performance enhancing drugs in sport were to be introduced, it would need to be clarified who should determine the permissible limits of use of such drugs. This could not be left to the discretion of an individual physician, as assessments of the acceptability of risks in doping may vary. Moreover, athletes who were willing to take risks would search for the most helpful doctor. Thus, the concept of equal opportunities for all athletes would be compromised by a physician factor. To avoid this, the limits of doping would need to be specified in advance and independent of the physician-patient relationship. Furthermore, to ensure comparability of conditions for athletes, such rules would need to be established and ratified internationally. Thus, an international organization with the required expertise and authority to make such evaluations would still be needed to determine the permissible limits of doping in sport. All limits on doping would then be based on whether the risk to the athlete was still considered acceptable. Ensuring the safety of athletes in the event of a legalization of performance-enhancing drugs would require internationally coordinated, costly and complicated regulations that would require considerable effort and result in extensive controls. There might be rare instances of doping in sport that had acceptable side effects, but making these permissible would add further difficulties in terms of defining limits and introducing further controls. The “natural lottery” of athletic talents would be compensated for only partially by use of performance-enhancing agents. It would also be complemented by another “natural lottery” of variable responses to doping measures, combined with the inventiveness of doping doctors. There would be no gain in “justice” (i.e. fairer results that reflected efforts made) for athletes as a result of legalizing doping. Legalization would not reduce restrictions on athletes’ freedom; the control effort would remain the same, if not increased. Extremely complicated international regulations would have to be adopted. The game of the “tortoise and the hare” between doping athletes and inspectors would remain because prohibited but not identifiable practices could still provide additional benefits from use of permissible drugs. Audience mistrust, particularly toward athletes who achieved outstanding feats, would remain because it would still be possible that these athletes were reliant on illegal doping practices. Doping entails exposing the athletes to avoidable risks that do not need to be taken to increase the appeal of a sport. Most importantly, the function of sport as a role model would definitely be damaged. It is not necessary to clarify the question of what constitutes the “spirit of sport” and whether this may be changed. From a practical point of view, a legalization of performance-enhancing drugs in sport should not be considered for the simple reason that it has no advantages but many disadvantages [11308].

What role would be left for physicians if performance-enhancing drugs were to be legalized under medical supervision? Medical supervision would be concerned only with the development and, if necessary, production of performance enhancing drugs, their use within the permissible boundaries and controlling their effects. Further responsibilities and, in particular, assessing which risks are acceptable for the athlete to take, should not be assigned to the physician. Nevertheless, even with pre-determined limits on the permissibility of performance-enhancing drugs, physicians would always retain a degree of autonomy in terms of determining the optimal doping regimen for their athletes. Enforcement of the present ban on performance enhancing drugs depends on extensive and logistically complex

2158
controls which, if they are effective at all, considerably limit the athlete's freedom. Supporters of a legalization of performance enhancing drugs might believe that concerns about athletes' health would lead to more tests when the use of such drugs is permitted. However, it is important to note that certain health problems manifest themselves only over the long term and cannot be detected by close medical inspection. Furthermore, if proponents of limited legalization advocate banning all doping practices with potential long-term risks, the great temptation remains for athletes to do these things illegally. In this respect, nothing would be gained. Thus, the key argument against limited legalization under medical supervision becomes clear, namely, that no advantage would be obtained in terms of reducing the current difficulties of implementing doping controls. There would always be those who attempted to use new, performance-enhancing methods that are not permitted and have yet to be discovered. For these reasons, if limited legalization of performance-enhancing drugs did ensue, an unpleasant development in athletic sports, which has spoiled the relationship between the audience and the sport, would continue. This refers to the fact that when an athlete achieves outstanding results in sport, the suspicion is automatically raised that this was achieved by doping. In other words, sports fans have become skeptical. This situation would probably not change with a limited legalization; there would still be the possibility that an excellent performance was achieved through the use of doping practices that are not within the rules. Thus, even the lack of trust in athletes would not disappear.

The argument for legalization is usually based on the fact that athletes take risks in sport anyway, and banning doping therefore smacks of unacceptable paternalism. However, this argument fails to observe an important distinction: the risks of doping in sport are additional and avoidable, whereas other risks in sport are unavoidable. It is impossible to play football or other kinds of sport without risk of injury. Furthermore, while in many other kinds of sport the precautions taken can lower the risks, they cannot eliminate them completely. Conversely, as noted, the risks of performance enhancing drugs add to those that already exist in sport and can be completely avoided by doing without drugs all together. However, this raises the question of whether it is beneficial to take extra, avoidable risks in sport, e.g. to make the sport more attractive, an issue that is discussed further in the next section. The concept of “inherent coerciveness” would assume greater importance if limited legalization of performance-enhancing agents in sport were to come into effect. All competitive athletes have to make adjustments in many areas of their lives if they want to be successful in their given sports. Thus, the athlete has liberty to act but in the knowledge that his/her actions will have certain consequences. If the athlete were to forgo certain performance-enhancing behaviour, he/she would be less successful. If one allows the use of performance-enhancing drugs within certain boundaries, then all athletes who wished to be successful would have no choice but to use the substances that are allowed by the rules. They would have “free choice under pressure” in this respect. They would be forced to take actions that entail risks that are unnecessary in sport and confer no advantages upon their sport. In this respect, a limited legalization of performance-enhancing drugs would unnecessarily put further pressure on athletes to do more risky things. Conversely, “an effective policy for eliminatin performance-enhancing drug use would harm no one, except those who profit from it”.

The fact that a legalization of performance enhancing drugs would not be advantageous to sport can be seen by analysing how the use of these drugs would affect performance. The following possibilities arise:

1. All athletes respond to the approved doping measures in the same way and their performance improves in the same way. In that case, the finishing order of cyclists in the Tour de France, for example, would remain unchanged. The event would be slightly shorter in duration with the use of performance-enhancing drugs than without them. However, that would be of no benefit to the competition. All of the athletes
would have put in considerably greater effort and would have been required to take more risks with no change in the result of the event. Furthermore, the generations of athletes who were not allowed to use performance-enhancing substances would have more difficulty being included on lists of the “alltime best athletes” based on absolute values of times, weights, lengths, etc.

2. The previous point explores the consequences of legalized doping in sport if the upshot is that all athletes respond in the same manner. However, it is improbable that all athletes will respond to doping options in the same way because different measures would probably be used; and athletes respond differently to performance-enhancing drugs. The use of different doping measures is possible when there is flexibility in prescription (e.g. dosage) and enforcement of permissible doping practices. In such circumstances, athletes will strive, with the help of their physicians, to identify and use the best method for enhancing performance within the permitted limits. Thus, the outcome would reflect not only the athletes' performance, training methods, discipline and talent, but also the cleverness of their supervising physicians in finding and using the optimal doping aids within permissible boundaries. This technical extension of the competition in addition to current medical care in sport would become increasingly complicated and expensive as more substances were included on the permitted list. Different responses of athletes to performance enhancing drugs would also be expected to occur because of genotypic differences alone.

“Naturalness” in sports

If a legalization of performance enhancing drugs became a reality, the new lottery of differences in response to performance enhancing drugs discussed in the previous section would be combined in most cases with the ingenuity of the particular sports physician and other existing factors (natural talent, discipline, training) to determine the outcomes of athletic events. It must also be reiterated that a limited legalization would not exclude continued use of prohibited doping methods, perhaps in addition to use of permitted agents. It also seems likely that the more the permitted drugs were limited to minimize risks, the greater the temptation would be to use prohibited doping measures. Crucially, a legalization of performance enhancing drugs would have a massive impact on the perception of sport. It would ultimately compromise the currently, widely accepted “spirit of sport”. Sport is an artificial setting, created by human beings, in which the competitor is required to perform, at least according to current, widely prevalent belief, with a degree of “naturalness”. The sports-watching audience is interested in “athletic performance, not biochemistry.” “The fascination of sports mainly comes from the demonstration of what people are able to do on their own. Doping destroys this fascination. "However, it is important to clarify three aspects of “naturalness of performance” in sport. The first is the difficulty of determining what constitutes a “natural” measure of improving performance. Many permissible training methods and food supplements are in some ways less “natural” than other things that athletes may do. However, the fact that defining an acceptable limit for such measures, particularly when the dividing line appears to be opinion-based and is established on a more or less continuous spectrum, does not necessarily mean that we should dispense with such limits. Furthermore, this is not the approach taken in other areas of life. The difficulties inherent in putting forward arguments as to why one substance or another should or should not be on the World Anti-Doping Agency list are not sufficient reasons to characterize this list as completely arbitrary and, therefore, irrelevant. Second, it must be clearly stated that “naturalness” in sport is not considered as a value in itself, but only as a value in this specific context. In sport, great importance is attached to the “naturalness” of achievements, whereas in other areas of life this is not necessarily the case. Third, according special attention and value on
“naturalness” of performance in sport means that sport is considered different to other areas of life, in which certain “artificial” measures of obtaining improvement are allowed [11308].

An example for the society

Another argument for the need to protect the “spirit of sport” can be put forward. There can be no doubt that sport, playful in nature, but still in accordance with the rules, sets an example for society. As Albert Camus once said, “After many years during which I saw many things, what I know most surely about morality and the duty of man I owe to sport.” Sport shows, with its rules and requirement for fairness, how to deal with other problems in society. It conveys an attitude that acts as a role model in many other areas of human life and “in a sense, it can be a model for a better society.” On this issue, three different facets of the functions of role models need to be distinguished. An aspect of society can take on the function of a role model if it sets special, exemplary standards; respects certain standards in a special and exemplary way; or controls or ensures compliance with the standards in an exemplary way. First, it is useful to consider the ongoing impact of sport as a role model in these three areas if the ban on performance-enhancing drugs remained in place as follows:

- with respect to setting standards, sport would remain a model at least for most citizens
- continued violations of the norm (standard) would be anticipated and the function of sport as a role model in terms of respecting standards in a special and exemplary way would continue to be debatable
- the system of controlling or ensuring compliance with standards in sport is currently unconvincing, with many doping violations remaining undiscovered. Improving the control system would depend on further technical developments (e.g. in the collation of indirect evidence), which would make the system more convincing and therefore more able to act as a role model

Second, it is important to consider how the exemplary role of sport could change if doping were legalized as follows:

- at least for a significant part of society, sport would lose its function as a role model because the model standard it exemplifies would be abolished
- with a limited legalization of performance enhancing agents, continued violations of the new norm would be anticipated because the potential for the use of additional banned but performance-enhancing substances would remain
- the cost of control would remain unchanged and the suspicion that the control system was not effective would remain. Again, therefore, nothing would be gained in these respects compared with the existing ban. The function of the role model would also still be dependent on further technical development.

To summarize, a legalization of performance enhancing drugs would definitely result in some lessening of the exemplary role of sport in terms of setting standards. The same challenges that exist at present would need to be faced with respect to the other facets of role model function. Overall, the function of sport as a role model would be reduced. A legalization of performance enhancing drugs would diminish the function of sport as a role model, and this would particularly be the case with respect to children and adolescents. Self-restraint would be abandoned and the message would be that one must be willing to do anything for success. This boundless willingness is not a preferred role model, especially for youth. It is doubtful whether one should raise children according to a life plan which links life satisfaction to the boundless willingness to provide peak performance.” A total ban on doping for children and adolescents when there is simultaneous legalization for adults is impracticable and
would not seem to be feasible. Also, the manner in which children and adolescents under the age of 18 years (which is an advanced age in many sports) react to performance enhancers is not known. However, supporters of a legalization of performance enhancing drugs do not exclude children and adolescents. Rather, they justify approval of doping in these age groups by pointing out that competitors at this stage are taking various other risks in sports anyway [11208].

**Law versus ethics**

There have been several sceptical challenges to the legitimacy of the antidoping position arising from both philosophers working in the field of medical ethics and sports ethics. They argue, typically, that the bans on certain performance-enhancing processes and substances rest on principles that are inconsistently applied. Criticisms of the ethical justification of antidoping legislation are not uncommon in the literatures of medical ethics, sports ethics and sports medicine. Critics of antidoping point to inconsistencies of principle in the application of legislation and the unjustifiability of ethical postures enshrined in the World Anti-Doping Code, a new version of which came into effect in January 2009. Critical discussion has focused on the legitimacy of the use of steroids, genetic manipulation and other forms of illicit performance enhancement. Surprisingly little discussion has been had on the interface between law, medicine and ethics as they converge in sports medicine and elite sports. Athletes, whether they choose to or not, provide role models for society, and their better performances are not morally admirable when their record-breaking feats are not the product of natural talents. Developing coherent ethical and legal responses to the use of doping is difficult partly because of the challenges ethical postures have traditionally created for the law [10270].

The World Anti-Doping Agency (WADA), established in 1999, is based on the cooperation between sports organisations and governments, and is financed by sports organisations and governments on an equal basis. Concerning doping, therefore, WADA and the WADC enjoy a hegemonic position with respect to medical, policy, scientific, as well as juridical matters. The WADC is predicated upon the evidence of sports medicine and sports science experts who determine which substances or processes are to be prohibited. The determination of the resultant list of prohibited substances and methods is juridically final. This position has, however, never been challenged in any court. Antidoping rules are juridical norms and belong to the area of sports law, whose relationship with public legislation is not always clear. This means not only that athletes are uncertain of the legitimacy of the legislation, but so too are physicians and healthcare professionals who are part of the sports medical entourage. Typically sports are organised nationally, but fall under the jurisdiction of international sports federations and, when relevant, the International Olympic Committee. Nearly all major sports federations now have their own antidoping rules; nevertheless, the WADC obliges them to apply the obligatory articles of the WADC and thus to follow the principles of the WADC. In this way the juridical norms of the WADC now concern not only the athletes but also other people taking part in one or other way in the sport concerned under the jurisdiction of a signatory of the WADC. Doping has been criminalised in some European countries (e.g. France, Italy, Slovenia), but seldom beyond there. Doping in criminal law is often more limited in scope than in sports legislation. The differences between public law and private law have kept these two sets of procedures juridically distinct. On one hand, sports organisations or their tribunals apply their antidoping rules with disciplinary sanctions, whereas on the other hand criminal courts apply the state law concerning doping punishments. One significant consequence of this dual legal economy has been that the same antidoping violation can now be, and often is, punishable both as a crime in a criminal court and as a disciplinary offence in a sports organisation or its tribunal (or a surrogate such as the CAS). The
incidence of these cases has greatly increased in recent years [10270].

When cases of the dual prosecution of athletes and their medicoscientific support systems become more common, the question of whether the principles of criminal law should be applied to doping disciplinary processes will be accentuated. At least three ethicolegal dimensions of doping prosecution deserve critical scrutiny:

- notions of guilt, negligence and liability
- aspects of punishment
- privacy

Rule breaking exhibits degrees of intentionality. It also typically elicits guilt. Culpability is not limited to premeditated acts. The rugby player who stretches his foot out to trip his opponent, or the football player who swears at the referee in response to a perceived poor judgement intend their acts and may or may not feel guilty. Equally, players may feel guilty for accidental career-ending injuries they inflict unintentionally on another professional. The establishment of an offence without mens rea is not without precedent in sports rules, but a particularly strong variant arises in antidoping policy when a prohibited substance is found to be present within an athlete’s body tissue or fluids. Ought then the principle of nulla poena sine culpa (no punishment without guilt) to be applied in doping offences without intention? It is important to note that culpa, guilt, may be constituted by an act or omission. The common omission is that of negligence. Guilt may indeed be relatively blameless in the case of the accidental or unwitting ingestion of banned substances provided by other parties. Yet beyond the legal sphere, it could be argued that this strict liability is in conflict with the more general presumption of innocence. It might be argued that doping offences ought not to be considered an exception to the general ethical norm of demonstrating culpability [10270].

One of the leading principles in criminal law and part of everyday morality is that for one and the same crime only one punishment can and ought to be imposed, ne bis in idem (not twice for the same). Only the punishment by a criminal court is a punishment in the sense of criminal law, whereas the other is better conceived of as a sanction. The doping sanction by a sports organisation or its tribunal is a thus disciplinary measure. The first one belongs to the area of public law, the latter of private law. Can the separateness of these two sanctions still stay without offending the principle ne bis in idem? It seems not. Rather, the athlete merely has to pay twice for one act, only the recipient is separate. From the athlete’s point of view there is no difference in fines between public and private law because the consequence is the same. One potential exception arises in the case of the private sponsorship of sports teams and individual athletes therein. Athletes and members of the support team frequently make an agreement with third parties for different kinds of economic and other benefits and entitlements (such as state-funded health care). In these agreements it is possible that the athletes commit themselves to compensate to their federation for the loss to their image and/or economic losses in the case of a doping offence of which the athlete is found guilty. There may be cases then when there can be in one and the same doping offence three different sanctions: punishment according to criminal law; disciplinary sanctions according to the national sports federations antidoping rules and contractual penalties (financial compensation) according to private law and the agreement between the athlete and his/her sports federation [10270].

In the area of genometry—the nascent field of science and technology that proposes to apply enhanced understanding of the human genetic code to reshaping our individual and collective destinies—no topic has generated more interest among the general public, as well as in the athletic community, than the potential for physical enhancement of the human body and its performance. Genometric experiments have produced physically enhanced mice, and
the production of similarly enhanced humans may not be far off. Although it is not the
objective of most genometric research, the day will come when gene-based "treatments" will
enable individuals to build muscle or increase endurance faster than is possible through
conventional methods. One article describes developments in the area of physical
enhancement that may find application in the "gene doping" of athletes. For example, human
performance-related genes may be delivered to athletes using tools developed for research
in gene therapy; the protein products of these genes may be administered in recombinant
form; and recently discovered small-molecule activators of the major genetic regulatory
pathways of physical prowess may be taken orally, providing "exercise in a pill". This article
also describes US and international attempts to regulate and punish the use of prohibited
techniques for performance enhancement among athletes. As science advances, defining
and detecting "gene doping" becomes increasingly complex. Thus, the study of physical
enhancement provides an ideal starting point for the interdisciplinary Redefined Destinies
Colloquium's examination of the intersection between law and science [10271].

Undoubtedly, what has been the most contentious of the revised WADC has related to the
privacy of the athlete. There are two cases that may be thought problematical: urine sample
provision and the athlete's location and availability for testing. The ability of police officers to
undertake bodily searches is forbidden in law in all but highly serious criminal cases. By
contrast, in doping control all athletes have an obligation to give a urine or blood sample in
any place at any time upon request. These samples have to be given under the supervision
of a doping control officer so that he/she can observe urination at all times. This ruling was
established in order to prevent athletes using a catheter to provide "clean"urine samples,
which were stored at a time when they were not on a doping cycle. Clearly, there is no
privacy in this operation. In democratic societies one may move freely without announcing to
public authorities where one is going. Yet elite athletes belonging to a doping testing pool in
each sport are required to give whereabouts information. Typically, this means nominating
one hour per day months in advanced when they must consider it their duty to be present.
The information must thus be accurate and predictive. Three failures to comply within an 18-
month period constitute an antidoping violation. The surrender of certain freedoms of
movement from surveillance is a highly controversial issue. One justification for the privacy
limitations is that the athletes have agreed to observe all the sports rules when they
compete. They cannot select which to observe and which not. A rejoinder might be that they
have no choice other than compliance but that they do not assent to its legitimacy. It may
well be thought that the request for whereabouts information is a coercive offer; that elite
sportsmen and women can only ply their trade effectively in one arena, and thus that the
controls undermine autonomous assent [10270].

The idea that sport is somehow beyond the ethics of everyday living and law has long been
jettisoned. The legal peculiarities highlighted here and their ethical implications reveal how
antidoping legislation and rules appear to differ from other areas of law and commonsense
morality. Although it has sometimes been argued that doping legislation is unacceptably
burdensome, it has been attempted to disambiguate certain apparent tensions between
criminal law, private law and everyday ethics. Nevertheless, the nearly unanimous decision
of national sports federations and sports federations to regulate against doping has been a
response to a genuine crisis in the representation of the values of sport themselves and
serves to protect the interests of all engaged therein. Sports physicians, medical ethicists
and technologists generally have recently argued for the liberalising of regulations
concerning human enhancement in sports. Critical discussion has focused on the legitimacy
of the use of steroids, genetic manipulation and other forms of illicit performance
enhancement. Surprisingly little discussion has been had on the interface between law,
medicine and ethics as they converge in sports medicine and elite sports. The use of a wide
variety of banned and non-banned supplements is prevalent among casual gym users, those
active in sports, and is by no means restricted to elite athletes. This development is to be seen in the context of changes in social attitudes to medical and pharmaceutical products and services that have developed in relation to the supratherapeutic goals of medicine. Athletes, whether they choose to or not, provide role models for society, and their better performances are not morally admirable when their record-breaking feats are not the product of the “virtuous perfection of our natural talents”. Developing coherent ethical and legal responses to the use of doping is difficult partly because of the challenges ethical postures have traditionally created for the law. Therefore, criticisms of the ethical justification of antidoping legislation are not uncommon in the literatures of medical ethics, sports ethics and sports medicine. Critics of antidoping point to inconsistencies of principle in the application of legislation and the unjustifiability of ethical postures enshrined in the World Anti-Doping Code, a new version of which came into effect in January 2009. One article explored the arguments concerning the apparent legal peculiarities of antidoping legislation and their ethically salient features in terms of: notions of culpability, liability and guilt; aspects of potential duplication of punishments and the limitations of athlete privacy in antidoping practice and policy. It is noted that tensions still exist between legal and ethical principles and norms that require further critical attention. Doping has been criminalised in some European countries (e.g. Sweden, France, Italy, and Slovenia), but seldom beyond there. In some member states of the European Union related acts such as drug abuse or the smuggling of medicines are criminal acts. In other cases, tax law, for example, has been used by state authorities to use coercive powers such as search and seizure for the detection of crimes to prosecute the case. It is important to note that doping has not, however, usually been defined in criminal codes exactly in the same way as in sport. Doping in criminal law is often more limited in scope than in sports legislation. The validity of a doping violation is established by a sports tribunal, which is convened by national sports federations or international sports federations. Sanctions normally take the form of ineligibility and the loss of medals, prize money and so forth. These override rights to compete and reward, which are internal to the sport and its governance. In cases of dispute, and in accordance with the WADC, the Court of Arbitration for Sport (CAS) arbitrates between parties. It is noteworthy that this is obligatory in cases of antidoping under the auspices of the WADC, whereas in other disputes both parties must normally consent for the case to be heard there and to abide by its rulings. By contrast, antidoping rules of sports organisations have emerged within the sphere of private law and, in most cases, remain there.

International doping policy: the WADA and the WADC

The World Anti-Doping Agency (WADA), established in 1999, is based on the cooperation between sports organisations and governments, and is financed by sports organisations and governments on an equal basis. Its most notable achievement has been the worldwide WADC in 2003 and its revision implemented in 2009. The rationale behind the WADC is the harmonisation of antidoping rules and measures. Nearly all international sports federations have accepted the WADC. Concerning doping, therefore, WADA and the WADC enjoy a hegemonic position with respect to medical, policy, scientific, as well as juridical matters. The WADC is predicated upon the evidence of sports medicine and sports science experts who determine which substances or processes are to be prohibited. The determination of the resultant list of prohibited substances and methods is juridically final. This position has, however, never been challenged in any court. Antidoping rules are juridical norms and belong to the area of sports law, whose relationship with public legislation is not always clear. This means not only that athletes are uncertain of the legitimacy of the legislation, but so too are physicians and healthcare professionals who are part of the sports medical entourage. Despite co-funding and legitimation by national sports federations and governments on an equal basis, the juridical norms of the WADC are not generally binding and apply only to the international sports federations who are signatories to the WADC. They do not apply directly
to athletes themselves. Instead, athletes fall under the jurisdiction of their own national sports federation antidoping rules, which themselves are governed by the WADC. Typically sports are organised nationally, but fall under the jurisdiction of international sports federations and, when relevant, the International Olympic Committee. Nearly all major sports federations now have their own antidoping rules; nevertheless, the WADC obliges them to apply the obligatory articles of the WADC and thus to follow the principles of the WADC. In this way the juridical norms of the WADC now concern not only the athletes but also other people taking part in one or other way in the sport concerned under the jurisdiction of a signatory of the WADC. This development is an important development because it is now well known that the athlete engages in doping as part of a systemic effort between coaches, masseuse, physicians, physiotherapists, team management and so on. The clearest examples of this systemic sports medicine and sports science development in former times was the East German state sports science apparatus, and more recently the Tour de France 2007, in which, it is widely thought, that the majority of professional teams were engaged in doping practices to some degree. Article 2.8 may now be employed to hold sports physicians to account for their complicity with doping violations. A sports organisation may impose a sanction for this violation in accordance with its rules. This is not a punishment in the same sense as in the criminal code, but is better thought of as a disciplinary consequence or sanction. These sanctions are limited to the powers of a private organisation. So, if these doping rules are broken, the question is not typically one of a crime being committed but rather the lesser one of a violation of the rules of a private organisation.

Public law versus sport's law

More countries have included doping offences in their criminal legislation, the supervision of doping offences has expanded and, when previously only athletes were punished, now the focus is also on the elite sports support system including sports physicians. This dual legal economies have sometimes converged. A cursory examination of this convergence is found in the following recent and high profile examples. The Tour de France doping scandal in cycling in 1998 started from police investigations, and the Chinese doping scandal in swimming in 1998 started from Australian customs officers’ seizure of human growth hormones entering Australia for the world championships of that year. Similarly, in Spain Operation Puerto was conducted by the Spanish authorities into doping practices that followed the seizure of prohibited substances and other material by the Spanish police in 2006. It should be noted, here, however, that tax evasion legislation initially uncovered an illegal pharmacology trade, and the fallout led to the revelations that led to UK sprinter Dwayne Chambers’ ban from international athletic competition for 2 years and his lifetime ban from representing Britain at the Olympic Games. In the Athens Olympic Games in 2004 two Greek sprinters, Kenteris and Thánou, evaded doping control officers. Perhaps the most recent high profile case are the investigations in the USA into Balco Laboratories, where tax investigations uncovered widespread illicit doping, which saw the greatest ever American female athlete being imprisoned. We will comment on this below. These high profile cases seem to reinforce the idea that there is a certain dependency on the powers of state authorities if antidoping rules are to be effective. In the following section we probe this dual legal economy between sports antidoping rules and criminal law.

Guilt, negligence and liability

Rule breaking exhibits degrees of intentionality. It also typically elicits guilt. Culpability is not limited to premeditated acts. The rugby player who stretches his foot out to trip his opponent, or the football player who swears at the referee in response to a perceived poor judgement intend their acts and may or may not feel guilty. Equally, players may feel guilty for accidental career-ending injuries they inflict unintentionally on another professional. Moreover, there are
many instances during games when the official decides that a pattern of rule-breaking behaviour, which, although it does not appear premeditated, exhibits intentionality in the habitual nature of the act. Aside from these cases, there are others in which a rule is determined to have been broken when the athlete fails in some other duty. So, the relay runner who steps on or over the inside lane marking is deemed not to have completed a minimum of 400 m thereby and is disqualified irrespective of the absence of *mens rea* (guilty mind). The establishment of an offence without *mens rea* is not without precedent in sports rules, but a particularly strong variant arises in antidoping policy when a prohibited substance is found to be present within an athlete's body tissue or fluids. Article 2.1 in the WADC wording states that an offence is merely for “the presence of a prohibited substance or its metabolites or markers in an athlete's sample". The wording of this violation indicates that this type of doping offence can include wider liability than other types of doping offences in which liability is estimated in accordance with normal juridical principles. Ought then the principle of *nulla poena sine culpa* (no punishment without guilt) to be applied in doping offences without intention? It is important to note that *culpa*, guilt, may be constituted by an act or omission. Guilt may indeed be relatively blameless in the case of the accidental or unwitting ingestion of banned substances provided by other parties. Yet beyond the legal sphere, it could be argued that this strict liability is in conflict with the more general presumption of innocence. It might be argued that doping offences ought not to be considered an exception to the general ethical norm of demonstrating culpability. The application of strict liability is not unique. There exist numerous other examples in which the level of care demanded is as high when imposing sanctions; such as in the transport of radioactive substances and wastes, or in dealing with hazardous chemicals or medicines, in dangerous building works and so on. Here the sanction operates to prevent harm to others and the self engaged in dangerous practices. The concept of “strict liability”, as it has been used in doping cases, as it has been argued, does not entail intentionality. The sanction is an inevitable consequence, if a doping offence has been established [10270].

**Privacy**

Undoubtedly, what has been the most contentious of the revised WADC has related to the privacy of the athlete. There are two cases that may be thought problematical: urine sample provision and the athlete's location and availability for testing.

- The ability of police officers to undertake bodily searches is forbidden in law in all but highly serious criminal cases. By contrast, in doping control all athletes have an obligation to give a urine or blood sample in any place at any time upon request. These samples have to be given under the supervision of a doping control officer so that he/she can observe urination at all times. This ruling was established in order to prevent athletes using a catheter to provide "clean" urine samples, which were stored at a time when they were not on a doping cycle. Clearly, there is no privacy in this operation, and this is confounded in the case of athletes who are legal minors, when a chaperone is required.
- In democratic societies one may move freely without announcing to public authorities where one is going. Yet elite athletes belonging to a doping testing pool in each sport are, according to Article 14.3 of the WADC, required to give whereabouts information. Typically, this means nominating one hour per day and 3 months in advance when they must consider it their duty to be present. The information must thus be accurate and predictive.

One justification for the privacy limitations is that the athletes have agreed to observe all the sports rules when they compete. They cannot select which to observe and which not. A rejoinder might be that they have no choice other than compliance but that they do not
assent to its legitimacy. It may well be thought that the request for whereabouts information is a coercive offer; that elite sportsmen and women can only ply their trade effectively in one arena, and thus that the controls undermine autonomous assent. The imposition on the athlete to surrender an aspect of their privacy non-elite athletes enjoy may also comprise a coercive offer. This contextual element increases the burden upon the WADA to justify its application. A key concern here will be one of proportionality. Does the surrender of one's privacy need to be so severe? Clearly the issue of weighing the demand to surrender privacy and to examine the latitude of testers merits further discussion [10270].

**Physician's responsibility**

The manner in which healthcare and medical professionals serve their athlete patients is governed by a variety of relevant codes of conduct. A range of codified rules is presented that refer both the welfare of the patient and the maintaining of confidentiality, which is at the heart of trustworthy relations. The 2009 version of the World Anti-Doping Code (WADC), however, appears to oblige all healthcare professionals not to assist athletes if they are known to be engaged in doping behaviours under fear of removal from working with athletes from the respective sports. In contrast, serving the best interests of their athlete patients may oblige healthcare professionals to give advice and guidance, not least in terms of harm minimisation. In so far as the professional conduct of a healthcare professional is guided both by professional code and World Anti-Doping Code, they are obliged to fall foul of one or the other [10272].

Sport is conducted in a highly charged and emotional environment. Doctors who engage in sports medicine frequently get involved in sport because they too are passionate about the sport. This passion may conflict with the necessity to be dispassionate about the outcome of the game when dealing with injured players. Sports-medicine doctors must remember at all times the importance of ethical medical practice and professional conduct. Prior to each game or session, team doctors should remind themselves of the basic principles of virtuous practice and the paramount importance of player autonomy. The sports team doctor should avoid becoming overinvolved with the team management to ensure that ethical principles do not get overlooked in the pursuit of victory. It is imperative that a degree of professional distance is maintained in order to achieve this [10272].

“I am in blood Stepp’d in so far that, should I wade no more, returning would be as tedious as to go o’er” is from Macbeth: Act III, Scene IV, by William Shakespeare. There are many ethical dilemmas that are unique to sports medicine because of the unusual clinical environment of caring for players within the context of a team whose primary objective is to win. Many of these ethical issues arise because the traditional relationship between doctor and patient is distorted or absent. The emergence of a doctor-patient-team triad has created a scenario in which the team’s priority can conflict with or even replace the doctor’s primary obligation to player well-being. As a result, the customary ethical norms that provide guidelines for most forms of clinical practice, such as patient autonomy and confidentiality, are not easily translated in the field of sports medicine. As a result, the ethical norms that provide guidelines for most forms of clinical practice, such as patient autonomy and confidentiality, are not easily translated in the field of sports medicine. The team doctor, as an individual, is not infallible, and medical decisions may be influenced by the appeal of status, admiration and gratitude. Such behaviour is self-gratifying, and it should be remembered that passion must not be allowed to compromise judgement. Sports doctors are thus frequently under intense pressure, whether implicit or explicit, from management, coaches, trainers and agents, to improve performance of the athlete in the short term rather
than considering the long-term sequelae of such decisions. A myriad of ethical dilemmas are encountered, and for many of these dilemmas there are no right answers. Informed consent in clinical sports medicine takes on a greater level of importance than in normal clinical circumstance because of all the extra pressures and influences. The ramifications of the player’s decision extend further than his own well-being and have an effect on his team and coach. The consent process may be threatened by the fact that different parties in the triad of relationships may have different values and priorities, and therefore might choose different options. This case raises a number of important questions: should informed consent be aimed at the team authority, such as the coach or owner? In what ways can the sports-medicine doctor recognize that the team has a legitimate stake in the outcome and yet remain loyal to the player? Should the physician seek consensus with all the parties involved? Although these are all very relevant and sensible questions, the answer is simple. The primary obligation of the sports-medicine doctor is to the patient. Patient autonomy always supplants the doctor’s partiality. Although the paymaster in professional sport is the team, sports-medicine doctors cannot abdicate their responsibility to the individual player. The burden of obligation to the team should be removed from the team doctor, as it is the player’s right to determine what is in their best interest. However, as a patient advocate, the doctor must be cognisant of the fact that the player is often under external pressure from teammates, coaches and agents as well as internal drives and goals that may influence their treatment decisions. In fact, there is a responsibility on the team doctor to tease out the extent of influence on a player to make a certain decision in the process of informed consent [10269].

The cliché – there’s no “I” in team – is a frequent exhortation in prematch team talks. It advocates neutralising individualism which might detract from the team effort. While there’s no ‘I’ in team, there is an “a,” and “a” stands for autonomy. Respect for a patient’s autonomy is considered a fundamental ethical principle. Autonomy refers to the capacity of a rational individual to make an informed, uncoerced decision. This belief forms the central premise of the concept of informed consent. Included in this concept also is the principle of confidentiality. The player must give informed consent for confidential information to be divulged to the management team. This area is of particular relevance to sports-medicine doctors when dealing with an injured player who is faced with a treatment choice. The sports doctor must work as a patient advocate to ensure the player understands the risks and benefits of all possible treatment options [10269].

Healthcare professionals should always aim to "do good" and promote the interest of their patients. It is one of the core values of healthcare ethics and is important in elucidating the nature and goals of medicine as a social practice. The very nature of sport is that it can occasion harm and involve various degrees of risk, and thus raises the question of how of a doctor can stand idly by and watch this happen without intervening. Players participate in sport of their own volition and need to be aware of the inherent risks they face. The principal motivation of the sports-medicine doctor is one of beneficence, and the primary aim is to "do good" for the patient by treating any injuries that may occur and prevent any further harm. One can find conflicts between beneficence and non-maleficence in almost any clinical situation. The dichotomy between the two principles is the foundation for “risk/benefit” analysis. The principle of beneficence and non-maleficence should be considered together and aim at producing a net benefit over harm, in keeping with traditional Hippocratic moral obligation. The obligation to provide net benefit to patients requires that there is a clear distinction between risk and probability when an assessment of harm and benefit is made [10269].

Healthcare professionals should also act fairly when the interests of different individuals or groups are in competition. The obligations of justice may be divided into three categories: fair
distribution of scant resources (distributive justice), respect for people's rights (rights based justice) and respect for moral acceptable laws (legal justice). Distributive justice is relevant to sports medicine in the context of limited resources. If resources are scarce, they should be distributed equally based on need and not on the basis of star talent. Concerning rights-based justice, the team doctor should respect each individual's right to treatment, should they require it. Failure to act because of personal bias or contrary beliefs would be unjust. Finally, the principle of legal justice holds that the team doctor must not willfully cause bodily harm to a player or do anything in contravention of morally acceptable laws [10269].

Self-reporting on doping

In spite of the limited validity of self-reports in socially sensitive behaviour being well documented, how this discrepancy affects the conclusions drawn on the differences in social cognitive measures between those involved versus those who are abstinent remains unknown. Whereas social psychology research routinely considers the effect of social desirability on explicitly assessed data, we are unaware of studies that investigated differences in related social cognition under different scenarios where user vs. non-user groups were established based on self-report admissions, chemical findings or validated self-reports, and used both explicit and implicit assessments. Social science studies of doping practices in sport rely predominantly on self-reports. The majority of the quantitative research into doping behaviour has been based on self-reports, where athletes are not only asked to report on their own attitudes, perceived injunctive and/or descriptive norms but also asked to confess their compromising behaviour (i.e. taking prohibited substances). Self-reports among athletes in Olympic sports have yielded prevalence data ranging between 1 and 30 percent, which itself is higher than the yearly rate (about 2 percent) of adverse analytical findings in the World Anti-Doping Agency accredited laboratories [274]. Studies of psychoactive drug use indicate that self-reporting is characterised by under-reporting. Likewise doping practice is likely to be equally under-reported, if not more so. This calls for more sophisticated methods for such reporting and for independent, objective validation of its results. Social science doping research has a long standing tradition in investigating social cognition (attitudes, norms, beliefs) and personality traits in a quest to find a set of characters that clearly distinguishes athletes who engage in doping practices and those who do not [10275-10280]. Based on these differences, past research has strived to establish behavioural models [10281-10286] with the ultimate aim of being able to predict doping use and to inform anti-doping programmes for potential intervention points and strategies. Previously, researchers assumed that social cognitive determinants of behaviour are accessible and explicitly endorsed by individuals, hence relied exclusively on individual's self-reports when investigating thoughts and feelings that underlie human behaviour. However, over the past two decades, convincing evidence has led to suggestions that the human mind operates in dual, conscientious and unconscious modes [10287-10289], therefore key components of the cognitive processes influencing behaviour are partially hidden from people's awareness or under limited ability to control. Owing to this phenomenon, it has been acknowledged that self-report measures are restricted in capturing the complexity of the cognitive processes that underlie social actions, thus social psychologists have turned to incorporating implicit assessment of the relevant cognitions. This approach has particularly intrigued researchers in socially sensitive domains where it is fair to assume that socially desirable responding is likely to confound explicit assessments [10267] A new doping attitudes questionnaire was developed and combined with a response latency-based implicit association test and hair sample analysis for key doping substances in 14 athletes selected from a larger sample (n=82) to form contrast comparison groups. Results indicate that patterns of group differences in social projection, explicit attitude about and perceived pressure to use doping,
vary depending on whether the user and non-user groups are defined by self-report or objectively verified through hair analysis. Thus, self-confessed users scored higher on social projection, explicit attitude to doping and perceived pressure. However, when a doping substance was detected in the hair of an athlete who denied doping use, their self-report evidenced extreme social desirability (negative attitude, low projection and low perceived pressure) and contrasted sharply with a more positive estimate of their implicit doping attitude. It was concluded that hair analysis for performance enhancing substances has shown considerable potential in validating athletes' doping attitude estimations and admissions of use. Hair samples from the participants in a previous study were tested for performance enhancing and social drugs. Of the 82 athletes, 12 (15%) reported having personal experience with prohibited performance enhancing substances, one with therapeutic use exemption. Twelve hair samples were positive for anabolic steroids and/or erythropoietin (EPO), of which 10 (12%) were confirmed with no overlap between confessed lifetime experience and current use. None of the positives reported medical use of anabolic steroids or EPO. The pattern was very similar for social drugs with 15 percent overlap between self-reported use (27, 33%) and current use (12, 15%). Three of the confirmed doping positives also tested positive for social drugs. The observed discrepancies between self-reports and objectively verified social drug taking behaviour is in line with previous research and although not surprising, they highlight the fact that a significant proportion of respondents simply choose to deny their real current or recent behaviour, even under circumstances when the verification is known to the participants. This phenomenon that has already cast doubt over drug use survey research expands to, or even magnifies the unreliability of doping use epidemiology surveys. The evaluation of anti-doping interventions is seriously hindered by the absence of reliable information on athletes’ true behaviour; opens the field to wild guesses and speculations, often about other athletes, sports and nations. Devising more reliable ways to gauge this crucial information is an important issue but beyond the foci of this research and shall be addressed by future research. The results not only confirm the need for improved self-report methodology for future research in socially-sensitive domains but also indicate where the improvements are likely to come from: as chemical validation remains expensive, a more realistic promise for large scale studies and online data collection efforts is held by measures of implicit social cognition. It may be concluded that incorporating developments in hair sample analysis for the detection of performance enhancing substances, this initial study examined the prospects of objective validation of athletes’ doping attitude estimations and admissions of use. Overall the results indicate that patterns of group differences in deliberately expressed attitudinal outcomes, such as social projection, explicit attitude to doping and perceived pressure to use, vary depending on whether the user and non-user groups are defined by self-report or by objective verification such as hair sample analysis. When user and non-user groups were defined by self-report, the differences between them on several attitudinal outcomes were observed in the expected direction (i.e. self confessed user groups scored higher on social projection, explicit attitude to doping and perceived pressure to use). However, data from hair analysis revealed that deliberate response distortion may have biased these results. Subjects, whose hair sample returned positive for doping but who denied doping use in self-reports, were observed to manipulate their questionnaire responses to a greater degree than all other groups. Implicit doping attitude and its correlation to the explicit attitude towards doping are indicative of this distorted responding. Therefore, the observed discrepancy between self-report and objectively (e.g. chemically) validated behavioural data needs to be considered when drawing conclusions from self-report findings [10267].

It is important to note the distinction between two types of information. In the questionnaire phase, participants are asked if they have ever used performance enhancing substances or social drugs. Hence an affirmative to this question does not necessary mean current or recent use. Hair analysis covered approximately the last 3 months (minus the last 2 weeks
when the hair is still in the body); therefore results reflect relatively recent use. It should be noted that the hair analysis at this stage was limited to the list of most often used performance enhancing and social drugs. Contradicting answers can be derived from two legitimate sources: respondent answered truthfully about having an experience but the last occasion when drugs were used happened before the 6-month maximum detection window; or the drugs used were not among those tested for. Theoretically there is also a possibility that a respondent did not answer truthfully but there is very little reason to admit a socially unacceptable behaviour when it in fact did not happen. On the contrary, a “no” answer on the questionnaire coinciding with positive analytical results in the matching hair sample can be seen as a denial on the self-report because the denied ‘ever use’ is contradicted by the very presence of a drug or drugs in the hair. Three out of 4 of the self-admitted users believed that most high-performing athletes used performance enhancing substances in training and competition with the 4th athlete believing that doping is used by most athletes in training but not in competition. Of those who denied doping use but their hair samples indicated otherwise, half (3/6) agreed that performance enhancing substances are used in both training and competition by most high performing athletes, followed by 2/6 stating that most athletes do not use doping (1 in each “denier” group) with 1 athlete believing that doping is used by most high performing athletes but used only in competition. This view was generally shared by the clean athletes, where 2 of the 4 thought that doping is used in both training and competition with the remaining 2 votes being split between training only and competition only. Theoretically there are two fundamental and mutually exclusive assumptions underpinning the observed low scores on explicit social cognition measures among verified doping users. On the one hand, it may be reduced introspective accessibility of the constructs in question: having no insight into their feelings and biases the respondents produced low scores are no reflection of their actual doping-related cognition, but instead represent an extraneous influence, such as generic social desirability. On the other hand, answers on the explicit tests are consciously and deliberately distorted in order to create a favourable (but false) impression. There are results, however, that suggest that objectively verified doping users had, in fact not only introspective access to the construct (doping attitude) but also had positive feelings toward it [10267].

Despite the widespread use, self-report techniques come with considerable limitations. With regard to self-reported behaviour, it must be assumed that individuals are willing to disclose this, often discriminating, information to the researcher. When self-reports are used to assess social cognitive processes, it is further assumed that people have introspective access to the construct in question (e.g. attitude) and have no intention of distorting their responses. Violations of either of these two assumptions negate the validity of self-report assessment and conclusions derived solely from self-declared data. Doping is a decidedly ostracised behaviour. Admitting use or even expressing supportive opinions against the general view is likely to prompt many athletes to conceal their true behaviour and thoughts about doping if they could be discriminating for the person or the group he/she represents. Recently, researchers have recognised this problem and made attempts to use indirect methods to obtain information on doping behaviour. One notable example being the use of the Random Response Technique (RRT) where estimation of doping prevalence is made on aggregated levels [10290]. Another line of research has made attempts to estimate the likelihood of self-involvement in doping utilising the False Consensus Effect (FCE) which has been evidenced in various socially sensitive situations [10291]. Despite the advances these latter approaches have brought to doping behaviour research, results still carry the inevitable caveat of being based on self-declarations. Independent validation or calibration [10292] of these results remains an issue [10267].

**Hypoxic tents**
The influence of altitude training (i.e. hypoxic environments plus exercise training regimens) was first realized in 1968 during the Mexico City Olympics, and has been used by many competitive athletes since that time. Today, there is an active debate in the sport community around the moral nature of various hypoxic devices used in coordination with training regimens by athletes to enhance aerobic performance. The World Anti-Doping Agency (WADA) had considered banning the use of these devices. WADA's Ethical Issues Review Panel determined that hypoxic tents are performance enhancing and are a violation of the spirit of sport. However, WADA appears to be somewhat ambivalent about its stance on the matter of health risks regarding hypoxic tents. Despite the “two out of three” rule being met, WADA chose to gather further input. The result, announced in September 2006, was that WADA has cautiously refrained from banning hypoxic tents and intends to monitor their health risk. WADA was clear about its stance on the spirit of sport, claiming that the passive use of technology was lacking in virtue and thus was not in line with its perspective of the spirit of sport. Although the first two criteria WADA employs are available for physiological evidence and fact, its third criteria, the spirit of sport, is open for philosophical debate. In one paper, it was provided an overview of the physiological background and then discussed the moral aspects of the “spirit of sport” from three disparate perspectives. In addition, we address the concept of authentic physiology in an effort to provide a foundation for a reasoned debate that can endure the rigours of logic and tests of consistency for other ergogenic aids [07133].

However, the appeal to empiricism fails when we look to the spirit of sport. Here, one must look toward philosophy in general, morals and logic in particular, to fully understand the meaning of and criteria for the spirit of sport. WADA (2003) defines the spirit of sport as the celebration of the human spirit, body and mind, and is characterized by the following values: ethics, fair play and honesty; health; excellence in performance; character and education; fun and joy; teamwork; dedication and commitment; respect for rules and laws; respect for self and other participants; courage; community and solidarity. Each of the components of this definition falls under the general heading of morality. Further, each component is addressed by one or more of the following theories that attempt to explain morality (i.e. how we ought to live). Therefore, to clarify the understanding of what constitutes the spirit of sport, it is needed to comprehend the ways in which morality is approached theoretically. Generally, there are two dominant schools of thought, and at least one non-traditional perspective worthy of our attention. Each comes from a disparate philosophical background and each manifests a distinct intent in the pursuit of how one should live. The first school of thought is broadly termed teleology (from the Greek root telos, the end purpose) or consequentialism. This perspective considers the outcome or consequence of our action as the criteria for what is considered moral (e.g. "excellence in performance"); the means or intent we select to accomplish these goals are of secondary importance. Utilitarianism is a sub-theory of teleology that argues that should select actions and (or) rules that will lead to the greatest pleasure (or happiness/good) and the least pain for the greatest number. This theory is flexible enough to accommodate the will and perception of the majority’s rule with each individual counting as one. It is a theory that argues that the primary intent of behavior should be outcome based and situational as opposed to a process-based duty. The second classical approach to morality is termed deontology (from the Greek root deon, an obligation or duty) or non-consequentialism. This theory, and its related subtheories, is focused on our duty to adhere to particular principles, policies, and codes. In contrast to teleology, the outcome of our action is less important than our intent to do our duty. In a sport context, the old adage that "it is not whether you win or lose, but how you play the game" can be construed as a deontological statement in so far as it suggests that we ought to focus on our duty by abiding by the spirit and rules of the game, with the outcome as a secondary. The principles that must be followed as deontologists may come from a variety of sources: divine, intuitive, and
societal or cultural. Divine deontology is based in religious contexts. The laws or principles or commandments based upon the words of deity form our understanding of duty (e.g. duty based upon the Bible or Koran). Intuitive deontology places emphasis on our capacity to reason. Because humans have this ability, we are able to establish universal laws of moral conduct. Kant's categorical imperative is such an example. It states that one should "act only according to that maxim by which you can at the same time will that it should become a universal law". Both divine and intuitive approaches attempt to be universal in their application.

Social contract morality establishes principles through societal agreement (e.g. the "rules and laws" governing any sport are based upon social contract theory). Once principles or codes are established, it is then incumbent on the members of the community (or organisation) to adhere to (or to modify when necessary) these rules as their duty. If one agrees to be a member of the organisation, then he or she is formally or informally agreeing to follow the rules governing that organisation (this stance is played out in detail in Plato’s dialogue, the Crito). To do otherwise would contravene a societal code that encourages us to negotiate in good faith. In the case of sport, the community “agrees” to the formal rules of play, the size of fields, the weight of implements, etc, and assumes that all competitors have implicitly agreed to abide by them. It must be pointed out that deontology is not necessarily based in any empirical findings (as teleology often is), or science-based logic, but rather is a dictum or a code of conduct sent down from a position of authority or one designed by and committed to by the group (e.g., the World Anti-Doping Agency). If the rules of the particular social contract fail to meet the needs of the current context, then rules can and should be modified to more accurately reflect situational demands. For example, in a landmark work of social psychologist identified a cognitively heightened stage of moral reasoning in terms of social contract deontology. In this stage of development, the individual comes to appreciate that rules can be modified if they are deemed to be unjust (e.g. Dr. Martin Luther King Jr.’s rejection of unjust laws of segregation in the USA). In the same manner, if an athlete, coach, or administrator perceives a rule to be unjust, he or she has a duty to voice disapproval and to challenge the legitimacy of the rule. A case in point was the protest against racism in the United States by Americans Tommie Smith and John Carlos in the 1968 Mexico City Olympics. Under the spectre of significant public criticism and pressure, they “voiced” their dissatisfaction with the state of American race relations. To summarize, the intent of deontological conduct is to adhere to one’s duty in its various forms and to recognise that the outcome of our behaviour is secondary.

A third approach to moral conduct rejects the intent of both the teleological and deontological perspectives. The existentialist suggests that focusing on pleasure, either individual pleasure or the will of the majority, as the criteria for moral conduct is demeaning (i.e. a lower order good), and that to blindly follow rules issued by an external authority (e.g. the church or the coach) is to fail to take full responsibility for one’s own actions and to acknowledge one’s freedom to choose. Rather, moral conduct is linked to the acknowledgement of our free will, our responsibility for all decisions we make, and our intent to act authentically. It must be noted that authenticity, freedom, and responsibility are not the exclusive domain of this school of thought. However, unlike other moral approaches, these concepts are the primary focus of existentialism (i.e. as opposed to outcome or duty). Authenticity, as a central feature of this school of thought, implies an awareness of one’s own reality as distinct not only from the reality of other individuals, but also from the perception of the illusion of a reality that all share. In other words, the existentialist will argue that I grasp the meaning of sport as seen through my unique perspective, coloured by my feelings, values, etc, and that this perspective is not the same as any other, though on the surface it may appear to be so. Further, there is not and cannot be one exclusive perspective or meaning that we all share. Having said this, there is something called “sport”, in as much as there is something we call
“music”; however, the existentialist will view these two phenomena as “my sport” and “my music”. The essence of this line of thinking is that what we ought to do is to constantly strive to be aware of our own essence (philosophical or physical) without being deluded into thinking that we must adopt the perspective of the norm. In terms of authentic behavior, argues that the existentialist recognises his or her own “radical” freedom to choose a particular course of action. This action is true for him or her, and not necessarily what is expected, predicted, or valued by others; he terms this acting “good faith”. Further, the individual cannot choose anything, in good faith, as better unless it is better for all. This sense of universality can be found in the following quotation from Sartre: “When we say that man chooses his own self, we mean that every one of us does likewise; but we also mean by that that in making this choice he also chooses all men. In fact, in creating the man that we want to be, there is not a single one of our acts which does not at the same time create an image of man as we think he ought to be.” In contrast, “bad faith” reveals itself when an individual acts not according to his or her own will, but denies his or her freedom and does the bidding or will of others. For example, an athlete who acts morally or immorally as a result of the dictum of the coach demonstrates his or her rejection of freedom to choose [07133].

To meet the teleological standard, the spirit of sport must demonstrate that it will result in the greatest pleasure or happiness and least amount of pain or unhappiness for the greatest number. Deontologically, the spirit of sport must foster our sense of duty, both in a universal sense of respect for the dignity of others and to our adherence to establish socially acceptable norms, rules, and laws. Finally, the spirit of sport ought to provide a medium through which individuals may develop authentically. The specific concern for WADA is that the use of passive technology (hypoxic tents) violates this spirit or moral ideal. Passivity implies that there is little or no external physical interaction with a device; it acts on the athlete’s internal physiology and requires nothing more than involuntary physiological adaptation (i.e. “a flick of a switch”). Thus a device like a hypoxic tent is considered passive by WADA, as it claims that the athlete needs to do nothing but sleep to have gains in performance and this contravenes WADA’s notion of the spirit of sport. In the following section we discuss this concern from the viewpoint of moral theory. The criterion for moral non-consequentialism is then whether or not a particular behavior has contravened an accepted notion of divine, intuitive, or social contract duty. In the case of hypoxic tents, invoking a religious duty seems remote. Though some religious orders may rule out the use of any technology whatsoever, passive or otherwise (e.g. the Amish). However, the Kantian imperative and social contract theory do resonate in this case. The categorical imperative suggests that if an act is moral it must be universalisable. In other words, would we wish that all athletes, who could gain relevant physiological effect from passive technology, be able to do so? Or would we condemn globally the practice? If we condemn the practice universally, then a myriad of “acceptable” regimens must be banned in addition to devices such as hypoxic tents, such as listening to music as a motivational device. Is this a reasonable principle? Is it a principle that WADA will or could apply universally? One would have to consider the “spirit” of other forms of hypoxic and passive training to determine if WADA’s criticism can stand up to the logic of consistency and Kantian universalisability. For example, is training at sea level during the day and being driven to altitude in the mountains to sleep at night a regimen that goes against the spirit of sport because it is perceived to be “passive”? If so, then this too must be considered a breach of the spirit of sport. Further, based upon the premise of passivity, one might also believe that the intake of a well-balanced meal, meditation, mental imagery, or rest and recovery as passive approaches to performance enhancement [07133].

From an existential perspective, training that awakens or encourages self-discovery and “opens-up” the individual to his or her own (social, emotional, cognitive) possibilities would be
training in “good faith”. For example, this would be the case for an athlete who is able to discover more about his or her abilities, standards of excellence, levels of commitment, etc. as a function of this training. The test of authenticity fails at the philosophical level when the athlete participates in a training regimen to satisfy an agenda that is not his or her own, or one that does not lead to an awareness of the authentic self (bad faith). For example, suppose an athlete succumbs to pressure to participate in a particular training regimen and also fails to see any meaning behind participation, then he or she fails the test of authenticity. These two scenarios are reasonably straight forward if the training regimen is not contentious (e.g. lifting weights). However, if the method is out of the ordinary, then another test of authenticity concerning physiological adaptation can be considered. To answer this question, we begin by proposing the following definition of authentic physiology: the adaptation of the human body, through its genetic potential, to an external stimulus that is non-hormonal, non-drug and non-invasive. This definition places emphasis upon the natural or authentic response to a stimulus. Further it places particular emphasis on the natural limit to adaptation. For example, weight training without steroid use results in strength gains mediated by the body’s genetic potential, including its own hormone synthesis; weight training with steroids, which may be natural or synthetic analogues, leads to levels that cannot be self-produced, and therefore would be classified as inauthentic according to this definition. The use of either increased elevation or hypoxic tents creates a stimulus and causes the body to naturally adapt to this stimulus. It is not invasive, hormonal, or synthetic and it does not push the athletes’ physiological response past its genetic potential. In contrast, the use of recombinant EPO is invasive, it is a manufactured agent and it does stimulate the body’s response past its genetic potential and thus is physiologically inauthentic. It cannot be convincingly argued that hypoxic tents are physiologically inauthentic in terms of the body’s adaptation to the stimulus provided. It is true that the environment in the tent is not in itself a natural environment, but rather it is a replication of a natural environment. However, similarly, a fitness centre in not a natural environment and the athlete is allowed to train in this environment and is not forced to chop wood or pick up large rocks to induce a training effect, though an axe in the example given could be considered non-natural [07133].

The teleologist’s concern is outcome. In particular, the utilitarian’s standard for moral conduct is the greatest goodness for the greatest number. Thus, the utilitarian must demonstrate that the use of passive technology generally and hypoxic tents specifically either fails or meets the test of maximizing pleasure or happiness. In the case of hypoxic tents, the empirical evidence available shows overwhelmingly that these devices do no harm when used as directed and that enhanced performance outcomes, though not dramatic, do occur. Further, proponents argue that these devices enable all athletes to have access to this training regimen at a significantly reduced cost in travel and intrusiveness (i.e. time away from employment and family to train at sites where there is sufficient altitude available), and reduced incentive to seek out illegal and potentially harmful means (e.g. synthetic EPO) to bypass the resource issues of traditional altitude training. The third criterion for WADA, the “spirit of sport”, is less obvious from a utilitarian perspective, as it is difficult to judge not only the process of establishing that the spirit has been transgressed, but also that this transgression will result in the greatest unhappiness. Although a survey of athletes has not been conducted by this research team or by WADA, it would seem that WADA is the only public voice making this claim, whereas those disputing this interpretation seem to be wide and varied [07133].

Hypoxic devices in combination with proper physical training regimens have been associated with significant improvements in performance and have been shown to pose no known health risk to the user. The controversy stems primarily from the interpretation of WADA’s principle, “spirit of sport”, and the extent to which a training regimen is employed, either passively or
actively, by the athlete. It has been suggested that many training regimens are passive in nature and are accepted by the sport community implicitly and explicitly. The position taken in this paper is that the concept of the “spirit of sport” needs to be refined and we argue that this should include the notion of authenticity, both physiological and philosophical. Combined with the criteria of consequentialism and non-consequentialism, authenticity can more clearly define “natural” or “genuine” competition in a much more philosophically sound manner than the current documentation [07133].

Pain medication

Many athletes have promoted a culture of acceptance regarding pain and sport. “No pain, no gain” resonates in athletic venues worldwide. Although some exertion-related soreness is to be expected, there also is a degree of pain that is abnormal when attributed to exercise. Pain may be a sign of injury that if ignored or masked by analgesics could lead to worsening of the root condition. To some extent, the perception of pain is an evolutionary adaptation to prompt the injured to take time for rest and allow for healing. In recent years, television commercials have touted the use of various over-the-counter (OTC) and prescription medications for treating pain. Many people of all activity levels take widely available analgesics in response to pain resulting from athletic activity. The general acceptance of exercise coexisting with pain has led to regular use of medications, prescribed or not, to alleviate physical activity related pain, especially when related to acute injury [07216].

A survey of 563 National Collegiate Athletic Association (NCAA) Division I athletes demonstrated that 165 (29 %) felt there was nothing wrong with using painkillers on the day of competition to cope with injury-related pain. Ethical considerations include the voluntary nature of sports, treating athletes under the legal age of consent, and blurring the lines between what is most important to a player: short-term pain relief for performance or longer-term consequences of potential increases in injury severity [07216].

Injection therapy with local anaesthetics

Sports medicine physicians often use injections for the targeted delivery of medications. Whether it is anesthetics, corticosteroids, NSAIDs, or other injectables, this treatment method is akin to a “smart bomb.” Injections avoid most of the systemic side effects and deliver higher doses to the intended tissue compared with the same medication administered orally. However, the provider must be aware that some agents may have some absorption beyond the target tissue, although the systemic levels typically are much lower than levels achieved via enteral administration. Injecting requires skill, familiarity with anatomy, and knowledge of the medication’s benefits and risks. Sterile preparation may minimize infection risk but often proves challenging in the training room. Injections pose a risk of bleeding and injury to adjacent structures. As with dispensing medications, informed consent may be obtained prior to administering injections. Most high school athletes are too young to legally provide consent; thus, parents must be consulted if injections are considered as a part of the pain management plan [07216].

Injections often are viewed as more aggressive treatment typically reserved for higher-level athletes. Using injections to treat sports-related injury pain is not unheard of, yet the medical literature does not lend support to the practice. It was published the results of a case series involving professional Australian football and rugby players treated with injection of local anesthetics (without corticosteroid) to facilitate quicker return to play. This was an important first step to try to adequately describe the benefits and risks associated with injections for
sports injury. Over the course of 6 years, in his role as team physician, the author treated 2851 injuries in professional Australian football and rugby players. Of the 2851 injuries, 221 (8 %) were treated with injection of the local anesthetic bupivacaine. These included injuries to the ribs, iliac crest (hematomas), acromioclavicular joint, fingers, thumb, ankles, metacarpals, sternum, and toes. A total of 86 (39 %) of these injections were administered to treat acute injury with the game in progress, whereas the other treatments were administered for both acute and chronic pain prior to a game to allow the athlete to participate. Complications were broken down into major or minor. Out of 221 injections, there were 17 complications (8 %); six (3 %) were major and 11 (5 %) minor. All six of the major complications did well with eventual return to play. Some of these injuries needed surgery to provide definitive treatment, but the author believed it was possible that some of those injuries would have needed surgery regardless of the injection to allow for return to play, and the injections allowed the athlete to continue playing until season’s end [07216].

Injection therapy with local corticosteroids

Various corticosteroids often are used in combination with anesthetics. Steroids’ role in these types of injections is controversial. Steroid injections can produce pain relief of much longer duration than anesthetic alone can produce in certain conditions. Such relief has even been demonstrated in conditions in which inflammation plays little role (such as chronic tendinopathy), which suggests the mechanism of action is beyond anti-inflammatory action alone. Although these injections yield less-than-ideal results for providing cure or extended pain relief in chronic tendinopathy, many physicians still use these treatments in noninflammatory processes. Steroid injections also carry more risks than nonsteroid percutaneous injections. Adverse events include fat atrophy, skin lightening, blood sugar elevation (particularly in diabetics), and a theoretical risk of suppression of immune response to an infection mistaken for inflammation. Whereas teaching in medicine commonly discourages injection of corticosteroids directly into tendons and muscles, it has been done successfully in the highest level of athlete. After a retrospective review of the records for one NFL team, it was noted that 58 players suffered a severe hamstring injury with associated palpable defect in the muscle. These players were treated with corticosteroids and analgesics injected directly into the injury site. The injection was administered after the injured player identified the area of pain by pointing. The physician then prepped the area in sterile fashion and injected intramuscularly 3 mL of 1 percent lidocaine and 1 mL of dexamethasone (4 mg) via a 25-gauge needle. A compressive device was applied afterward, and the player held out of all activities for 48 hours. None of the players had any complications, and only nine (16 % of the 58) missed any games as a result of treating the hamstring injury this way [07216].

Concern of delayed healing or facilitating tendon rupture has prompted many practitioners to avoid injecting tendons directly, so the reporting of such treatments was a bold and significant contribution to the literature. However, it should be noted that a retrospective review had no control group or blinding to the procedure. Furthermore, the NFL athlete represents a unique patient population with different pressures and responsibilities than the average athlete; thus, generalization to all athletes might be unwise [07216].

Concluding remarks

High-quality pain control and sports injury studies are lacking. Although there are some prospective, double-blind, randomized controlled trials, the sample sizes are often small, and the study designs flawed. As with many aspects of musculoskeletal medicine, there is no iron-clad, evidence-based medicine regarding pain. This requires the sport medicine
physician to synthesize the available data and combine it with experience, anecdotal reports, and common sense. Therein lies the art of medicine. When using NSAIDs, the aim should be to use the lowest effective dose for the shortest duration to avoid adverse events. More aggressive treatments, such as anesthetic injections, depend on the injury’s location and severity and the level of competition [216].

Publication ethics
For research that involves human participants or animals to be published in the International Journal of Sports Medicine (IJSM) it has been recognised ethical standards and national and international laws. Authors are required to confirm that these standards and laws have been adhered to by formally citing this editorial within the methods section of their own manuscript. Authors should confirm that research using human participants has been conducted ethically according to the principles of the Declaration of Helsinki. The Declaration is intended to be read as a whole and every principle is equally important, but those points most-commonly considered by sport and exercise scientists may be summarized [09391]:

- **Basic principles.** Respect the rights and welfare of participants which must take precedence over all other interests
- **Ethical review.** Before research begins and before amendments are applied, research must be reviewed and approved by an appropriate ethics committee
- **The research protocol.** The study, research design and statistical analysis must be clearly described, justifiable and appropriate. In drawing up the research protocol, the researcher must
  o consider ethical issues in accordance with the Declaration of Helsinki,
  o provide information regarding funding, sponsors, institutional affiliations, other potential conflicts of interest,
  o consider the contribution to new knowledge and consider the environment,
  o include details of any incentives for participants and provisions for treating and/or compensating participants who are harmed as a consequence of participation in the research study,
  o describe the arrangements for post-study access by all participants to interventions identified as beneficial in the study or access to other appropriate care or benefits.
- **Consent.** Informed consent should be provided freely by the participant and should ideally be in writing. If written consent cannot be obtained, or is not appropriate, then oral consent should be formally documented and witnessed. Research that involves children or other populations that cannot consent (e.g. vulnerable populations) should seek consent from an appropriate person and assent from the participant. Research involving participants who are physically or mentally incapable of giving consent may be undertaken only if the physical or mental condition that prevents giving informed consent is a necessary characteristic of the research population. Informed consent/assent must include the
  o aims of the research
  o methods
  o sources of funding
  o conflicts of interest
  o institutional affiliations
  o anticipated benefits and potential risks
  o potential discomfort
  o right to refuse to participate or withdraw consent without reprisal
- **Conduct.** Research must be conducted
  o in accordance with appropriate risk management
  o by appropriately qualified researchers and support staff
  o with skill and care
  o in an appropriate setting
  o in order to protect the privacy of participants and confidentiality of their personal information
  o in accordance with laws and regulations of the country or countries in which the research is to be performed as well as international norms and standards.
Specific laws relevant to research ethical may regulate the collection, use and/or storage of human tissue; the protection of individuals that lack the capacity to consent; data protection; and the use of drugs in research.

- **Governance.** Serious adverse events occurring during the study must be reported to the ethics committee that ethically reviewed and approved the research.

Specific issues relevant to sports medicine are [09391]:

- **Retrospective ethics.** Data are routinely collected from individuals for various purposes. For example, sport scientists may monitor physiological function of an athlete in order for him or her to gain an edge over their rivals. Data collected exclusively for one purpose cannot be used for another purpose (research) unless consent for the use in research is subsequently given and the research ethically approved. An exception to this would be where the data collected for the primary purpose is anonymised prior to use in a research study (second purpose) which has ethical approval. Retrospective ethical approval cannot be granted for any research study.

- **The use of placebo.** The inclusion of a placebo group in a research study may challenge the principle of equipoise. Ideally, participants should be randomly assigned to experimental or placebo groups. In healthy volunteers, where possible, the type of treatment should be blinded, for example, comparator (control) participants could be given a fitness or lifestyle information sheet. After a finite length of time those participants in the comparator group could be offered the experimental condition, or an experiment could be halted if at any point it became clear that the placebo group was fairing more poorly. In more medical research where participants are patients or clients extreme care must be taken to avoid the abuse of placebo. In this type of research, the use of placebo is acceptable when no current proven intervention exists, or when the participant will not be at risk of serious or irreversible harm.

- **Deception.** The use of deception in research (e.g. in a pacing strategy study in which time trial distance is deceived) must be merited such that there are no reasonable alternatives for obtaining the data, as long as there is no reasonable expectation to cause pain or severe emotional distress. If deception is to be used, then the participants must be accurately informed of the risks and be debriefed at the conclusion of the study with the option to withdraw their data.

- **Good research practice.** The UK Medical Research Council has outlined some basic principles of good research practice which may help to ensure that research is conducted ethically. These include planning, conduct, recording data, reporting results, applying the results. Relevant issues not specifically raised in the Declaration of Helsinki include the use, calibration and maintenance of equipment, COSHH, documentation of standard operating procedures, retention of data, publication policy, authorship, correction of errors and retraction of published findings and intellectual property rights.
Bacterial hydrolysis of urine without doping substances

For almost two decades it has been known that enzymatic hydrolysis of "normal" urine samples from the entire male horse using Escherichia coli (E. coli) followed by solvolysis (ethyl acetate:methanol:sulphuric acid) results in the detection of significant amounts of estr-4-ene-3,17-dione (19-norandrost-4-ene-3,17-dione) along with estr-4-en-17beta-ol-3-one (19-nortestosterone, nandrolone) in extracts of the hydrolysed urine and that both steroids are isolated from the solvolysis fraction. This solvolysis process is targeted at the steroid sulphates. Also it has been shown that 19-norandrost-4-ene-3,17-dione and 19-nortestosterone are isolated from testicular tissue extracts. Subsequently, evidence was obtained that 19-nortestosterone detected in extracts of "normal" urine from male horses may not be derived from the 17beta-sulphate conjugate. However, following administration of 19-nortestosterone based proprietary anabolic steroids to all horses (males, females and castrates), the urinary 19-nortestosterone arising from the administration is excreted primarily as the 17beta-sulphate conjugate. Thus, if the 19-nortestosterone-17beta-sulphate conjugate arises only following administration this has interesting implications for drug surveillance programmes to control administration of 19-nortestosterone based anabolic preparations to male horses. These results have led to consider that the precursors to 19-nortestosterone and 19-norandrost-4-ene-3,17-dione, present in the urine prior to the hydrolysis steps, have the same basic structure except for the functionality at the 17-position. It has been used preparative high pressure liquid chromatography (LC) and LC fractionation to separate these precursors from the high amounts of oestrogenic sulphates present in "normal" urine from the entire male horse. Purified fractions have then been studied by liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) to identify the precursors [06312].

Contaminated food for animals

Presence of drugs is completely prohibited in post racing urine samples by most of racing and competition authorities, even if environmental contamination might occur. To assess the daily dose of several contaminants absorbed through the diet that would result in detectable concentrations in urine caffeine, theobromine, theophylline, atropine, scopolamine, bufotenine, DMT or morphine were administered orally to 6 horses, in different dosages, for 3 days before their urine was sampled for regular anti-doping tests. Theobromine, theophylline, bufotenine and morphine daily intake >10 mg, 2 mg, 10 mg and 200 microg, respectively, by a performance horse, were found to result in detectable urinary concentrations. At the 2 tested doses, atropine (5 and 15 mg) and dimethyltryptamine (3 and 10 mg) were not detected in urine. For caffeine and scopolamine, even the lowest dosage tested (5 mg/horse/day and 2 mg/horse/day respectively) induced detectable concentrations of the molecule in urine. It was concluded that horses fed dietary contaminants, even at level much below the effective dosage, may be positive to antidoping urine analysis. Thus, selection of feed materials appears to be of great importance to prevent non voluntary positive result to anti-doping tests [06313].

Screening
It was presented at one time employed four liquid chromatography/mass spectrometric (LC/MS) methods for the detection of a large variety of drugs in equine urine. Drug classes covered by these methods included anti-diabetics, anti-ulcers, cyclooxygenase-2 (COX-2) inhibitors, sedatives, corticosteroids, anabolic steroids, sulfur diuretics, xanthines, etc. With the objective to reduce labour and instrumental workload, a new ultra performance liquid chromatography/tandem mass spectrometric (UPLC/MS/MS) method has been developed, which encompasses all target analytes detected by the original four LC/MS methods. The new method has better detection limits than the superseded methods. In addition, it covers new target analytes that could not be adequately detected by the four LC/MS methods. The new method involves solid-phase extraction (SPE) of two aliquots of equine urine using two Abs Elut Nexus cartridges. One aliquot of the urine sample is treated with beta-glucuronidase before subjecting to SPE. A second aliquot of the same urine sample is processed directly using another SPE cartridge, so that drugs that are prone to decomposition during enzyme hydrolysis can be preserved. The combined eluate is analysed by UPLC/MS/MS using alternating positive and negative electrospray ionisation in the selected-reaction-monitoring mode. With this newly developed UPLC/MS/MS method, the simultaneous detection of 140 drugs at ppb to sub-ppb levels in equine urine can be achieved in less than 13 min inclusive of post-run equilibration. Matrix interference for the selected transitions at the expected retention times is minimised by the excellent UPLC chromatographic separation. The method has been validated for recovery and precision, and is being used regularly in the authors' laboratory as an important component of the array of screening methods for doping control analyses of equine urine samples [11311].

Liquid chromatography-mass spectrometry (LC-MS) has been widely used in doping control laboratories over the last two decades. Currently, simple quadrupole, triple quadrupole and ion trap are the most commonly employed analyzers in toxicological analysis. Nevertheless, the main lack of these technologies is the restricted number of target compounds simultaneously screened without loss of sensitivity. In one article it was presented an innovative screening approach routinely applied in the French horse doping control laboratory based on high resolution (50000) and high mass accuracy (<5 ppm) in full scan MS mode for more than 235 target analytes screened from an initial volume of 5mL of urine. The sample preparation was classically founded on solid phase extraction by means of reverse phase C18 cartridges. LC-MS analyses were carried out on a Shimadzu binary HPLC pumps linked to a C18 Sunfire column associated with the high resolution exacteive benchtop orbitrap mass spectrometer. This screening was performed alternatively in positive-negative ionization mode during the same run. Thus, the identification of compounds of interest was made using their exact mass in positive-negative ionization mode at their expected retention time. All data obtained were processed by ToxID software (Thermo-FisherScientific) which is able to identify a molecule by theoretical mass and retention time. In order to illustrate this innovative technology applied in our laboratory, sample preparation, validation data performed on 20 target compounds from 16 different horse urine samples, chromatograms and spectra was discussed [11312].

In one study a general screening protocol was developed to detect prohibited substances and metabolites for doping control purposes in equine sports. It was based on the establishment of a unified sample preparation and on the combined implementation of liquid and gas chromatographic MS analysis. The sample pretreatment began with two parallel procedures: enzymatic hydrolysis of sulfate and glucuronide conjugates, and methanlysis of the 17-beta-sulfate steroid conjugates. The extracts were treated for LC-TOF-MS, GC-HRMS and GC-MS assays. The majority of the prohibited substances were identified through a high mass accuracy technique, such as LC-TOF-MS, without prior derivatization. The sample preparation procedure included the formation of methylated and trimethylsilylated derivatives common in toxicological GC-MS libraries. The screening method was enhanced by post-run
library searching using automated mass spectral deconvolution and identification system (AMDIS) combined with deconvolution reporting software (DRS). The current methodology is able to detect the presence of more than 350 target analytes in horse urine and may easily incorporate a lot of new substances without changes in chromatography. The full scan acquisition allows retrospective identification of prohibited substances in stored urine samples after reprocessing of the acquired data. Validation was performed for sixty representative compounds and included limit of detection, matrix interference - specificity, extraction recovery, precision, mass accuracy, matrix effect and carry over contamination. The suitability of the method was demonstrated with previously declared positive horse urine samples [13779].

Anabolic steroids, general

One paper describes the application of gas chromatography-mass spectrometry (GC-MS) for in vitro and in vivo studies of 6-OXO in horses, with a special aim to identify the most appropriate target metabolite to be monitored for controlling the administration of 6-OXO in racehorses. In vitro studies of 6-OXO were performed using horse liver microsomes. The major biotransformation observed was reduction of one keto group at the C3 or C6 positions. Three in vitro metabolites, namely 6alpha-hydroxyandrost-4-ene-3,17-dione (M1), 3alpha-hydroxyandrost-4-ene-6,17-dione (M2a) and 3beta-hydroxyandrost-4-ene-6,17-dione (M2b) were identified. For the in vivo studies, two thoroughbred geldings were each administered orally with 500 mg of androst-4-ene-3,6,17-trione (5 capsules of 6-OXO®) by stomach tubing. The results revealed that 6-OXO was extensively metabolized. The three in vitro metabolites (M1, M2a and M2b) identified earlier were all detected in post-administration urine samples. In addition, seven other urinary metabolites, derived from a further reduction of either one of the remaining keto groups or one of the remaining keto groups and the olefin group, were identified. These metabolites included 6alpha,17beta-dihydroxyandrost-4-en-3-one (M3a), 6,17-dihydroxyandrost-4-en-3-one (M3b and M3c), 3beta,6beta-dihydroxyandrost-4-en-17-one (M4a), 3,6-dihydroxyandrost-4-en-17-one (M4b), 3,6-dihydroxyandrostan-17-one (M5) and 3,17-dihydroxyandrostan-6-one (M6). The longest detection time observed in urine was up to 46 h for the M6 metabolite. For blood samples, the peak 6-OXO plasma concentration was observed 1 h post administration. Plasma 6-OXO decreased rapidly and was not detectable 12 h post administration [10424].

Anabolic and androgenic steroids (AASs) are synthetic substances related to the primary male sex hormone, testosterone. AASs can be abused in both human and equine sports and, thus, are banned by the International Olympic Committee and the Association of Racing Commissioners International (ARCI). Enforcement of the ban on the use of AASs in racehorses during competition requires a defensible and robust method of analysis. To address this requirement, a high-throughput ultra high-performance liquid chromatography-mass spectrometric (UHPLC-MS) method was developed for the detection, quantification and confirmation of 55 AASs in equine plasma. AASs were recovered from equine plasma samples by liquid-liquid extraction with methyl tert-butyl ether (MTBE). Analytes were chromatographically separated on a sub-2 microm particle size C18 column with a mobile phase gradient elution and detected by selected-reaction monitoring (SRM) on a triple quadrupole mass spectrometer. AASs with isobaric precursor ions were either chromatographically resolved or mass spectrometrically differentiated by unique precursor-to-product ion transitions. A few of them that could not be resolved by both approaches were differentiated by intensity ratios of three major product ions. All the epimer pairs, testosterone and epitestosterone, boldenone and epiboldenone, nandrolone and epitestosterone, were chromatographically base-line separated. The limit of detection and that of quantification was
50 pg/ml for most of the AASs, and the limit of confirmation was 100-500 pg/ml. Full product ion spectra of AASs at concentrations as low as 100-500 pg/ml in equine plasma were obtained using the triple quadrupole instrument, to provide complementary evidentiary data for confirmation. The method is sensitive and selective for the detection, quantification and confirmation of multiple AASs in a single analysis and will be useful in the fight against doping of racehorses with AASs [10425].

It was developed a method for the simultaneous analysis of 11 anabolic steroids: fluoxymesterone, 17alpha-methyltestosterone, mestanolone, methandienone, methandriol, oxymetholone, boldenone, furazabol, methenolone, nandrolone, and stanozolol, for possible application to a doping test in racehorses. It was selected 15 kinds of target substances for a doping test from the main metabolites of these anabolic steroids, and established a method for simultaneous analysis. Urine was hydrolyzed and subjected to solid-phase extraction. Then, the residue from the extracts was derivatized by trimethylsilylation. The derivatized samples were subjected to ion-trap gas chromatography-tandem mass spectrometry, and their mass chromatograms and product ion spectra were obtained. The limit of detection of the target substances was 5-50 ng/mL, and the mean recovery and coefficient of variation were 71-105 percent and 1.1-10 percent, respectively [08186].

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In 2008, Pennsylvania became the first State in the USA to ban and enforce the ban on the use of anabolic and androgenic steroids (AAS) in equine athletes by using plasma for analysis. To enforce the ban, a rapid and high-throughput method for analysis of 60 AAS in equine plasma was developed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Analytes were recovered from plasma by liquid-liquid extraction (LLE) using methyl tert-butyl ether, separated on a reversed-phase C₁₈ column and analyzed by electrospray ionization mass spectrometry. Multiple-reaction monitoring (MRM) scan was employed for screening. When the MRM signal of an analyte exceeded 1000 counts per second (cps), information-dependent acquisition (IDA) triggered generation of an enhanced product ion (EPI) scan of the analyte. A library for the analytes was simultaneously established using the EPI spectrum. Unambiguous identification of any of the 60 AAS in a test sample was based on both the presence of MRM response within the correct retention time (tR) window and a
qualitative match between EPI spectrum of the test sample and that of the reference drug standard stored in the library. Total analysis time was 7 min. The limit of detection (LOD) and limit of confirmation (LOC) for most of the analytes were 0.01-2 ng/mL and 0.1-10 ng/mL, respectively. Recovery of the analytes from plasma by LLE was 74-138 percent. The method was successfully verified and is routinely used in the screening of post-race equine plasma samples for the presence of these 60 AAS. The method is rapid, sensitive, reproducible, and reliable [11313].

It was presented at one time employed four liquid chromatography/mass spectrometric (LC/MS) methods for the detection of a large variety of drugs in equine urine. Drug classes covered by these methods included anti-diabetics, anti-ulcers, cyclooxygenase-2 (COX-2) inhibitors, sedatives, corticosteroids, anabolic steroids, sulfur diuretics, xanthines, etc. With the objective to reduce labour and instrumental workload, a new ultra performance liquid chromatography/tandem mass spectrometric (UPLC/MS/MS) method has been developed, which encompasses all target analytes detected by the original four LC/MS methods. The new method has better detection limits than the superseded methods. In addition, it covers new target analytes that could not be adequately detected by the four LC/MS methods. The new method involves solid-phase extraction (SPE) of two aliquots of equine urine using two Abs Elut Nexus cartridges. One aliquot of the urine sample is treated with beta-glucuronidase before subjecting to SPE. A second aliquot of the same urine sample is processed directly using another SPE cartridge, so that drugs that are prone to decomposition during enzyme hydrolysis can be preserved. The combined eluate is analysed by UPLC/MS/MS using alternating positive and negative electrospray ionisation in the selected-reaction-monitoring mode. With this newly developed UPLC/MS/MS method, the simultaneous detection of 140 drugs at ppb to sub-ppb levels in equine urine can be achieved in less than 13 min inclusive of post-run equilibration. Matrix interference for the selected transitions at the expected retention times is minimised by the excellent UPLC chromatographic separation. The method has been validated for recovery and precision, and is being used regularly in the authors' laboratory as an important component of the array of screening methods for doping control analyses of equine urine samples [11314].

During last decades, the use of natural steroids in racing and food producing animals for doping purposes has been flourishing. The endogenous or exogenous origin of these naturally occurring steroids has since remained a challenge for the different anti-doping laboratories. The administration of these substances to animals is usually made through an intra-muscular pathway with the steroid under its ester form for a higher bioavailability and a longer lasting effect. Detecting these steroid esters would provide an unequivocal proof of an exogenous administration of the considered naturally occurring steroids. A quick analytical method able to detect at trace level (below 50 pg/mL) a large panel of more than 20 steroid esters in serum and plasma potentially used for doping purposes in bovine and equine has been developed. Following a pre-treatment step, the sample is submitted to a solid phase extraction (SPE) before analysis with UPLC-MS/MS. The analytical method's efficiency has been probed through three different in vivo experiments involving testosterone propionate intra-muscular administration to three heifers, 17-estradiol benzoate intra-muscular administration to a bull and a heifer and nandrolone laurate intra-muscular administration to a stallion. The results enabled detecting the injected testosterone propionate and 17-estradiol benzoate 2 and 17 days, respectively, post-administration in bovine and nandrolone laurate up to 14 days post-administration in equine. The corresponding elimination profiles in bovine serum and equine plasma have been established. The first bovine experiment exhibited a maximal testosterone propionate concentration of 400 pg/mL in one of the three heifer serum within 5h post-administration. The second bovine experiment reported a maximal 17-estradiol benzoate concentration of 480 pg/mL in the same matrix recorded 9 days after its administration. The last equine experiment resulted in a maximal nandrolone laurate
concentration of 440 pg/mL in horse plasma 24h after administration [13782].

**Anabolic steroids in dogs**

Effective control of the use of anabolic-androgenic steroids (AASs) in animal sports is essential in order to ensure both animal welfare and integrity. In order to better police their use in Australian and New Zealand greyhound racing, thorough metabolic studies have been carried out on a range of registered human and veterinary AASs available in the region. Canine metabolic data are presented for the AASs boldenone, danazol, ethylestrenol, mesterolone, methandriol, nandrolone and norethandrolone. The principal Phase I metabolic processes observed were the reduction of A-ring unsaturations and/or 3-ketones with either 3alpha,5beta- or 3beta,5alpha-stereochemistry, the oxidation of secondary 17beta-hydroxyl groups and 16alpha-hydroxylation. The Phase II beta-glucuronylation of sterol metabolites was extensive [13783].

**In hair**

The detection of the abuse of anabolic steroids in equine sport is complicated by the endogenous nature of some of the abused steroids, such as testosterone and nandrolone. These steroids are commonly administered as intramuscular injections of esterified forms of the steroid, which prolongs their effects and improves bioavailability over oral dosing. The successful detection of an intact anabolic steroid ester therefore provides unequivocal proof of an illegal administration, as esterified forms are not found endogenously. Detection of intact anabolic steroid esters is possible in plasma samples but not, to date, in the traditional doping control matrix of urine. The analysis of equine mane hair for the detection of anabolic steroid esters has the potential to greatly extend the time period over which detection of abuse can be monitored. Equine mane hair samples were incubated in 0.1M phosphate buffer (pH 9.5) before anabolic steroids (testosterone, nandrolone, boldenone, trenbolone and stanozolol), anabolic steroid esters (esters of testosterone, nandrolone, boldenone and trenbolone) and associated compounds (fluticasone propionate and esters of hydroxyprogesterone) were extracted by liquid-liquid extraction with a mix of hexane and ethyl acetate (7:3, v:v). Further sample clean up by solid phase extraction was followed by derivatisation with methoxylamine HCL and analysis by UHPLC-MS/MS. Initial method development was performed on a representative suite of four testosterone esters (propionate, phenylpropionate, isocaproate and decanoate) and the method was later extended to include a further 18 compounds. The applicability of the method was demonstrated by the analysis of mane hair samples collected following the intramuscular administration of 500 mg of Durateston® (mixed testosterone esters) to a Thoroughbred mare (560 kg). The method was subsequently used to successfully detect boldenone undecylenate and stanozolol in hair samples collected following suspicious screening findings from post-race urine samples. The use of segmental analysis to potentially provide additional information on the timing of administration was also investigated [13784].

**Differentiation between endogenous steroids and synthetic homologues in cattle**

Although substantial technical advances have been achieved during the past decades to extend and facilitate the analysis of growth promoters in cattle, the detection of abuse of synthetic analogs of naturally occurring hormones has remained a challenging issue. When it became clear that the exogenous origin of steroid hormones could be traced based on the $^{13}$C/$^{12}$C isotope ratio of the substances, GC/C/IRMS has been successfully implemented to this aim since the end of the past century. However, due to the costly character of the instrumental setup, the susceptibility of the equipment to errors and the complex and time
consuming sample preparation, this method is up until now only applied by a limited number of laboratories. In this review, the general principles as well as the practical application of GC/C/IRMS to differentiate between endogenous steroids and exogenously synthesized homologous compounds in cattle will be discussed in detail, and will be placed next to other existing and to be developed methods based on isotope ratio mass spectrometry. Finally, the link will be made with the field of sports doping, where GC/C/IRMS has been established within the World Anti-Doping Agency (WADA) approved methods as the official technique to differentiate between exogenous and endogenous steroids over the past few years [13785].

Testosterone

In one work, a disposable immunosensor for the detection of testosterone, an endogenous steroid hormone, in bovine urine has been developed using screen-printed electrodes (SPEs). Due to concerns over the use of steroid hormones as growth promoters, the EU prohibits their use in food producing animals. Consequently, rigorous screening procedures have been implemented in all member states to detect the illegal administration of such compounds. Competitive immunoassays were developed, initially by enzyme linked immunosorbent assay (ELISA), and subsequently transferred to an electrochemical immunosensor format using disposable screen-printed carbon electrodes. Horseradish peroxidase (HRP) was the enzyme label of choice and chronoamperometric detection was carried out using a tetramethylbenzidine/hydrogen peroxide (TMB/H₃O₂) substrate system, at +100 mV. The EC₅₀ values obtained for the assay in buffer and urine gave relatively comparable results, 710 pg/mL and 960 pg/mL, respectively. The linear range obtained for the assay in buffer extended from 0.03 ng/mL to 40 ng/mL; while that in urine ranged from 0.03 ng/mL to 1.6 ng/mL. The corresponding limits of detection (LOD) in buffer and urine were 26 pg/mL and 1.8 pg/mL. Cross reactivity profiles of the antibody have been examined, with notable cross reactivities with 19-nortestosterone (11.6 %) and boldenone (9.9 %). Precision studies for the sensor demonstrated adequate reproducibility (CV<13 %, n=3) and repeatability (CV<9 %, n=3). Recovery data obtained showed good agreement between spiking studies and known concentrations of analyte. Sensors showed stability for 4 days at +4 degrees C. A sensitive, highly specific, inexpensive, disposable immunosensor, showing excellent overall performance for the detection of testosterone in bovine urine, has been developed [06317]

Doping control of anabolic substances is normally carried out with urine samples taken from athletes and horses. Investigation of alternative specimens, e.g. hair samples, is restricted to special cases, but can also be worthwhile, in addition to urine analysis. Moreover, hair material is preferred in cases of limited availability or complicated collection of urine samples, e.g. from horses. In this work, possible ways of interpretation of analytical results in hair samples are discussed and illustrated by practical experiences. The results demonstrate the applicability of hair analysis to detect anabolic steroids and also to obtain further information about previous abuse. Moreover, the process of incorporation of steroids into hairs is described and the consequences on interpretation are discussed, e.g. on the retrospective estimation of the application date. The chosen examples deal with the detection of the anabolic agent testosterone propionate. Hair samples of an application study, as well as a control sample taken from a racing horse, were referred to. Hair material was investigated by a screening procedure including testosterone, nandrolone and several esters (testosterone propionate, phenylpropionate, decanoate, undecanoate, cypionate; nandrolone decanoate, dodecanoate and phenylpropionate; limits of detection (LODs) between 0.1 and 5.0 pg/mg). Confirmation of testosterone propionate (LOD 0.1 pg/mg) was carried out by an optimised sample preparation. Trimethylsilyl (TMS) and tert-butyl dimethylsilyl derivatives were
detected by gas chromatography-high-resolution mass spectrometry (GC-HRMS) and gas chromatography-tandem mass spectrometry (GC-MS/MS) [08437].

Pennsylvania State Racing Commissions regulate the endogenous androgenic steroid, testosterone (TES), in racing intact males (RIM) by quantification of TES in post-race samples. Post-race plasma samples (2209) collected between 2008 and 2010 were analyzed for TES, nandrolone (NAN), and other anabolic steroids (ABS). Of the 2209 plasma samples, 2098 had quantifiable TES ≥25 pg/mL. Plasma (mean ± SD) concentrations of TES and NAN in RIM were 329 ± 266 and 96 ± 68 pg/mL, respectively. Only 65 percent of RIM had quantifiable concentration of NAN, and there was no relationship between TES and NAN. Plasma TES concentrations were significantly higher during the months of April, May, June, July, and August. A significantly higher plasma TES was observed in thoroughbred (TB) versus. that in standardbred (STB). Plasma concentrations of TES from breeding stallions (BS) were 602 ± 357 pg/mL. Statistically significant lower plasma concentrations of the two steroids were observed in RIM horses. Based on quantile distribution of TES in the RIM and BS populations, 99.5 percent were at or below 1546 and 1778 pg/mL, respectively. Based on this population of RIM, the suggested upper threshold plasma concentration of endogenous TES in horses competing in PA should remain at 2000 pg/mL [11315].

Analysis of equine plasma samples to detect the abuse of anabolic steroids can be complicated when the parent steroid is endogenous to the animal. Anabolic steroids are usually administered intramuscularly as synthetic esters and therefore detection of the exogenous esters provides unequivocal proof of illegal administration. An ultra high performance liquid chromatography tandem mass spectrometric (UPLC-MSMS) method for the analysis of esters of testosterone (propionate, phenylpropionate, isocaproate, and decanoate) and boldenone (undecylenate) in equine plasma has been developed. Esters were extracted from equine plasma using a mixture of hexane and ethyl acetate and treated with methoxyamine hydrochloride to form methyloxime derivatives. Metenolone enanthate was used as an internal standard. After chromatographic separation, the derivatized steroid esters were quantified using selected reaction monitoring (SRM). The limit of detection for all of the steroid esters, based on a signal to noise ratio (S/N) of 3:1, was 1-3 pg/mL. The lower limit of quantification (LLOQ) for the all of the steroid esters was 5 pg/mL when 2 mL of plasma was extracted. Recovery of the steroid esters was 85-97% for all esters except for testosterone decanoate which was recovered at 62%. The intra-day coefficient of variation (CV) for the analysis of plasma quality control (QC) samples was less than 9.2 percent at 40 pg/mL and less than 6.0 percent at 400 pg/mL. The developed assay was used to successfully confirm the presence of intact testosterone esters in equine plasma samples following intramuscular injection of Durateston® (mixed testosterone esters) [11316].

1-Testosterone (17beta-hydroxy-5alpha-androst-1-en-3-one), a synthetic anabolic steroid, has been described as one of the most effective muscle-building supplements currently on the market. It has an anabolic potency of 200 as compared to 26 for testosterone. Apart from its abuse in human sports, it can also be a doping agent in racehorses. Metabolic studies on 1-testosterone have only been reported for human in the early seventies, whereas little is known about its metabolic fate in horses. One paper described the studies of in vitro and in vivo metabolism of 1-testosterone in horses, with the aim of identifying the most appropriate target metabolites to be monitored for controlling the misuse or abuse of 1-testosterone in racehorses. Six in vitro metabolites, namely 5alpha-androst-1-ene-3alpha,17beta-diol (T1a), 5alpha-androstane-3beta,17beta-diol (T2), epiandrosterone (T3), 16,17-dihydroxy-5alpha-androst-1-ene-3-one (T4 & T5), and 5alpha-androst-1-ene-3,17-dione (T6), were identified. For the in vivo studies, two thoroughbred geldings were each administered orally with 800 mg of 1-testosterone by stomach tubing. The results revealed that the parent drug and eight metabolites were detected in urine. Besides the four in vitro metabolites (T1a, T2, T3, and
T5), four other urinary metabolites, namely 5α-androst-1-ene-3β,17α-diol (T1b), 5α-androst-1-ene-3β,17β-diol (T1c), 5α-androstane-3α,17α-diol (T7) and 5α-androstane-3β,17α-diol (T8) were identified. The study shows that the detection of 1-testosterone administration is best achieved by monitoring the parent drug, which could be detected for up to 30 h post-administration [12492].

**Testosterone and nandrolone**

Pennsylvania (PA) State Racing Commissions regulate the endogenous androgenic steroid, testosterone (TES), in racing intact males (RIM) by quantification of TES in post-race samples. Post-race plasma samples (2209) collected between 2008 and 2010 were analyzed for TES, nandrolone (NAN), and other anabolic steroids (ABS). Of the 2209 plasma samples, 2098 had quantifiable TES ≥ 25 pg/mL. Plasma (mean ± SD) concentrations of TES and NAN in RIM were 329 ± 266 and 96 ± 68 pg/mL, respectively. Only 65 percent of RIM had quantifiable concentration of NAN, and there was no relationship between TES and NAN. Plasma TES concentrations were significantly higher during the months of April, May, June, July, and August. A significantly higher plasma TES was observed in Thoroughbred (TB) (348 ± 289 pg/mL) versus that in Standardbred (STB) (315 ± 248 pg/mL). Plasma concentrations of TES from breeding stallions (BS) were 602 ± 357 pg/mL. Statistically significant (P < 0.0001) lower plasma concentrations of the two steroids were observed in RIM horses. Based on quantile distribution of TES in the RIM and BS populations, 99.5% were at or below 1546.1 and 1778.0 pg/mL, respectively. Based on this population of RIM, the suggested upper threshold plasma concentration of endogenous TES in horses competing in PA should remain at 2000 pg/mL [12493].

**Nandrolone**

19-Norandrostenedione (NAED) and nandrolone are anabolic-androgenic steroids (AASs). Nandrolone was regarded solely as a synthetic AAS until the 1980s when trace concentrations of apparently endogenous nandrolone were detected in urine samples obtained from intact male horses (stallions). Since then, its endogenous origin has been reported in boars and bulls; endogenous NAED and nandrolone have been identified in plasma and urine samples collected from stallions. More recently, however, it was suggested that NAED and nandrolone detected in urine samples from stallions are primarily artifacts due to the analytical procedure. The present study was undertaken to determine whether NAED and nandrolone detected in plasma and urine samples collected from stallions are truly endogenous or artifacts from sample processing. To answer this question, fresh plasma and urine samples from ≥8 stallions were analyzed for the two AASs, soon after collection, by liquid chromatography hyphenated to tandem mass spectrometry (LC-MS/MS). NAED and nandrolone were not detected in fresh plasma samples but detected in the same samples post storage. Concentrations of both AASs increased with storage time, and the increases were greater at a higher storage temperature (37°C versus 4°C, and ambient temperature versus 4°C). Although NAED was detected in some fresh stallion urine samples, its concentration (<407 pg/mL) was far lower (<0.4 %) than that in the same samples post storage (at ambient temperature for 15 days). Nandrolone was not detected in most of fresh urine samples but detected in the same samples post storage. Based on these results, it is concluded that all NAED and nandrolone detected in stored plasma samples of stallions and most of them in the stored urine samples are not from endogenous origins but spontaneously generated during sample storage, most likely from spontaneous decarboxylation of androstenedione-19-oic acid and testosterone-19-oic acid. It was the first time that all NAED and nandrolone detected in plasma of stallions and most of them detected in the urine have
been shown to be spontaneously generated in vitro during sample storage. This finding would have significant implications with regard to the regulation of the two steroids in horse racing [12494].

A method to quantify metabolites of 17beta-nandrolone (17betaN) in boar and horse urine has been optimized and validated. Metabolites excreted in free form were extracted at pH 9.5 with tert-butylmethylether. The aqueous phases were applied to Sep Pak C18 cartridges and conjugated steroids were eluted with methanol. After evaporation to dryness, either enzymatic hydrolysis with beta-glucuronidase from Escherichia coli or solvolysis with a mixture of ethylacetate:methanol:concentrated sulphuric acid were applied to the extract. Deconjugated steroids were then extracted at alkaline pH with tert-butylmethylether. The dried organic extracts were derivatized with MSTFA:NH4I:2-mercaptoethanol to obtain the TMS derivatives, and were subjected to analysis by gas chromatography mass spectrometry (GC/MS). The procedure was validated in boar and horse urine for the following metabolites: norandrosterone, noretiocholanolone, norepiandrosterone, 5beta-estrane-3alpha, 17beta-diol, 5alpha-estrane-3beta, 17beta-diol, 17alpha-diol, 17alpha-nandrolone, 17betaN, 5(10)-estrane-3alpha, 17alpha-diol, 17alpha-estradiol and 17beta-estradiol in the different metabolic fractions. Extraction recoveries were higher than 90 percent for all analytes in the free fraction, and better than 80 percent in the glucuronide and sulphate fractions, except for 17alpha-estradiol in the glucuronide fraction (74 %), and 5alpha-estrane-3beta, 17alpha-diol and 17betaN in the sulphate fraction (close to 70 %). Limits of quantitation ranged from 0.05 to 2.1 ng/mL in the free fraction, from 0.3 to 1.7 ng/mL in the glucuronide fraction, and from 0.2 to 2.6 ng/mL in the sulphate fraction. Intra- and inter-assay values for precision, measured as relative standard deviation, and accuracy, measured as relative standard error, were below 15 percent for most of the analytes and below 25 percent, for the rest of analytes. The method was applied to the analysis of urine samples collected after administration of 17betaN laurate to boars and horses, and its suitability for the quantitation of the metabolites in the three fractions has been demonstrated [06318].

The Commission Decision 2002/657/EC is a fundamental reference document for the UE laboratories involved in residue analysis although its implementation has caused some difficulties in the requirements interpretation. In this work a pragmatic validation approach of a quantitative confirmatory method for the detection of 17-alpha-(alpha-NT) and 17-beta-19-nortestosterone (beta-NT) in bovine urine by gas chromatography mass spectrometry is proposed. The 19-nortestosterone is a banned anabolic steroid for which no minimum required performance limit (MRPL) has been laid down, therefore the limit reported in Italian Residue Monitoring Plan (2 microg/L) has been considered the reference level to evaluate the method performances. The decision limit (CCalpha) and the detection capability (CCbeta) were obtained by the calibration curve procedure. The minimum required performance level (MRPL), which represents the starting concentration of the calibration curves, was preliminary fixed estimating the results dispersion of blank urine samples fortified at 2 microg/L for each isomer. The found CCalpha and CCbeta were 1.5 and 1.9 microg/L for alpha-NT and 1.2 and 1.4 microg/L for beta-NT. The precision (repeatability and within-laboratory reproducibility) and recoveries were suitable for the investigated concentration range (1-3 microg/L). Finally, the method ruggedness (minor and major changes) has been also demonstrated [06319].

Following administration of the anabolic steroid 19-nortestosterone or its esters to the horse, a major urinary metabolite is 19-nortestosterone-17beta-sulphate. The detection of 19-nortestosterone in urine from untreated animals has led to it being considered a naturally occurring steroid in the male horse. Recently, it was demonstrated that the majority of the 19-nortestosterone found in extracts of normal urine from male horses arises as an artefact through decarboxylation of the 19-carboxylic acid of testosterone. The aim of one
investigation was to establish if direct analysis of 19-nortestosterone-17β-sulphate by liquid chromatography/tandem mass spectrometry (LC/MS/MS) had potential for the detection of 19-nortestosterone misuse in the male horse. The high concentrations of sulphate conjugates of the female sex hormones naturally present in male equine urine were overcome by selective hydrolysis of the aryl sulphates using glucuronidase from Helix pomatia; this was shown to have little or no activity for alkyl sulphates such as 19-nortestosterone-17β-sulphate. The free phenolic steroids were removed by solid-phase extraction (SPE) prior to LC/MS/MS analysis. The method also allowed for the quantification of the sulphate conjugate of boldenone, a further anabolic steroid endogenous in the male equine with potential for abuse in sports. The results indicate that while 19-nortestosterone-17β-sulphate is present at low levels as an endogenous substance in urine from normal male horses, its use as an effective threshold substance may be viable [08436].

5α-Estrane-3β,17α-diol is the major metabolite of nandrolone in horse urine. The presence of 5α-estrane-3β,17α-diol in female and gelding urines is prohibited by Racing Rules and its natural presence in male urine led regulation authorities to establish a concentration threshold of 45 ng/mL. One paper described a rapid, simple and stereoselective synthesis of 5α-estrane-3β,17α-diol, providing horseracing laboratories with an essential reference material for their antidoping performance [11317].

**Stanozolol**

Within equine drug surveillance, there is significant interest in analyzing intact phase II conjugates of drugs in urine, but progress has been limited by a lack of reference material. In one study, in vitro techniques using equine liver fractions were employed to produce glucuronide and sulfate conjugates of stanozolol, 16β-hydroxystanozolol and nandrolone, the glucuronide conjugate of morphine and the glutathione metabolite of chlorodinitrobenzene for the first time in equine sports drug surveillance. The glucuronide conjugate of the synthetic progestagen altenogest was also produced in vitro, removing the requirement for sample hydrolysis during routine urinalyses. It was concluded that these results highlight the potential of in vitro studies for the production of phase II reference material, allowing the development of assays based on intact conjugates [10537].

**Methyltestosterone and mestranolone**

Anabolic steroids with the 17α-methyl,17β-hydroxyl group, which were developed as oral formulations for therapeutic purposes, have been abused in the field of human sports. These anabolic steroids are also used to enhance racing performance in racehorses. In humans, structurally related 17α-methyltestosterone (MTS) and mestanolone (MSL), which are anabolic steroids with the 17α-methyl,17β-hydroxyl group, have metabolites in common. The purpose of this study was to determine metabolites common to these two steroids in horses, which may serve as readily available screening targets for the doping test of these steroids in racehorses. Urine sample collected after administering MTS and MSL to horses was treated to obtain unconjugated steroid, glucuronide, and sulfate fractions. The fractions were subjected to gas chromatography/mass spectrometry (GC/MS), and 17α-methyl-5α-pregn-3β,17β-diol, 17α-hydroxymethyl-5α-pregn-3β,17β-diol, 17α-methyl-5α-pregn-3β,16β,17β-triol, and 17α-methyl-5α-pregn-3β,16α,17β-triol were detected as the common metabolites by comparison with synthesized reference standards. The urinary concentrations of these metabolites after dosing were determined by GC/MS. 17α-Methyl-
methyl-5alpha-androstan-3beta,16beta,17beta-triol was mainly detected in the sulfate fractions of urine samples after administration. This compound was consistently detected for the longest time in the urine samples after dosing with both steroids. The results suggest that 17alpha-methyl-5alpha-androstan-3beta,16beta,17beta-triol is a very useful screening target for the doping test of MTS and MSL in racehorses [06322].

**Mesterolone**

Mesterolone (1alpha-methyl-5alpha-androstan-17beta-ol-3-one) is a synthetic anabolic androgenic steroid (AAS) with reported abuses in human sports. As for other AAS, mesterolone is also a potential doping agent in equine sports. Metabolic studies on mesterolone have been reported for humans, whereas little is known about its metabolic fate in horses. One paper described the studies of both the in vitro and in vivo metabolism of mesterolone in racehorses with an objective to identify the most appropriate target metabolites for detecting mesterolone administration. In vitro biotransformation studies of mesterolone were performed by incubating the steroid with horse liver microsomes. Metabolites in the incubation mixture were isolated by liquid-liquid extraction and analysed by gas chromatography-mass spectrometry (GC-MS) after acylation or silylation. Five metabolites (M1-M5) were detected. They were 1alpha-methyl-5alpha-androstan-3alpha-ol-17-one (M1), 1alpha-methyl-5alpha-androstan-3beta-ol-17-one (M2), 1alpha-methyl-5alpha-androstane-3alpha-17one (M3), 1alpha-methyl-5alpha-androstane-3beta-17one (M4), and 1alpha-methyl-5alpha-androstane-3,17-dione (M5). Of these in vitro metabolites, M1, M3, M4 and M5 were confirmed using authentic reference standards. M2 was tentatively identified by mass spectral comparison to M1. For the in vivo metabolic studies, Proviron (20 tablets x 25 mg of mesterolone) was administered orally to two thoroughbred geldings. Pre- and post-administration urine samples were collected for analysis. Free and conjugated metabolites were isolated using solid-phase extraction and analysed by GC-MS as described for the in vitro studies. The results revealed that mesterolone was extensively metabolised and the parent drug was not detected in urine. Three metabolites detected in the in vitro studies, namely M1, M2 and M4, were also detected in post-administration urine samples. In addition, two stereoisomers each of 1alpha-methyl-5alpha-androstane-3,17alpha-diol (M6 and M7) and 1alpha-methyl-5alpha-androstane-3,16-diol-17-one (M8 and M9), and an 18-hydroxylated metabolite 1alpha-methyl-5alpha-androstane-3,18-diol-17-one (M10) were also detected. The metabolic pathway for mesterolone is postulated. These studies have shown that metabolites M8, M9 and M10 could be used as potential screening targets for controlling the misuse of mesterolone in horses [07399].

**Methandienone**

Methandienone, methandriol, and oxymetholone, which are anabolic steroids possessing 17alpha-methyl and 17beta-hydroxy groups, were developed as oral formulations for therapeutic purposes. However, they have been used in racehorses to enhance racing performance. In humans, it has been reported that structurally related anabolic steroids having the 17alpha-methyl and 17beta-hydroxy groups, including 17alpha-methyltestosterone, mestanolone, methandienone, methandriol, and oxymetholone, have metabolites in common. In one study, it was found that metabolites common to those of 17alpha-methyltestosterone and mestanolone were detected in horse urine after the administration of oxymetholone, methandienone, and methandriol. Based on analytical data, it was confirmed these to be the common metabolites of five structurally related steroids, 17alpha-methyltestosterone, mestanolone, oxymetholone, methandienone, and methandriol.
Furthermore, it was detected hitherto unknown urinary metabolites of methandriol and oxymetholone in horses. The parent steroid itself was detected in horse urine after the administration of methandriol, other than metabolites common to 17alpha-methyltestosterone and mestanolone. On the other hand, the major metabolite of oxymetholone was mestanolone, aside from metabolites presumed to be the stereoisomers of 2-hydroxymethyl-17alpha-methyl-5alpha-androstan-3,17beta-diol and 2,17alpha-dihydroxymethyl)-5alpha-androstan-3,17beta-diol. The simultaneous detection of common metabolites and other main metabolites would help narrow down the candidate-administered steroid for the doping tests in racehorses [08438].

**Boldione, boldenone and boldenone esters**

Conflicting findings regarding the boldenone content of bovine faeces suggest it may be synthesized de novo in emitted faeces. It was tested this hypothesis by analysing uncontaminated urine, fresh and various forms of dried faeces from 10 calves (not given boldenone) by liquid chromatography/tandem mass spectrometry for 17alpha- and 17beta-boldenone (alpha and beta BOL); 1,4-androstadiene-3,17-dione (ADD); 4-androstone-3,17-dione (AED), testosterone (T) and epitestosterone (ET). Urine contained no alpha BOL, beta BOL or ADD. The analysed substances were variably present in the rectal faeces, and at generally higher levels in faeces scraped from skin or stall floor. In pooled rectal faeces naturally dried for 13 days, alpha BOL, ADD, AED and ET levels were extremely high (much higher than accounted for by increases due to drying), and beta BOL and T were absent. It is concluded that de novo synthesis of alpha BOL and metabolites occurs naturally in bovine faeces and only uncontaminated urine should be analysed for illegal boldenone [06320].

Electrochemical based immunosensors for the detection of boldenone and methylboldenone in bovine urine were described in this paper. The immunosensors were fabricated by immobilizing boldenone-bovine serum albumin conjugate on the surface of screen-printed electrodes (SPEs), and followed by the competition between the free analyte and coating conjugate with corresponding antibodies. The use of anti-species IgG-horseradish peroxidase conjugate determined the degree of competition. The electrochemical technique chosen was chronoamperometry, performed at a potential of +100 mV whereby the product of the catalysis of 3,3',5,5'-tetramethylbenzidine undergoes reduction produced by the enzyme label. The limits of detection of assay were 31 pg/mL for boldenone and 120 pg/mL for methylboldenone, respectively. Results of repeated analysis of each androgen carried out using three different batches of electrodes indicate suitable repeatability. Urine samples were determined directly after a single dilution step, omitting extraction and hydrolysis. This method offers the advantage to pick up both boldenone and its major metabolites in an efficient manner due to the high cross-reactivity pattern of alpha-boldenone with this antibody. The concentration of methylboldenone in urine detected by developed methods does indicate methylboldenone administration to heifers. Gas chromatography coupled to mass spectrometry analysis was performed to quantitate the individual metabolites present in urine samples, and results were validated with both ELISA and immunosensor data [06321].

Boldenone is banned in the European Union (Directive 96/22/EC) as growth promoter for meat producing animals. Boldione (ADD), boldenone and boldenone esters (mainly the undecylenate form) are commercially available as anabolic preparations, either to the destination of human, horse or cattle. Since the late 90s, the natural occurrence of boldenone metabolites has been reported in cattle. According to EU regulation, the unambiguous demonstration of boldenone administration in bovine urine should be provided on the basis of boldenone identification in the corresponding conjugate fraction. An analytical
The method has been developed and validated according to current standards with main concern to the measurement of intact 17beta-boldenone-sulphate. The analytical procedure included direct extraction-purification of target analyte on octadecylsilyl cartridges and direct detection of phase II metabolite by liquid chromatography (negative electrospray), tandem mass spectrometry (QqQ) or high resolution mass spectrometry. Decision limit (CC-alpha) and detection capability (CC-beta) were respectively 0.2 microg/L and 0.4 microg/L on triple quadrupole and 0.1 microg/L and 0.2 microg/L on hybrid system. The method was successfully applied to the analysis of incurred samples collected in different experiments. 17beta-Boldenone-sulphate was measurable up to 36h after oral administration of boldione, and 30 days after 17beta-boldenone undecylenate intra-muscular injection. This conjugate form was never detected in non-treated animals, confirming its status of definitive candidate marker for boldenone administration in calf [09399].

**Trenbolone and estradiole**

The increasing size of concentrated animal feeding operations has led to a concomitant increase in the land-application of manure, which has spawned research on the concentrations and environmental risk assessment of natural and synthetic hormones in animal manures. 17beta-trenbolone acetate (TBA) is widely used in the United States for improving daily gains in beef cattle and is often administered in combination with 17beta-estradiol (17beta-E2). Trenbolone (TB) and E2 isomers and their metabolites were quantified in manure collection pits and lagoon effluent from beef cattle implanted with the commercial anabolic preparation Ravoler-S (containing 140 mg 17beta-trenbolone acetate and 28 mg 17beta-E2). Manure pit and lagoon effluent samples were collected weekly for 9 weeks post implanting and analyzed using reverse-phase liquid chromatography tandem mass spectrometry. 17alpha-TB was the most abundant androgen with the highest concentration observed 2 weeks post implant. 17beta-TB and trendione peaked at the end of week 2 and 4, respectively. For the estrogens, the highest concentrations for estrone (E1), estriol (E3), and 17alpha-E2 were observed after week 4, 6, and 8, respectively. 17beta-E2 concentrations were the lowest of the estrogens and erratic over time. In lagoon water, which is used for irrigation, 17alpha-TB and E1 had the highest detected hormone concentrations (1.53 and 1.72 microg/L, respectively). Assuming a 1-2 order dilution during transport to surface water, these hormone levels could lead to concentrations in receiving waters that exceed some of the lowest observable effect levels (LOELs) reported for hormones (e.g. 0.01-0.03 microg/L) [12495].

**Fluoxymesterone**

Fluoxymesterone, an anabolic steroid with the 17alpha-methyl,17beta-hydroxy group, has been developed as an oral formulation for therapeutic purposes. However, it is also used illegally in racehorses to enhance racing performance. In this study, it was detected 9alpha-fluoro-17,17-dimethyl-18-norandrostane-4,13-dien-11beta-ol-3-one by gas chromatography/mass spectrometry (GC/MS), which has not been reported as a fluoxymesterone metabolite so far in horse. It was synthesized for use as a reference standard, and characterized on the basis of $^1$H NMR and $^{13}$C NMR spectra, as well as GC/MS EI mass spectra of TMS derivatives. It was excreted as the main metabolite in horse urine, and its reference standard could be synthesized easily. Therefore, this metabolite could be a useful target for a doping test of fluoxymesterone in racehorses [08435].
Selective androgen receptor modulators (SARMs)

Selective androgen receptor modulators (SARMs) are a group of substances that have potential to be used as doping agents in sports. Being a relatively new group not available on the open market means that no reference materials are commercially available for the main metabolites. In one study, the in vitro metabolism of SARMs by the fungus Cunninghamella elegans has been investigated with the purpose of finding out if it can produce relevant human and equine metabolites. Three different SARMs, S1, S4 and S24, were incubated for 5 days with C. elegans. The samples were analysed both with and without sample pretreatment using ultra performance liquid chromatography coupled to high resolution mass spectrometry. All the important phase I and some phase II metabolites from human and horse were formed by the fungus. They were formed through reactions such as hydroxylation, deacetylation, O-dephenylation, nitro-reduction, acetylation and sulfonation. The study showed that the fungus produced relevant metabolites of the SARMs and thus can be used to mimic mammalian metabolism. Furthermore, it has the potential to be used for future production of reference material [12496].

Growth hormone

Growth hormone (GH) is a polypeptide suspected of being used in horse racing to speed up physical performances. Despite scientific advances in the recent years, the control of its administration remains difficult. In order to improve it, a metabolomics study through LC-high resolution mass spectrometry measurements was recently initiated to assess the metabolic perturbations caused by recombinant equine growth hormone administration. Few tens of ions not identified structurally were highlighted as compounds responsible for the modification of metabolic profiling observed in treated animals. This previous work was based on the use of Uptisphere Strategy NEC as the chromatographic column. In parallel, more and more metabolomics studies showed the interest of the use of new chromatographic supports such as hydrophilic interaction chromatography for the analysis of polar compounds. It is in this context that an investigation was conducted on Uptisphere HDO and Luna hydrophilic interaction chromatography stationary phases to generate and process urinary metabolomics fingerprints, which could allow to establish a comparison with Uptisphere Strategy NEC. The chromatographic column the most adapted for the detection of new biomarkers of GH administration has been used to set up a relevant statistical model based on the analysis of more than hundred biological samples [11318].

Erythropoietin

Horse

Doping of horses with recombinant human erythropoietin (rHuEPO) to illegally enhance their endurance capacity in horseracing has been reported during the last years. This leads to increased blood viscosity which can result in sudden death and is of concern for the horse welfare. Additionally, the horse can start production of rHuEPO antibodies, which cross-reacts with endogenous equine EPO and can lead to severe anaemia and even death. In this study, a novel micro-chromatographic method, EPO WGA MAIIA, has been tested for the capability in plasma and urine samples to detect administration of erythropoiesis-stimulating agents, like the rHuEPO glycoprotein varieties Eprex and Aranesp, to horses. After administration of 40 IU Eprex peer kg/day to seven horses during 6 days, the presence of Eprex in horse plasma was detected up to 2-5 days after last injection. In urine samples
collected from two horses, Eprex was detected up to 3 days. A single injection of Aranesp (0.39 microg/kg) was detected up to 9 days in plasma and up to 8 days, the last day of testing, in the urine sample. The LC-FAIMS-MS/MS system, with 1 day reporting time, confirmed the presence of Eprex up to 1 day after last injection for six out of seven horses and the presence of Aranesp up to 5 days after last injection in plasma samples. The MAIIA system showed to be a promising tool with high sensitivity and extremely short reporting time (1 h) [12497].

Erythropoietin (EPO) and its recombinant analogues are suspected to be illicitly administered to horses for performance enhancing purposes and, consequently, prohibited in equine sports. Recently, a new erythropoiesis-stimulating agent, peginesatide (Omontys, formerly referred to as Hematide), belonging to the upcoming class of EPO-mimetic peptides, received approval for the treatment of anaemia in humans with chronic kidney disease on dialysis. As the pegylated dimeric peptide of approximately 45 kDa without sequence homology to EPO is not detectable by conventional EPO detection assays, specific methods are bound to be established for horse sports drug testing. Thus, by fortifying equine serum with peginesatide, an approach consisting of a proteolytic digestion with subtilisin after protein precipitation was developed, eventually targeting a proteotypic and xenobiotic pentapeptide which is easily accessible to liquid chromatography-tandem mass spectrometry analysis. The method was validated for qualitative purposes and demonstrated to be specific, precise (relative standard deviations below 14 %), sensitive (limit of detection 10 ng/mL) and linear. Being simple, cost-effective and readily transferable to other doping control laboratories, a mass spectrometric assay for the detection of therapeutic concentrations of peginesatide in equine serum is, in terms of preventive doping research, applicable to routine analysis shortly after approval of the drug [12498].

Recombinant human erythropoietin (rhEPO), darbepoetin alfa (DPO) and methoxy polyethylene glycol-epoetin beta (PEG-EPO) are synthetic analogues of the endogenous hormone erythropoietin (EPO). These erythropoiesis-stimulating agents have the ability to stimulate the production of red blood cells and are commercially available for the treatment of anaemia in humans. These drugs are understood to have performance-enhancing effects on human athletes due to their stimulation of red blood cell production, thereby improving delivery of oxygen to the muscle tissues. The method officially adopted by the International Olympic Committee (IOC) and World Anti Doping Agency (WADA) for the confirmation of rhEPO and/or DPO (rhEPO/DPO) in human urine is based on electrophoresis in combination with Western blotting. A shortcoming of the WADA method is the lack of definitive mass spectral data for the confirmation of a positive finding. Recently, a liquid chromatography-tandem mass spectrometry (LC/MS/MS) method for the detection and confirmation of rhEPO/DPO in equine plasma was reported. One paper presents a method for the detection and confirmation of rhEPO/DPO, as well as the newly released PEG-EPO, in equine plasma. The procedures involve immunoaffinity extraction using anti-rhEPO antibody-coated Dynabeads followed by trypsin digestion. The injected extract was further purified and concentrated using an on-line trap column in the nano-LC system. Detection and confirmation were achieved by monitoring a unique peptide segment of rhEPO/DPO/PEG-EPO using nano-liquid chromatography-tandem mass spectrometry equipped with a nanospray ionisation source operated in the selected reaction monitoring mode. rhEPO, DPO and PEG-EPO can be confirmed at 0.1, 0.2 and 1.0 ng/mL, respectively, in equine plasma [10297].

Recombinant human erythropoietin (rhEPO) and darbepoetin alfa (DPO) are protein-based drugs for the treatment of anemia in humans by stimulating erythrocyte production. One paper described the first method for differentiation and identification of rhEPO and DPO in equine plasma by liquid chromatography coupled to tandem mass spectrometry (LC-
MS/MS). The limit of identification was 0.1 ng/mL for DPO and 0.2 ng/mL for rhEPO in equine plasma, and the limit of detection was 0.05 ng/mL for DPO and 0.1 ng/mL for rhEPO. Analyte carryover problem encountered was solved by adding 20 percent acetonitrile to the solvent of the sample digest to increase solubility of the peptides. This method was successfully applied to identification of DPO in plasma samples collected from a research horse following DPO administration and from racehorses out of competition in North America [08440].

Recombinant human erythropoietins (rHuEPOs) are glycoproteins drugs, produced by the pharmaceutical industry to restore production of red blood cells by stimulating human bone marrow for which this pathology has been diagnosed. It is suspected that these molecules are diverted as doping agents in horseracing to enhance oxygen transport and aerobic power in racehorses. Although indirect double-blotting or direct liquid chromatography-mass spectrometry (LC-MS) methods have been developed to confirm the presence of rHuEPO in a sample, the short detection time (48 h) is still a problem for doping control. In this context, gene profiling investigation through Serial Analysis of Gene Expression (SAGE) has been conducted on seven thoroughbreds treated with Eprex. This functional genomic method has been performed from total blood cells collected from each animal to assess the mRNA expression consecutive to rHuEPO injections. Sample pooling was chosen as a powerful, cost-effective, and rapid means of identifying the most common and specific changes in terms of gene expression profile and to eliminate individual variation. Consequently, three SAGE libraries were constructed, before, during, and after Eprex® treatment. More than 71 440 mRNA signatures were observed and subjected to statistical analysis; 49 differentially expressed genes were identified and analyzed by qPCR. From the selected gene list, were defined as potential biomarkers in terms of their low inter-individual variation and capacity as strong markers of rHuEPO administration up to 60 days after the beginning of the doping period. In one paper, a new strategy is proposed to the horseracing industry to prevent rHuEPO abuse [10427].

Methods have been developed to screen for and confirm darbepoetin alfa, recombinant human EPO, and methoxy polyethylene glycol-epoetin beta (PEG-epoetin beta) in horse plasma. All three methods screen samples with an enzyme-linked immunosorbent assay (ELISA) and confirm by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This report focuses on PEG-epoetin beta. The ELISA assay was able to detect PEG-epoetin beta at 0.02 ng/mL in 50 µL of horse plasma. Many samples had high background levels of immunoreactivity; however, introducing polyethylene glycol 6000 (PEG 6000) into the samples before the ELISA assay removed the high background and increased the apparent concentrations of PEG-epoetin β. In samples collected following the administration of 100 µg of PEG-epoetin β by the intravenous (IV), intramuscular (IM) and subcutaneous (SC) routes, PEG-epoetin beta was detectable up to 72, 144, and 120 h, respectively. The samples were prepared for LC-MS/MS analysis by extraction with anti-rHuEPO-antibodies-coated Dynabeads followed by digestion with trypsin. The LC-MS/MS confirmation method used the multiple reaction monitoring (MRM) scan mode to monitor four precursor-product ion transitions of the EPO-derived peptide T₆. All four transitions of T₆ were detectable with S/N > 3. The limit of confirmation for PEG-epoetin beta was 1.0 ng/mL in 2 mL of horse plasma. The method successfully confirmed the presence of PEG-epoetin beta in a sample collected from a Mircera®-treated horse. Compared to PEG-epoetin beta, better sensitivity was achieved for darbepoetin alfa and recombinant human EPO. Darbepoetin alfa was detected in horse plasma four days after IM administration of 100 microg [11319].

Recombinant human erythropoietin (rHuEPO) causes an increase in red blood cell production and aerobic capacity in other species; however, data are lacking on effects in the horse. One study tested the hypothesis that rhuEPO administration would alter red cell volume (RCV),
aerobic capacity (VO_{2\text{max}}) and indices of anaerobic power. Eight healthy, unfit mares accustomed to the laboratory and experimental protocols were randomly assigned to either a control (n=4; 3 ml saline 3 times/week for 3 weeks) or EPO group (EPO, n=4, 50 iu/kg bwt rhuEPO/3 ml saline 3 times/week for 3 weeks). Exercise tests (GXT) were performed on a treadmill (6 % incline), 1 week before and 1 week after treatment. The GXT started at 4 m/sec, with a 1 m/sec increase every 60 sec until the horse reached fatigue. Oxygen uptake was measured via an open flow indirect calorimeter. Blood samples were collected before, during (each step) and 2 and 15 min post GXT to measure packed cell volume (PCV), haemoglobin concentration (Hb), blood lactate concentration (LA) and plasma protein concentration (TP). Plasma volume (PV) was measured using Evans Blue dye. Blood volume (BV) and RCV were calculated using PCV from the 8 m/sec step of the GXT. There were no alterations in any parameters in control horses. By week 3, EPO produced increases in resting PCV and Hb (37 %), RCV (26 %) and VO_{2\text{max}} (19 %) increased, but BV did not change due to decreased PV. There was a significant increase in velocity at VO_{2\text{max}} and LA\text{peak} for horses treated with rhuEPO and substantial decrease in VO_{2} recovery time when the pretreatment GXT was compared to the post treatment GXT. No differences were detected for TP, VLA4, run time or V_{\text{max}}. It was concluded that low dose rhuEPO administration increases RCV and aerobic capacity without altering anaerobic power. The study demonstrates that rhuEPO enhances aerobic capacity and exercise performance, a question relevant to racing authorities [06327].

**Dog**

Recombinant human erythropoietin (rHuEPO) doping is prohibited in animal (canine and equine) sport. The effectiveness of a range of immunoassay screening methods for the detection of rHuEPO in canine urine was evaluated. The excretion profiles following rHuEPO administration to dogs were investigated. The presence of rHuEPO in postadministration samples was confirmed using the World Anti-Doping Agency (WADA)-approved isoelectric focusing immunoblotting confirmatory technique. Following the administration study, a screening program involving approximately 6000 greyhound sport (mostly racing) samples was undertaken for rHuEPO. This resulted in the detection of the first rHuEPO positives in the world of canine or equine sport. In an additional case, endogenous HuEPO was detected in a sample submitted as greyhound urine. It was determined that this arose from the submission to control stewards, as greyhound urine, of a substance that was, in fact, human urine. This was a particularly welcome development as definitive confirmatory evidence of such sample switching can be difficult to obtain in the case of greyhounds [06328].

**Darbepoetin**

Recombinant human erythropoietin (rhEPO) and darbepoetin alpha (DPO) are protein-based drugs for the treatment of anemia by stimulating red blood cell production. Consequently, they are abused in human and equine sports. To deter their abuse in the horse racing industry, a sensitive and reliable method for confirmation of these agents in equine plasma has been in urgent need. Such a method by LC-MS/MS is described in this paper. The method involved analyte enrichment by immunoaffinity separation using anti-rhEPO antibody linked to magnetic beads, digestion by trypsin, and analysis by LC-MS/MS. Two specific proteotypic peptides, 46VNFYAWK52 and 144VYSNFLR150 from rhEPO and DPO were employed for confirmation of the analytes based on chromatographic retention times and major product ions. The limit of confirmation of this method was 0.2 ng/mL, and the limit of detection was 0.1 ng/mL for rhEPO and DPO in equine plasma. This method was successful in confirming the presence of rhEPO and DPO in plasma samples collected from research
horses to which rhEPO or DPO was administered and from racehorses following competition and in noncompetition samples in North America. To our knowledge, this is the first LC-MS method with adequate sensitivity and specificity in providing unequivocal confirmation of rhEPO and DPO in equine plasma samples. This method provides a powerful enforcement tool that was lacking in the fight against the abuse of rhEPO and DPO in the horse racing industry [07400].

**Insulin**

Insulin administration can increase muscle glycogen by utilising hyperinsulinaemic clamps prior to sports events or during the recovery phases, and increase muscle size by its chalonic action to inhibit protein breakdown. In order to control insulin abuse in equine sports, a method to detect effectively the use of insulins in horses would be required. Besides the readily available human insulin and its synthetic analogues, structurally similar insulins from other species can also be used as doping agents. One study described a method for the simultaneous detection of bovine, porcine and human insulins, as well as the synthetic analogues Humalog (Lilly) and Novolog (Novo Nordisk) in equine plasma. Insulins were isolated from equine plasma by immunoaffinity purification, followed by centrifugal filtration, and analysed by nano-liquid chromatography-tandem mass spectrometry (LC/MS/MS). Insulin and analogues were detected and confirmed by comparing their retention times and major product ions. All five insulins (human insulin, Humalog, Novolog, bovine insulin and porcine insulin), which are exogenous in the horse, could be detected and confirmed at 0.05ng/mL. This method was successful in confirming the presence of human insulin in plasma collected from horses up to 4h after having been administered a single low dose of recombinant human insulin (Humulin, Eli Lilly) [08439].

Insulin and its analogues have been banned in both human and equine sports owing to their potential for misuse. Insulin administration can increase muscle glycogen by utilising hyperinsulinaemic clamps prior to sports events or during the recovery phases, and increase muscle size by its chalonic action to inhibit protein breakdown. In order to control insulin abuse in equine sports, a method to effectively detect the use of insulins in horses is required. Besides the readily available human insulin and its synthetic analogues, structurally similar insulins from other species can also be used as doping agents. The author's laboratory has previously reported a method for the detection of bovine, porcine and human insulins, as well as the synthetic analogues Humalog (Lispro) and Novolog (Aspart) in equine plasma. This study describes a complementary method for the simultaneous detection of five exogenous insulins and their possible metabolites in equine urine. Insulins and their possible metabolites were isolated from equine urine by immunoaffinity purification, and analysed by nano liquid chromatography-tandem mass spectrometry (LC/MS/MS). Insulin and its analogues were detected and confirmed by comparing their retention times and major product ions. All five insulins (human insulin, Humalog, Novolog, bovine insulin and porcine insulin), which are exogenous in horse, could be detected and confirmed at 0.05ng/mL. This method was successfully applied to confirm the presence of human insulin in urine collected from horses up to 4h after having been administered a single low dose of recombinant human insulin (Humulin R, Eli Lilly) [11320].

**Seven peptide hormones**

In recent years, there has been an ongoing focus for both human and equine doping control laboratories on developing detection methods to control the misuse of peptide therapeutics.
Immunoaffinity purification is a common extraction method to isolate peptides from biological matrices and obtain sufficient detectability in subsequent instrumental analysis. However, monoclonal or polyclonal antibodies for immunoaffinity purification may not be commercially available, and even if available, such antibodies are usually very costly. In our study, a simple mixed-mode anion exchange solid-phase extraction cartridge was employed for the extraction of seven target peptides (GHRP-1, GHRP-2, GHRP-6, ipamorelin, hexarelin, CJC-1295, and N-acetylated LKKTETQ (active ingredient of TB-500)) and their in vitro metabolites from horse plasma. The final extract was subject to ultra-high-performance liquid chromatographic separation and analysed with a hybrid high-resolution mass spectrometer. The limits of detection for all seven peptides were estimated to be less than 50 pg/mL. Method validation was performed with respect to specificity, precision, and recovery. The applicability of this multi-analyte method was demonstrated by the detection of N-acetylated LKKTETQ and its metabolite N-acetylated LK from plasma samples obtained after subcutaneous administration of TB-500 (10 mg N-acetylated LKKTETQ) to two thoroughbred geldings. This method could easily be modified to cover more bioactive peptides, such as dermorphin, beta-casomorphin, and desmopressin. With the use of high-resolution mass spectrometry, the full-scan data acquired can also be re-processed retrospectively to search for peptides and their metabolites that have not been targeted at the time of analysis. This is the first identification of in vitro metabolites of all the studied peptides other than TB-500 in horses [13794].

Relaxine

Relaxin (RLX) is a peptide hormone belonging to the relaxin-like peptide family. Relaxin-2 (RLX-2), a heteromeric polypeptide consisting of an A-chain (24 amino acids) and a B-chain (29 amino acids) linked together by two inter-chain disulfide bonds, is the main circulating RLX hormone in human. Due to its ability to dilate blood vessels surrounding the smooth muscles via induction of nitric oxide resulting in the increase of blood and oxygen supplies to the muscles, it may enhance athletic performance and is therefore banned in horseracing, equestrian competitions, and human sports. In order to control the abuse of rhRLX-2, a definitive method is required to detect and confirm the presence of rhRLX-2 in biological samples. One paper described, for the first time, the detection and confirmation of rhRLX-2 in equine plasma by liquid chromatography-high resolution mass spectrometry (LC-HRMS) after immunoaffinity extraction. rhRLX-2 could be detected at less than 0.1 ng/mL, and confirmed at less than 0.2 ng/mL in plasma samples [13795].

Thyroid hormones

The aim of one study was to determine if topical application of dexamethasone affected the serum concentrations of thyroid hormones (triiodothyronine T3 and thyroxine T4), glucose, triglycerides, total protein and insulin in normal horses. Ten horses were treated twice daily for 10 days with 50 g dexamethasone using an ointment formulation. Thyroid hormones and insulin were assayed using standard radioimmunoassay methods, while glucose, triglycerides and total protein were determined using a standard enzymatic method and the Biuret reaction, respectively. An increase in serum glucose and triglyceride concentrations was accompanied by 2-6-fold increases in serum insulin concentrations, but there was no change in serum total protein concentration. Insulin secretion increased with concomitant hyperglycemia and hypertriglyceridemia. A non-significant decline in T4 secretion was noted. Serum T3 and T4 concentrations declined continuously below baseline values from 48 h. Glucose and insulin levels returned to baseline values 3 days after treatment withdrawal,
whereas triglycerides reverted to baseline by 7 days. In contrast, baseline values of serum T₃ and T₄ were not reached by 20 days following drug withdrawal. The results indicated that topical administration of dexamethasone affected thyroid function and physiological metabolic functions, which may have implications for potential doping cases in racing horses [10296].

**Hydrocortisone**

A liquid chromatography/time-of-flight mass spectrometry (LC/TOFMS) method was developed using the latest high-resolution LC column technology, the ultraperformance liquid chromatography (UPLC), and electrospray ionization (ESI) in the positive ion mode. Gradient UPLC separation conditions were optimized for a group of 22 analytes comprising 17 glucocorticosteroids, specific designer steroids such as tetrahydrogestrinone (THG) and specific beta₂-agonists such as formoterol. The UPLC/TOFMS separation obtained required 5.5 min only for all the substances tested. Even the critical pair of dexamethasone and betamethasone isomers was almost completely resolved. Thanks to the over 10,000 full-width at half maximum (FWHM) mass resolution and high mass accuracy features of TOFMS 50 mDa window accurate mass chromatograms could be reconstructed for the individual analytes. Sensitive screening in human and calf urine samples fortified at the glucocorticosteroids minimum required performance limit (MRPL) of 30 microg/L (human urine, sports doping) and 2 microg/L (calf urine, veterinary control) could be obtained. The potential of UPLC/TOFMS for confirmatory analysis was shown by determining the accurate mass of all compounds and fragment ions upon in-source collision-induced dissociation (CID) at different energies. The exact mass measurement errors for all glucocorticosteroids were found to be within 6 ppm. Considering veterinary control, limits of detection (LOD) and limits of quantification (LOQ) were determined for most of the analytes in calf urine and found to range from 0.1 to 3.3 and from 0.4 to 4.4 microg/L, respectively. The method can be easily extended with other banned substances of interest, as demonstrated by the addition of 21 beta₂-agonists to the original analyte mixture in urine, without causing any interferences [07401].

Two simple and rapid LC/MS methods with direct injection analysis were developed and validated for the quantification and identification of hydrocortisone in equine urine using the same sample preparation but different mass spectrometric systems: ion trap mass spectrometry (IT-MS) and time-of-flight mass spectrometry (TOF-MS). The main advantage of the proposed methodology is the minimal sample preparation procedure, as particle-free diluted urine samples were directly injected into both LC/MS systems. Desonide was used as internal standard (IS). The linear range was 0.25-2.5 microg/mL for both methods. Matrix effects were evaluated by preparing and analyzing calibration curves in water solutions and different horse urine samples. A great variation of the signal both for hydrocortisone and the internal standard was observed in different matrices. To overcome matrix effects, the unavailability of blank matrix and the excessive cost of the isotopically labeled internal standard, standard additions calibration method was applied. This work is an exploration of the performance of the standard additions approach in a method where neither nonspecific internal standards nor extensive sample preparation is utilized and no blank matrix is available. The relative standard deviations of intra and interday analysis of hydrocortisone in horse urine were lower than 10.2 and 5.4 percent, respectively, for the LC/IT-MS method and lower than 8.4 and 4.4 percent, respectively, for the LC/TOF-MS method [08441].

*Topical glucocorticoids*
There are no data available regarding the systemic (adverse) effects which might be induced by topical dermal glucocorticoids application in the horse. Besides their widespread use for the treatment of a variety of peripheral inflammatory disorders such as atopic dermatitis, eczemas or arthritis in the horse, their surreptitious application has become a concern in doping cases in competition/performance horses. Assessing both basal and ACTH-stimulated plasma cortisol as well as basal ACTH concentrations following application of dexamethasone-containing dermal ointment is necessary to determine influences on hypothalamus-pituitary-adrenal (HPA) axis. Ten clinically healthy adult standardbred horses (6 mares, 4 geldings) were rubbed twice daily each with 50 g dexamethasone-containing ointment on a defined skin area (30 x 50 cm) for 10 days. RIA and chemiluminescent enzyme immuno-metric assay were used to determine resting and ACTH-stimulated plasma cortisol and basal ACTH concentrations, respectively. HPA feedback sensitivity and adrenal function were measured by a standard ACTH stimulation test. Dermal dexamethasone suppressed significantly the resting plasma cortisol level (to 75-98 %) significantly below baseline within the first 2 days and decreased further until day 10. ACTH stimulation test showed a significantly reduced rise in plasma cortisol concentrations. Plasma ACTH level decreased also during topical dexamethasone application. The number of total lymphocytes and eosinophil granulocytes was reduced, whereas the number of neutrophils increased. No significant change of serum biochemical parameters was noted. Dermal dexamethasone application has the potential to cause an almost complete and transient HPA axis suppression and altered leukocyte distribution in normal horses. The effects on HPA axis function should be considered in relation to the inability of animals to resist stress situations. The data further implicate that percutaneously absorbed dexamethasone may cause systemic effects relevant to “doping” [09396].

The aim of one study was to determine if topical application of dexamethasone affected the serum concentrations of thyroid hormones (triiodothyronine $T_3$ and thyroxine $T_4$), glucose, triglycerides, total protein and insulin in normal horses. Ten horses were treated twice daily for 10 days with 50 g dexamethasone using an ointment formulation. Thyroid hormones and insulin were assayed using standard radioimmunoassay methods, while glucose, triglycerides and total protein were determined using a standard enzymatic method and the Biuret reaction, respectively. An increase in serum glucose and triglyceride concentrations was accompanied by 2-6-fold increases in serum insulin concentrations, but there was no change in serum total protein concentration. Insulin secretion increased with concomitant hyperglycemia and hypertriglyceridemia. A non-significant decline in $T_4$ secretion was noted. Serum $T_3$ and $T_4$ concentrations declined continuously below baseline values from 48 h. Glucose and insulin levels returned to baseline values 3 days after treatment withdrawal, whereas triglycerides reverted to baseline by 7 days. In contrast, baseline values of serum $T_3$ and $T_4$ were not reached by 20 days following drug withdrawal. The results indicated that topical administration of dexamethasone affected thyroid function and physiological metabolic functions, which may have implications for potential doping cases in racing horses [11322].

Clenbuterol and hydrocortisone

Highly luminescent Eu(3+) and Tb(3+) complexes of 10-[4-(3-isothiocyantopropoxy) benzoylmethyl]-1,4,7,10-tetraazacyclododecane-1,4,7 triacetic acid Eu(3+) is a subset of 1 and Tb(3+) is a subset of 1 were conjugated with a goat anti-rabbit IgG and a rabbit anti-mouse IgG, respectively, and applied as markers in a time resolved immunoassay for simultaneous quantitative determination of anabolic compounds clenbuterol and hydrocortisone. The assay was performed in horse urine, using a monoclonal antibody specific to clenbuterol and a rabbit polyclonal antibody specific to the free hydrocortisone.
These lanthanide chelates are very stable and highly luminescent in aqueous solution and allowed to reach 10 microg/L and 40 microg/L sensitivities for clenbuterol and for hydrocortisone, respectively. Application to the horse urine, that is a very complex matrix, has a considerable interest in the control of illegal use of these compounds [09398].

**Clenbuterol**

The pharmacokinetics of clenbuterol in equine urine and blood was investigated. Urine and blood samples were collected following 3-day multiple oral administrations. The samples were examined using enzyme-linked immunosorbent assay and further confirmed by solid phase extraction and capillary electrophoresis. Urinary clenbuterol was detectable until day 14 after the last dose. The urinary excretion of clenbuterol was characterized by a biphasic pattern. The half-lives of the bi-exponential elimination ($t_{1/2alpha}$) and $t_{1/2beta}$ for urinary clenbuterol were about 12 and 48 hours. After a single oral administration (4 microg/kg) of clenbuterol, the half-life of serum clenbuterol was approximately 11.4 hours [10298].

A sensitive method using LC/ESI-MS(n) has been developed on a quadrupole linear ion trap mass analyser for the detection of nine beta$_2$ agonists (cimaterol, clenbuterol, fenoterol, formoterol, mabuterol, terbutaline, ractopamine, salbutamol and salmeterol) in horse urine. The method consists of solid-phase extraction on CSDAU cartridges before analysis by LC/ESI-MS(n) . The efficiency of extraction combined with the sensitivity and the selectivity of MS(n) allowed the detection of these compounds at pg/mL levels. Administration studies of fenoterol and formoterol are reported and show their possible detection after inhalation. The method is applicable for screening and confirmatory analysis [11321].

The use of clenbuterol in performance horses necessitates the establishment of appropriate withdrawal times. To describe plasma and urine concentrations of clenbuterol following administration of 2 commonly used dosing regimens to racing fit thoroughbreds 22 horses received an oral dose of 0.8 μg/kg bwt of clenbuterol twice daily for 30 days. A second group of 6 horses received clenbuterol according to the escalating dose protocol on the manufacturer’s label. Blood and urine samples were collected prior to, throughout and at various times up to 35 days post administration of the final dose. Drug concentrations were measured using liquid chromatography-mass spectrometry, and plasma data were analysed using noncompartmental analysis. Behavioural and physiological effects were monitored and heart rate was recorded throughout the course of the study. Clenbuterol plasma concentrations were below the limit of quantification (10 pg/ml) of the assay by Day 4 in all horses receiving the chronic low-dose regimen and by Day 7 in 5 of 6 horses receiving the escalating dosing protocol. Urine clenbuterol concentrations fell below the limit of quantification of the assay between Days 21 and 28 in all 22 horses in the low-dose group and in 5 of 6 of the horses in the escalating dose group. Muscle fasciculations, sweating and transient increases in heart rate were noted in a small number of horses following clenbuterol administration, but tolerance to these effects occurred rapidly. Establishment of appropriate withdrawal times for specific racing jurisdictions depends upon the threshold adopted by that specific jurisdiction. The study extends previous studies describing the pharmacokinetics of clenbuterol and describes plasma and urine concentrations following administration of 2 commonly used dosing regimens to racing fit thoroughbreds, which will allow jurisdictions to establish withdrawal times in order to prevent inadvertent positive regulatory findings [13788].

The aim of one study was to evaluate the concentrations of clenbuterol residues in the red hair of Chinese Simmental beef cattle following exposure to two doses of clenbuterol for 21
days. This experiment was conducted in six male red pied Chinese Simmental beef cattle which were randomly divided into two groups (n=3). Groups 1 and 2 were administered clenbuterol at a dose of 16 and 48 microg/kg body weight (BW)/day, respectively. Hair samples were collected on Days 7, 14 and 21 during treatment, and on Days 0, 14, 28, 42 and 70 after discontinuation of medication, using liquid chromatography tandem mass spectrometry (LC-MS-MS) method. About 500 mg hair samples spiked with 50 pg/mg D9-clenbuterol internal standard were analyzed with which the method recovery was from 89 to 117 percent. The results showed that clenbuterol was significantly accumulated in hair, with a concentration of 0.98 ± 0.56 pg/mg in Group 1 and 6.34 ± 3.21 pg/mg in Group 2 on Day 7 of treatment, and the residue concentrations increased as treatment proceeded. During the early withdrawal period, the residues increased from 13.52 ± 8.69 to 17.96 ± 6.94 pg/mg in Group 1 and from 55.15 ± 4.04 to 147.79 ± 15.35 pg/mg in Group 2. No significant differences were found in the later withdrawal period in both treatment groups. The results of the present study indicated that the red hair of Chinese Simmental beef cattle has high accumulation potential for clenbuterol residues. Hair, as a target matrix, even light-pigmented hair, can be used to monitor clenbuterol abuse over a long period [13789].

**THG**

The anti-doping rules of national and international sport federations ban any use of tetrahydrogestrinone (THG) in human as well as in horse sports. Initiated by the THG doping scandals in human sports a method for the detection of 3-keto-4,9,11-triene steroids in horse blood and urine was developed. The method comprises the isolation of the analytes by a combination of solid phase and liquid-liquid extraction after hydrolysis and solvolysis of the steroid conjugates. The concentrations of THG in blood and urine samples were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Following the administration of a single oral dose of 25 microg THG per kg bodyweight to 10 horses, samples were collected at appropriate intervals. The plasma levels of THG reached maximal concentrations of 1.5-4.8 ng/mL. Twenty-four hours after the administration plasma levels returned to baseline. In urine, THG was detectable for 36 h. Urinary peak concentrations of total THG ranged from 16 to 206 ng/mL. For the 10 horses tested, the mean plasma clearance of THG was 2250 mL/h/kg and the plasma elimination half-life was 1.9 h [09392].

**Theobromine**

In equine sport, theobromine is prohibited with a threshold level of 2 microg/mL in urine, hence doping control laboratories have to establish quantitative and qualitative methods for its determination. Two simple liquid chromatography/mass spectrometry (LC/MS) methods for the identification and quantification of theobromine were developed and validated using the same sample preparation procedure but different mass spectrometric systems: ion trap mass spectrometry (ITMS) and time-of-flight mass spectrometry (TOFMS). Particle-free diluted urine samples were directly injected into the LC/MS systems, avoiding the time-consuming extraction step. The relative standard deviations of intra- and inter-day analysis were lower than 8.6 and 7.2 percent, respectively, for the LC/ITMS method and lower than 5.7 and 5.8 percent, respectively, for the LC/TOFMS method. The bias was less than 8.7 percent for both methods. The methods were applied to two case samples, demonstrating simplicity, accuracy and selectivity [09393].

**Flurbiprofen**
Flurbiprofen and its main acidic metabolites were detected in equine urine after a single-dose administration of 500 mg flurbiprofen to two 2.5-3.5-years-old mares, in order to be used in equine doping control routine analysis. The urine levels of the parent drug were determined using GC/MS. Five acidic metabolites were found in the urine. The structure of the proposed metabolites was confirmed by HRMS accurate mass measurements. The highest flurbiprofen concentration was 204 μg per ml at 1-3 h post administration. Flurbiprofen could be detected for 24-37 hours in urine using the standard screening procedure. All metabolites were present 25 h post administration, while 4'-hydroxyflurbiprofen could be traced for more than 48 hours and it is regarded as the long-term metabolite of flurbiprofen in horse [08434].

**Phenylbutazone**

A sensitive liquid chromatographic-tandem mass spectrometric method was developed and validated for screening, quantification, and confirmation of phenylbutazone and oxyphenbutazone in equine plasma. Analytes were recovered from plasma by liquid-liquid extraction followed by separation in a reversed-phase column and identification by mass spectrometry with selected reaction monitoring in negative electrospray ionization mode. Extraction recovery for both analytes was >80 percent. Hemolysis of red blood cells decreased analyte signal intensity but did not affect quantification results because an isotope-labeled IS was used. Analytes were stable in plasma for 24 h at room temperature, 9 days at 4 degrees C, and 45 days at -20 degrees C and -70 degrees C. The method was successfully used in screening, quantification, and confirmation of phenylbutazone in post-competition plasma samples obtained from racehorses. The method is simple, rapid, and reliably reproducible [09394].

**Salicylic acid**

In equine sport, salicylic acid is prohibited with a threshold level of 750 microg/mL in urine; hence, doping control laboratories have to establish quantitative and qualitative methods for its determination. A simple and rapid liquid chromatographic/mass spectrometric method was developed and validated for the quantification and identification of salicylic acid. Urine samples after 900-fold dilution and addition of the internal standard (4-methylsalicylic acid) were directly injected to the liquid chromatography/quadrupole time-of-flight mass spectrometry system. Electrospray ionization in negative mode with full scan acquisition mode and product ion scan mode were chosen for the quantification and identification of salicylic acid, respectively. Run time was 2.0 min. The tested linear range was 2.5-50 microg/mL (after 100-fold sample dilution). The relative standard deviations of intra- and inter-assay analysis of salicylic acid in horse urine were lower than 2.5 percent and 2.8 percent, respectively. Overall accuracy (relative percentage error) was less than 3.3 percent. Method was applied to two real samples found to be positive for salicylic acid, demonstrating simplicity, accuracy, and selectivity [09395].

**Prednisolone**

After the detection of low concentrations of prednisolone in racehorse urine samples collected at Italian racetracks, a study was initiated to investigate the accuracy of the analytical protocol used and the possible endogenous origin of detected prednisolone. Multiple reaction monitoring (MRM) MS(2) acquisition with a triple quadrupole (n=780) and
full scan MS(2) and MS(3) (n=180) acquisition with a linear ion trap were checked. As a further confirmation, ten urine samples were analysed by high-resolution mass spectrometry (HRMS). The study showed the difficulty of identifying prednisolone, probably due to interfering compounds with the same molecular weight (360 Da) present in the matrix. The characteristic transitions for prednisolone were identified, both in MS(2) and MS(3), as the ions 187 and 280; the ion 295 was also used for identification. The concentrations detected with the triple quadrupole and the linear ion trap were not statistically different. The exact mass of prednisolone formiate (the adduct acting as a molecular ion) was identified by HRMS. The very high frequency of prednisolone detection in the samples (79%), the low concentration of this steroid and, importantly, the narrow range of the 95 percent confidence limits (0.97-1.05 in MS(2) mode and 0.88-1.04 in MS(3) mode), could represent evidence that its presence is endogenous. In the light of these results, this hypothesis seems the most probable, even if further studies are required to confirm it. Furthermore, a microbiological origin (i.e. fermentation of cortisol after sample collection) could not be disregarded [12500].

**Bupivacain**

Bupivacaine is a local anaesthetic prohibited in equine sports. It is highly metabolized in the horse but a thorough description of its metabolite profile is lacking. An administration study should find appropriate analytical targets for doping control. Furthermore, knowledge of an in vitro system for production of metabolites would be beneficial. Marcain® (bupivacaine hydrochloride) was administered subcutaneously to a horse and urine samples were collected. In vitro metabolic systems consisting of the fungi Cunninghamella elegans and Cunninghamamella blakesleeeana were incubated with bupivacaine and bupivacaine-d(9). Samples were analyzed directly after dilution or cleaned up using liquid-liquid extraction. Separation was achieved with liquid chromatography. Mass spectrometric analysis was performed using positive electrospray ionization with both a tandem quadrupole and an ion trap instrument using MS(n) and hydrogen/deuterium exchange. In horse urine, seven phase I metabolites were found: 3'- and 4'-hydroxybupivacaine, N-desbutylbupivacaine, two aliphatically hydroxylated metabolites, one N-oxide, and dihydroxybupivacaine. Sulfated hydroxybupivacaine and glucuronides of 3'- and 4'-hydroxybupivacaine and of dihydroxybupivacaine were also detected. All these metabolites were previously undescribed in the horse, except for 3'-hydroxybupivacaine. 3'- and 4'-Hydroxybupivacaine were designated as appropriate targets for doping control. Interestingly, all the equine phase I metabolites were also detected in the samples from C. elegans and C. blakesleeeana. The qualitative aspects of the metabolism of bupivacaine in the horse have been investigated with many novel metabolites described. The fungi C. elegans and C. blakesleeeana have proven to be relevant models for mammalian metabolism of bupivacaine and they may in the future be used to produce analytical reference materials [12501].

**Levamisole**

Administration studies of levamisole in horses were carried out using two different levamisole preparations, namely, levamisole hydrochloride oral bolus and levamisole phosphate injectable solution. These preparations were analysed in detail for the presence of aminorex-like impurities. Both levamisole preparations were found to contain 1-(2-mercaptoethyl)-4-phenyl-2-imidazolidinone (I) and 4-phenyl-2-imidazo-lidinone (II) as degradation impurities, but neither aminorex nor rexamino was detected in these preparations. It was been established unequivocally that the normal use of levamisole products in horses can lead to the presence of aminorex, rexamino and 4-phenyl-2-imidazolidinone (II) in their urine and
blood samples. As compound II has the longest detection time, the detection of aminorex (and in some cases reexamino) in some of the official samples from racehorses can be ascribed to the use of levamisole products as long as compound II is also present as a marker. These findings should be of direct relevance to the investigation of some of the cases of aminorex detection in official doping control samples from racehorses [09397].

3,4-Methylenedioxyxypovalerone (MDPV)

3,4-Methylenedioxyxypovalerone (MDPV) is a psychoactive drug with potent stimulant properties and potential for abuse and drug dependency. MDPV was recently classified as a Class I drug by Racing Commissioners International, indicating that it is a banned substance in equine athletes because it lacks therapeutic value in horses. To enforce this ban, a sensitive and fast liquid chromatography-tandem mass spectrometry method was needed. It is for this reason that this method was developed for quantification and confirmation of MDPV in equine plasma. Sample preparation involved liquid-liquid extraction. The analyte was analyzed by a triple-quadrupole linear ion trap mass spectrometer in positive multiple-reaction-monitoring and enhanced product ion scan modes. The method was validated for precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), linearity, stability, extraction recovery, matrix effect, dilution accuracy and selectivity. The extraction recovery was >90%. The linearity range was from 5 to 15,000 pg/mL. LOD and LOQ were 2 and 5 pg/mL, respectively. Intra-day and inter-day accuracies were nearly 100 percent. The method is suitable for screening, quantification and confirmation of MDPV in equine plasma and has been successfully used to detect and confirm the presence of MDPV in equine plasma obtained post-competition [12502].

Glycopyrrolate

It was described a validated, rapid, sensitive, and specific UHPLC-MS/MS method to detect and quantify glycopyrrolate in 0.5 mL of horse urine. Further, we investigated the elimination of glycopyrrolate in urine after both intravenous and oral administration of clinically relevant doses to Thoroughbred horses. Quantification was performed by weighted, linear regression analysis using a deuterated analogue of glycopyrrolate as internal standard (IS). The method was characterized by a linear range of 5-2500 pg/mL, a lower limit of quantification of 5 pg/mL and a limit of detection of 1 pg/mL. The intra and inter-batch imprecisions were <10 percent RSD and accuracy of the method ranged between 94 and 104%. Glycopyrrolate remained detectable in urine samples collected through 168 h after intravenous administration and through 24h after oral administration. Analytical method validation requirements for linearity, specificity, precision, accuracy, stability, dilution integrity, matrix effect, and ruggedness have been fulfilled. The urine method described in this report is simple and efficient and is the first reported method with sufficient sensitivity, accuracy, and precision to regulate the use of glycopyrrolate in urine samples collected more than one day after dosing of horses. Urine to plasma glycopyrrolate concentration ratios were calculated and were approximately 100:1 in samples collected from 24h through the end of sample collection [12503].

Dermorphine

Dermorphin, a hepta-peptide with potent analgesic properties, is classified as a doping agent in equine racing. Since its discovery, a number of biologically active structural analogs have
been synthesized and made commercially available so there is a need for reliable methods of detection. A sensitive detection method was developed for dermorphin and six analogs in equine urine. Peptide enrichment was achieved using weak cation exchange with subsequent separation and detection by nano-UHPLC-MS/MS. Method validation parameters included: specificity, linearity (5-10000 pg/mL), recovery (58-93 %), intra and inter-assay repeatability, LOD (5-50 pg/mL) and matrix effects. The presented method will facilitate the control of the abuse of dermorphin and selected analogs in equine sports [13790].

Capsaicin

A method involving ultra high-performance liquid chromatography-tandem mass spectrometry was developed and validated for the analysis of capsaicin and dihydrocapsaicin in equine plasma. The analytes were recovered from plasma by liquid-liquid extraction using methyl tert-butyl ether and separated on a sub-2 micron column. The mobile phase was composed of 2 mM ammonium formate and methanol. A triple quadrupole mass spectrometer was used to detect the analytes in positive electrospray ionization mode with selected reaction monitoring. The limits of detection, quantification and confirmation for both analytes were 0.5, 1.0 and 2.5 pg/mL, respectively. The linear dynamic range of quantification was 1.0-1,000 pg/mL. During storage, both analytes in equine plasma were unstable at room temperature but stable at -20 and -70°C. The retention time and product ion ratios were employed as the criteria for confirmation of the presence of the analytes in plasma. The total analysis time was 2 min. The method is fast, selectively sensitive, reproducible, reliable and fully validated [13791].

Acepromazine

It was described the population pharmacokinetics of an acepromazine (ACP) metabolite (2-(1-hydroxyethyl)promazine) (HEPS) in horses for the estimation of likely detection times in plasma and urine. ACP (30 mg) was administered to 12 horses, and blood and urine samples were taken at frequent intervals for chemical analysis. A bayesian hierarchical model was fitted to describe concentration-time data and cumulative urine amounts for HEPS. The metabolite HEPS was modelled separately from the parent ACP as the half-life of the parent was considerably less than that of the metabolite. The clearance (Cl/F(PM)) and volume of distribution (V/F(PM)), scaled by the fraction of parent converted to metabolite, were estimated as 769 L/h and 6874 L, respectively. For a typical horse in the study, after receiving 30 mg of ACP, the upper limit of the detection time was 35 h in plasma and 100 h in urine, assuming an arbitrary limit of detection of 1 lg/L and a small probability of detection. The model derived allowed the probability of detection to be estimated at the population level. This analysis was conducted on data collected from only 12 horses, but it was assumed that this is representative of the wider population [13792].

Antioxidants

The objective of this study was to determine if competition intensity would have an effect on antioxidant status in horses before and during a three-day event. Body weight, body condition score, and blood was sampled from CCI2* (n=19) and CCI3* (n=23) horses before the start of dressage, 20 to 30 min following cross-country, and 18-24 h after cross-country. Data were analyzed using a PROC MIXED in SAS. There were no differences between
CCI2* and CCI3* horses during competition for plasma cortisol, lactate, α-tocopherol, retinol, or erythrocyte glutathione peroxidase. After cross-country, CCI3* horses had higher serum creatine kinase and aspartate aminotransferase than the CCI2* horses. Plasma beta-carotene was higher in the CCI2* horses compared to the CCI3* horses. Total erythrocyte glutathione was also higher in the CCI2* horses versus CCI3* horses. These results are the first report of antioxidant status of horses competing in this level of a three-day event. The changes in antioxidant and muscle enzymes observed between divisions are likely due to the increased anaerobic and musculoskeletal demand on the upper level horses and the fitness required to compete at that level [12504].

Vitamin E

Vitamin E is a primary chain-breaking antioxidant that prevents cyclic propagation of lipid peroxidation. Across species, vitamin E is essential for normal neuromuscular function by acting as a potent antioxidant, as well as by modulating the expression of certain genes, inhibiting platelet aggregation and stabilizing plasma membranes. One review focused on vitamin E structure, absorption, metabolism, current equine dietary recommendations, the interplay between antioxidants and exercise, a discussion of the necessity of vitamin E supplementation in the horse above the Nutritional Research Council 2007 requirements, and a review of equine diseases that are associated with a vitamin E deficiency. Particular emphasis is placed on the proteins involved in vitamin E absorption, transport, and metabolism as potential candidates for vitamin E-associated diseases across species [12505].

Myo-inositol trispyrophosphate (ITPP)

Myo-inositol trispyrophosphate (ITPP) is a new drug capable of increasing the amount of oxygen in hypoxic tissues. Studies have shown that administration of ITPP increases the maximal exercise capacity in normal mice as well as mice with severe heart failure. The properties of ITPP make it an ideal candidate as a doping agent to enhance performance in racehorses. While there have been speculations in the horseracing industry that the covert use of ITPP is already widespread, no reported method exists for the detection of ITPP in equine biological samples. ITPP is a difficult-to-detect drug due to its hydrophilic nature; the complexity of equine biological matrices also adds to the problem. One paper described for the first time a method for the detection and confirmation of ITPP in equine urine and plasma. ITPP was isolated from the sample matrices by solid-phase extraction and the extract was analyzed by hydrophilic interaction chromatography-tandem mass spectrometry. ITPP could be detected at low ppb levels in both fortified equine plasma and urine with good precision, fast instrumental turnaround time, and negligible matrix interferences. This is the first report of a validated method for the detection and unequivocal confirmation of low levels of ITPP in any biological fluid [12506].

Alpha-cobratoxin

Cobra venom (Naja kaouthia) contains a toxin called alpha-cobratoxin (alpha-Cbtx). This toxin is a natural protein containing 71 amino acids (MW 7821 Da) with a reported analgesic potency greater than morphine. In 2007, in USA, this substance was found in the barns of a thoroughbred trainer and since then till date, the lack of a detection of this molecule has remained a recurring problem for the horseracing industry worldwide. To solve this problem,
the first method for the detection of alpha-cobratoxin in equine plasma has now been developed. Plasma sample (3 mL) was treated with ammonium sulfate at the isoelectric point of alpha-Cbtx, and the pellet was dissolved in a phosphate buffer and mixed with methanol for precipitation. The supernatant was then concentrated prior to its extraction on WCX SPE cartridges. The eluate was concentrated with two consecutive filtration steps before the trypsin digestion. The samples were analyzed using a LC-MS/MS Q Exactive instrument at 70,000 resolution on the product ions of the doubly charged precursor of the target peptide ((24)TWCDAFCSIR(33)). The method was validated (n=18) at 5 microg/L (640 pmol/L) according to the Association of Official Racing Chemists (AORC) requirements. The lower limit of detection was 1 microg/L (130 pmol/L). The present method has made it possible for us to confirm the presence of α-Cbtx in a horse plasma sample 24 h post the administration of alpha-Cbtx. Thus, the present method provides the first sensitive, specific, and reliable analytical method to confirm the presence of α-Cbtx in equine plasma [13793].

**Quaternary ammonium drugs**

Quaternary ammonium drugs (QADs) are anticholinergic agents some of which are known to have been abused or misused in equine sports. A recent review of literature shows that the screening methods reported thus far for QADs mainly cover singly-charged QADs. Doubly-charged QADs are extremely polar substances which are difficult to be extracted and poorly retained on reversed-phase columns. It would be ideal if a comprehensive method can be developed which can detect both singly- and doubly-charged QADs. One paper described an efficient liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for the simultaneous detection and confirmation of 38 singly- and doubly-charged QADs at sub-parts-per-billion (ppb) to low-ppb levels in equine urine after solid-phase extraction. Quaternary ammonium drugs were extracted from equine urine by solid-phase extraction (SPE) using an ISOLUTE® CBA SPE column and analysed by LC/MS/MS in the positive electrospray ionisation mode. Separation of the 38 QADs was achieved on a polar group embedded C18 LC column with a mixture of aqueous ammonium formate (pH 3.0, 10 mM) and acetonitrile as the mobile phase. Detection and confirmation of the 38 QADs at sub-ppb to low-ppb levels in equine urine could be achieved within 16 min using selected reaction monitoring (SRM). Matrix interference of the target transitions at the expected retention times was not observed. Other method validation data, including precision and recovery, were acceptable. The method was successfully applied to the analyses of drug-administration samples [12116].

**Ethanol**

Ethanol elimination was studied in camels (n=8) after a single bolus intravenous dose of 0.1g/kg bodyweight (BW). Blood samples were then collected at set intervals. Ethanol and ethyl glucuronide (EtG) in blood were analysed by validated static headspace gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry (LC-MS) methods, respectively. Blood-ethanol concentration-time profiles were plotted for each camel and these were evaluated. A simple linear regression model was fitted to the selected data points and the slope of the fitted line was used to estimate the elimination rate, the distribution factor and turnover rate, which were 5.15mg/dL blood/h, 0.55L/kg and 0.028g/h/kg, respectively. Blood EtG concentration-time profiles were also plotted for each camel. The elimination half-life of EtG, estimated by linear regression (using the values obtained after ethanol was completely eliminated) was 2.18h. The theoretical initial blood concentration of EtG (C(0)), obtained by extrapolation to time zero was 23.4μg/dL. The
results will be useful in monitoring alcohol doping in camels using either parent drug or metabolite [10428].

**Laboratory techniques**

*Direct-injection differential-gradient LC-LC coupled to hybrid tandem MS/MS*

A rapid, selective and robust direct-injection LC/hybrid tandem MS method has been developed for simultaneous screening of more than 250 basic drugs in the supernatant of enzyme hydrolysed equine urine. Analytes, trapped using a short HLB extraction column, are refocused and separated on a Sunfire C(18) analytical column using a controlled differential gradient generated by proportional dilution of the first column's eluent with water. Independent data acquisition (IDA) was configured to trigger a sensitive enhanced product ion (EPI) scan when a multiple reaction monitoring (MRM) survey scan signal exceeded the defined criteria. The decision on whether or not to report a sample as a positive result was based upon both the presence of a MRM response within the correct retention time range and a qualitative match between the EPI spectrum obtained and the corresponding reference standard. Ninety seven percent of the drugs targeted by this method met our detection criteria when spiked into urine at 100 ng/mL; 199 were found at 10 ng/mL, 83 at 1 ng/mL and 4 at 0.1 ng/mL [06314].

*Stable carbon isotope analysis*

The use of anabolic substances is prohibited in food-producing animals throughout the European Union. No method is available to reliably detect the misuse of natural hormones in cattle. A method was developed to detect the abuse of testosterone in cattle fattening. Synthesized testosterone is rather depleted in the $^{13}$C/$^{12}$C ratio. Hence, the method is based on gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) analysis of urine. To select testosterone metabolites and endogenous reference compounds (ERC), the concentration of urinary steroids of cattle was investigated. Dehydroepiandrosterone and androst-5ene-3beta,17alpha-diol were chosen as ERCs to show endogenous $^{13}$C/$^{12}$C ratios. Etioclanolone and 5alpha-androstane-3beta,17alpha-diol were chosen as the most important testosterone metabolites. Other metabolites known from literature like epitestosterone were less promising. In principle, GC/C/IRMS is a nonspecific method because finally carbon dioxide is analyzed. Therefore, a dedicated cleanup procedure for the selected steroids was developed. By means of proposed confidence intervals in the isotopic composition of ERCs and metabolites, the administration of testosterone to cattle could be detected reliably. Differences of up to 11 per thousand on the delta-scale between ERC and testosterone metabolites were found after testosterone administration, whereas endogenous differences did not exceed 2 per thousand [06315].

*Solid-phase extraction and liquid chromatography-mass spectrometry*

One paper reports two highly efficient liquid chromatography-mass spectrometry (LC-MS) methods for the screening of anabolic steroids, corticosteroids, and acidic drugs for the purpose of doping control in equine sports. Sample extraction was performed using a mixed-mode C8-SCX solid-phase extraction (SPE) cartridge. The first eluted fraction (acids/neutral fraction) was base-washed and the resulting organic extract was used for the screening of anabolic steroids and corticosteroids by LC-MS using multiple reaction monitoring (MRM) in the positive electrospray ionisation (ESI) mode. The remaining aqueous extract was re-adjusted to pH 6 and acidic drugs were recovered by liquid/liquid extraction. Detection was
again achieved using LC-MRM but in the negative ESI mode. A total of 40 anabolic steroids and corticosteroids, and over 50 acidic drugs, including some cyclooxygenase-2 (COX-2) inhibitors, oxicams, anti-diabetics, sedatives, diuretics and Delta(9)-tetrhydro-11-norcannabinol-9-carboxylic acid, could be covered by the two LC-MS methods. Both methods utilized a high efficiency reversed-phase column (3.3 cm L x 2.1 mm I.D. with 3 microm particles) coupled with a fast-scanning triple-quadrupole mass spectrometer to achieve fast turnaround times. The overall turnaround times for both methods were 10 min, inclusive of post-run and equilibration times [06316].

**Liquid chromatography – Orbitrap mass spectrometry**

A recent trend in the use of high resolution accurate mass screening (HRAMS) for doping control testing in both human and animal sports has emerged due to significant improvement in high resolution mass spectrometry in terms of sensitivity, mass accuracy, mass resolution, and mass stability. A number of HRAMS methods have been reported for the detection of multi-drug residues in human or equine urine. As blood has become a common matrix for doping control analysis, especially in equine sports, a sensitive, fast and wide coverage screening method for detecting a large number of drugs in equine blood samples would be desirable. One paper presented the development of a liquid chromatography-high resolution mass spectrometry (LC-HRMS) screening method for equine plasma samples to cover over 320 prohibited substances in a single analytical run. Plasma samples were diluted and processed by solid-phase extraction. The extracts were then analyzed with LC-HRMS in full-scan positive electrospray ionization mode. A mass resolution of 60 000 was employed. Benzyldimethylphenylammonium was used as an internal lock mass. Drug targets were identified by retention time and accurate mass, with a mass tolerance window of ±3 ppm. Over 320 drug targets could be detected in a 13-min run. Validation data including sensitivity, specificity, extraction recovery and precision are presented. As the method employs full-scan mass spectrometry, an unlimited number of drug targets can theoretically be incorporated. Moreover, the HRAMS data acquired can be re-processed retrospectively to search for drugs which have not been targeted at the time of analysis [13796].

**Proteomics et al**

The combat against misuse of growth promoting agents is a major topic in agricultural meat production and human sports. In routine screening, hormone residues of all known growth promoting agents are detected by immuno assays or chromatographical methods in combination with mass spectrometry. To overcome the detection by these routine screening methods new xenobiotic growth promoters and new ways of application were developed, e.g. the combination of different agents in hormone cocktails are employed. To enable an efficient tracing of misused anabolic substances it is necessary to develop new screening technologies for a broad range of illegal drugs including newly designed xenobiotic anabolic agents. The use of omic technologies like, transcriptomics, proteomics or metabolomics is a promising approach to discover the misuse of anabolic hormones by indirectly detecting their physiological action. With the help of biostatistical tools it is possible to extract the quested information from the data sets retrieved from the omic technologies. One review described the potential of these omic technologies for the development of such new screening methods and presents recent literature in this field [09400].

**In hair**

The abuse of synthetic esters of natural steroids such as testosterone and estradiol in cattle fattening and sports is hard to detect via routine urine testing. The esters are rapidly
hydrolysed in vivo into substances which are also endogenously present in urine. An interesting alternative can be provided by the analysis of the administered synthetic steroids themselves, i.e., the analysis of intact steroid esters in hair by liquid chromatography tandem mass spectrometry (LC/MS/MS). However, retrospective estimation of the application date following a non-compliant finding is hindered by the complexity of the kinetics of the incorporation of steroid esters in hair. In this study, the incorporation of intact steroid esters in hair following pour-on treatment has been studied and critically compared with results from intramuscular treatment. To this end animals were pour-on treated with a hormone cocktail containing testosterone cypionate, testosterone decanoate and estradiol benzoate in different carriers. The animals were either treated using injection and pour-on application once or three times having 1 week between treatments using injection and pour-on application. Animals were slaughtered from 10-12 weeks after the last treatment. Both hair and blood plasma samples were collected and analysed by LC/MS/MS. From the results, it is concluded that after single treatment the levels of steroid esters in hair drop to CCbeta levels (5-20 microg/kg) after 5-7 weeks. When treatment is repeated two times, the CCbeta levels are reached after 9-11 weeks. Furthermore, in plasma, no steroid esters were detected; not even at the low microgramme per litre level but – in contrast with the pour-on application – after i.m. injection, significant increase of 17beta-testosterone and 17beta-estradiol were observed. These observations suggest that transport of steroid esters after pour-on application is not only performed by blood but also by alternative fluids in the animal so probably the steroid esters are already hydrolysed and epimerized before entering the blood [09401].

Measurement of steroid esters in bovine hair samples, using sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS), provides a powerful tool for identifying animals treated illicitly with growth promoters. The successful application of such testing requires appropriate sampling of hair from treated animals. One paper described the results of hair analysis by LC-MS/MS for two animal studies in which animals were treated with estradiol-3-benzoate and nortestosterone decanoate. The results from the first animal study indicate that animals treated with these anabolic steroids may not always be identified from analysis of hair samples; positive test results occur sporadically and only for some of the treated animals. The results from the second animal study identify conditions attaching to positive hair samples, such as, that concentrations of steroid esters in hair are related to distance of sampling from point of injection and to time post-treatment, that concentrations of steroid esters in hair are related to dose given to the animal but that this relationship may vary over time post-treatment, and that steroid esters may be measured in regrowth hair taken some weeks after treatment. Steroid esters are determined along the length of the hair, confirming that accumulation of steroid esters into hair occurs from various sources, including blood, sweat and sebum. The reported research provides some useful insights into the mechanisms governing the persistence of steroid esters in bovine hair following illicit treatment with growth promoters [09402].

The detection of the abuse of anabolic steroids in equine sport is complicated by the endogenous nature of some of the abused steroids, such as testosterone and nandrolone. These steroids are commonly administered as intramuscular injections of esterified forms of the steroid, which prolongs their effects and improves bioavailability over oral dosing. The successful detection of an intact anabolic steroid ester therefore provides unequivocal proof of an illegal administration, as esterified forms are not found endogenously. Detection of intact anabolic steroid esters is possible in plasma samples but not, to date, in the traditional doping control matrix of urine. The analysis of equine mane hair for the detection of anabolic steroid esters has the potential to greatly extend the time period over which detection of abuse can be monitored. Equine mane hair samples were incubated in 0.1 M phosphate buffer (pH 9.5) before anabolic steroids (testosterone, nandrolone, boldenone, trenbolone...
and stanozolol), anabolic steroid esters (esters of testosterone, nandrolone, boldenone and trenbolone) and associated compounds (fluticasone propionate and esters of hydroxyprogesterone) were extracted by liquid-liquid extraction with a mix of hexane and ethyl acetate (7:3, v:v). Further sample clean up by solid phase extraction was followed by derivatisation with methoxylamine HCl and analysis by UHPLC-MS/MS. Initial method development was performed on a representative suite of four testosterone esters (propionate, phenylpropionate, isocaproate and decanoate) and the method was later extended to include a further 18 compounds. The applicability of the method was demonstrated by the analysis of mane hair samples collected following the intramuscular administration of 500 mg of Durastesten® (mixed testosterone esters) to a Thoroughbred mare (560 kg). The method was subsequently used to successfully detect boldenone undecylenate and stanozolol in hair samples collected following suspicious screening findings from post-race urine samples. The use of segmental analysis to potentially provide additional information on the timing of administration was also investigated [13781].

**Identifying individual horses urine by single-nucleotide polymorphisms (SNPs)**

To construct a system for identifying individual horses from urine samples that are submitted for postracing doping tests, we developed a genotyping assay based on 26-plex single-nucleotide polymorphisms (SNPs). DNA was isolated from urine using a commercially available DNA/RNA extraction kit, and SNP genotyping was achieved with a SNaPshot™ technique. DNA profiles including 26 SNPs were acquired from urine samples and blood/hair samples. Within the studied Thoroughbred population, the 26-plex assay showed a probability of identity of 5.80 × 10⁻¹¹. Compared to the conventional short tandem repeat assay, the SNP assay used less DNA, and the rate of successful genotyping was improved to 97% using aliquots of horse urine as small as 140 microL. The urinary DNA could be successfully genotyped under proper storage concerning refrigeration or freeze-thawing. This SNP assay can be used for individual identification when suspicious results are obtained from horse doping tests [13780].

**TB-500**

The formulation TB-500 is suspected to be used as doping agent in sport. This work describes the detection and the identification of the N-terminal acetylated 17-23 fragment of human thymosin beta 4 (Ac-LKKTETQ) in TB-500 by means of high-performance liquid chromatography/high resolution mass spectrometry using an Orbitrap Exactive benchtop mass spectrometer. Ac-LKKTETQ was also synthesized by solid-phase peptide synthesis, and an analytical strategy for detection in plasma and urine by high-performance liquid chromatography/low resolution triple-quadrupole mass spectrometry was suggested [12264].

A veterinary preparation known as TB-500 and containing a synthetic version of the naturally occurring peptide LKKTETQ has emerged. The peptide segment (17)LKKTETQ(23) is the active site within the protein thymosin beta (4) responsible for actin binding, cell migration and wound healing. The key ingredient of TB-500 is the peptide LKKTETQ with artificial acetylation of the N-terminus. TB-500 is claimed to promote endothelial cell differentiation, angiogenesis in dermal tissues, keratinocyte migration, collagen deposition and decrease inflammation. In order to control the misuse of TB-500 in equine sports, a method to definitely identify its prior use in horses is required. One study described a method for the simultaneous detection of N-acetylated LKKTETQ and its metabolites in equine urine and plasma samples. The possible metabolites of N-acetylated LKKTETQ were first identified from in vitro studies. The parent peptide and its metabolites were isolated from equine urine or plasma by solid-phase extraction using ion-exchange cartridges, and analysed by liquid
chromatography-mass spectrometry (LC/MS). These analytes were identified according to their LC retention times and relative abundances of the major product ions. The peptide N-acetylated LKKTETQ could be detected and confirmed at 0.02 ng/mL in equine plasma and 0.01 ng/mL in equine urine. This method was successful in confirming the presence of N-acetylated LKKTETQ and its metabolites in equine urine and plasma collected from horses administered with a single dose of TB-500 (containing 10 mg of N-acetylated LKKTETQ). This is the first identification of TB-500 and its metabolites in post-administration samples from horses [12499].

**Primary hepatocytes as a bioassay**

Cattle hepatocytes have already been used in veterinary in vitro toxicology, but their usefulness as a multi-parametric screening bioassay has never been investigated so far. In this study, cattle hepatocytes were incubated with illicit steroids/prohormones (boldenone, BOLD; its precursor boldione, ADD; dehydroepiandrosterone, DHEA; an association of ADD:BOLD), to characterize their transcriptional effects on drug metabolizing enzymes (DMEs) and related nuclear receptors (NRs), on cytochrome P450 3A (CYP3A) apoprotein and catalytic activity as well as to determine ADD and BOLD metabolite profiling. DHEA-exposed cells showed an up-regulation (higher than 2.5-fold changes) of three out of six NRs, CYP2B22 and CYP2C87; likewise, ADD:BOLD increased CYP4A11 mRNA levels. In contrast, a reduction of CYP1A1 and CYP2C87 mRNAs (lower than 2.5(-1)-fold changes) was noticed in ADD- and DHEA-incubated cells. No effect was noticed on CYP3A gene and protein expression, though an inhibition of 6beta-, 2beta- and 16beta-hydroxylation of testosterone (higher than 60 percent of control cells) was observed in ADD- and BOLD-exposed cells. Finally, 17alpha-BOLD was the main metabolite extracted from hepatocyte media incubated with ADD and BOLD, but several mono-hydroxylated BOLD and ADD derivatives were detected, too. Collectively, cattle hepatocytes can represent a complementary screening bioassay, useful to characterize growth promoters metabolite profiling and their effects upon DMEs expression, regulation and function [12488].

**Molecularly imprinted polymer applied to the selective isolation of urinary steroids**

The use of anabolic substances to promote growth in livestock is prohibited within the European Union as laid down in Directive 96/22/EC. Nowadays, efficient methods such as steroid profiling or isotopic deviation measurements allow to control natural steroid hormones abuse. In both cases, urine is often selected as the most relevant matrix and, due to its relatively high content of potential interferents, its preparation before analysis is considered as a key step. In this context, the use of a selective sorbent such as molecularly imprinted polymer (MIP) was investigated. A MIP was synthesized based on 17beta-estradiol, methacrylic acid and acetonitrile as template, monomer and porogen, respectively. Two approaches were then tested for non-conjugated (aglycons and glucuronides deconjugated) steroid purification: molecularly imprinted solid phase extraction (MISPE) and semi-preparative supercritical fluid chromatography with a commercial MIP as stationary phase (SFC-MIP). Parameters for both approaches were optimized based on the main bovine metabolites of testosterone, estradiol, nandrolone and boldenone. The MISPE protocol developed for screening purposes allowed satisfactory recoveries (upper 65 % for the 12 target steroids) with sufficient purification for gas chromatography-mass spectrometry (GC-MS) analysis. For confirmatory purposes, the use of isotopic ratio mass spectrometry (IRMS) requires a higher degree of purity of the target compounds, which can be reached by the SFC-MIP protocol with three steps less compared to the official and current method. Purity, concentration and absence of isotopic fractionation of target steroids extracted from urine of
treated cattle (treated with testosterone, estradiol, androstenedione, and boldenone) allowed the measurement of $^{13}$C/$^{12}$C isotopic ratios of corresponding metabolites and endogenous reference compounds (ERC) and proved the relevance of the strategy [12489].

**Single-nucleotide polymorphism assay**

To construct a system for identifying individual horses from urine samples that are submitted for post-racing doping tests, we developed a genotyping assay based on 26-plex single-nucleotide polymorphisms (SNPs). DNA was isolated from urine using a commercially available DNA/RNA extraction kit, and SNP genotyping was achieved with a SNaPshot(™) technique. DNA profiles including 26 SNPs were acquired from urine samples and blood/hair samples. Within the studied Thoroughbred population, the 26-plex assay showed a probability of identity of $5.80 \times 10^{-11}$. Compared to the conventional short tandem repeat assay, the SNP assay used less DNA, and the rate of successful genotyping was improved to 97 percent using aliquots of horse urine as small as 140 microL. The urinary DNA could be successfully genotyped under proper storage concerning refrigeration or freeze-thawing. This SNP assay can be used for individual identification when suspicious results are obtained from horse doping tests [12490].

**Orbitrap**

A recent trend in the use of high resolution accurate mass screening (HRAMS) for doping control testing in both human and animal sports has emerged due to significant improvement in high resolution mass spectrometry in terms of sensitivity, mass accuracy, mass resolution, and mass stability. A number of HRAMS methods have been reported for the detection of multi-drug residues in human or equine urine. As blood has become a common matrix for doping control analysis, especially in equine sports, a sensitive, fast and wide coverage screening method for detecting a large number of drugs in equine blood samples would be desirable. One paper presented the development of a liquid chromatography-high resolution mass spectrometry (LC-HRMS) screening method for equine plasma samples to cover over 320 prohibited substances in a single analytical run. Plasma samples were diluted and processed by solid-phase extraction. The extracts were then analyzed with LC-HRMS in full-scan positive electrospray ionization mode. A mass resolution of 60 000 was employed. Benzyldimethylphenylammonium was used as an internal lock mass. Drug targets were identified by retention time and accurate mass, with a mass tolerance window of ±3 ppm. Over 320 drug targets could be detected in a 13-min run. Validation data including sensitivity, specificity, extraction recovery and precision are presented. As the method employs full-scan mass spectrometry, an unlimited number of drug targets can theoretically be incorporated. Moreover, the HRAMS data acquired can be re-processed retrospectively to search for drugs which have not been targeted at the time of analysis [12491].

**Protein biomarkers**

The development of protein biomarkers for the indirect detection of doping in horse is a potential solution to doping threats such as gene and protein doping. A method for biomarker candidate discovery in horse plasma is presented using targeted analysis of proteotypic peptides from horse proteins. These peptides were first identified in a novel list of the abundant proteins in horse plasma. To monitor these peptides, an LC-MS/MS method using multiple reaction monitoring was developed to study the quantity of 49 proteins in horse plasma in a single run. The method was optimised and validated, and then applied to a population of race-horses to study protein variance within a population. The method was finally applied to longitudinal time courses of horse plasma collected after administration of
an anabolic steroid to demonstrate utility for hypothesis-driven discovery of doping biomarker candidates [09403].

Other aspects on forensic medicine

In order to detect switching and/or manipulation of samples, the owner of a stallion asked a lab to perform a DNA test on a positive doping urine sample. The objective was to compare the urine DNA profile versus blood and hair DNA profiles from the same stallion. At first, 10 microsatellite markers were investigated to determine the horse identity. No results were obtained when horse specific markers were typed in the urine sample. In order to confirm the species origin of this sample it was analyzed the mitochondrial cytochrome b gene. This analysis from blood and hair samples produced reproducible and clear PCR-RFLP patterns and DNA sequence match with those expected for horse, while the urine sample results were coincident with human. These results allowed us to exclude the urine sample from the questioned stallion and determine its human species origin, confirming the manipulation of urine sample [08442].

Horses (in general)

In racing and other equine sports, it is possible to increase artificially both the physical capability and the presence of a competitive instinct, using drugs, such as anabolic steroids and agents stimulating the central nervous system. However, an anti-doping policy must not impede the use of legitimate veterinary medications and most regulatory bodies in the world now distinguish the control of illicit substances (doping control) from the control of therapeutic substances (medication control). For doping drugs, the objective is to detect any trace of drug exposure (parent drug or metabolites) using the most powerful analytical methods (generally chromatographic/mass spectrometric techniques). This so-called “zero tolerance rule” is not suitable for medication control, because the high level of sensitivity of current screening methods allows the detection of totally irrelevant plasma or urine concentrations of legitimate drugs for long periods after their administration. Therefore, a new approach for these legitimate compounds, based upon pharmacokinetic/pharmacodynamic (PK/PD) principles, has been developed. It involves estimating the order of magnitude of the irrelevant plasma concentration (IPC) and of the irrelevant urine concentration (IUC) in order to limit the impact of the high sensitivity of analytical techniques used for medication control. The European Horserace Scientific Liaison Committee (EHSLC), which is the European scientific committee in charge of harmonising sample testing and policies for racehorses in Europe, is responsible for estimating the IPCs and IUCs in the framework of a risk analysis, i.e. risk assessment, risk management, and risk communication. For medication control, the main task of EHSLC in the risk management procedure is the establishment of harmonised screening limits (HSL). The HSL is a confidential instruction to laboratories from racing authorities to screen in plasma or urine for the presence of drugs commonly used in equine medication. The HSL is derived from the IPC (for plasma) or from the IUC (for urine), established during the risk assessment step. The EHSLC decided to keep HSL confidential and to inform stakeholders of the duration of the detection time of the main medications when screening is performed with the HSL. A detection time is the time at which the urinary (or plasma) concentration of a drug, in all horses involved in a trial conducted according to the EHSLC guidance rules, is shown to be lower than the HSL when controls are performed using routine screening methods. These detection time, as issued by the EHSLC (and adopted by the Fédération Equestre Internationale or FEI) provide guidance to veterinarians enabling them to determine a withdrawal time for a given horse under treatment. A
withdrawal time should always be longer than a detection time because the withdrawal time takes into account the impact of all sources of animal variability as well as the variability associated with the medicinal product actually administered in order to avoid a positive test. The major current scientific challenges faced in horse doping control are those instances of the administration of recombinant biological substances (EPO, GH, growth factors etc.) having putative long-lasting effects while being difficult or impossible to detect for more than a few days. Innovative bioanalytical approaches are now addressing these challenges. Using molecular tools, it is expected in the near future that transcriptional profiling analysis will be able to identify some molecular "signatures" of exposure to doping substances. The application of proteomic (i.e. the large scale investigation of protein biomarkers) and metabolomic (i.e. the study of metabolite profiling in biological samples) techniques also deserve attention for establishing possible unique fingerprints of drug abuse [10293].

It was reviewed drug and medication control in equestrian sports and addressed the rules of racing, the technological advances that have been made in drug detection and the importance of metabolism studies in the development of effective drug surveillance programmes. Typical approaches to screening and confirmatory analysis are discussed, as are the quality processes that underpin these procedures. It was also addressed four specific topics relevant to equestrian sports: substances controlled by threshold values, the approach adopted recently by European racing authorities to control some therapeutic substances, anabolic steroids in the horse and LC-MS analysis in drug testing in animal sports and metabolism studies. It was emphasised the importance of research and development and collaboration to further global harmonisation and the development and support of international rules [10294].

**Drug metabolism in horses**

A detailed understanding of equine drug metabolism is important for detection of drug abuse in horseracing and also in veterinary drug development and practice. To date, however, no comprehensive review of equine drug metabolism has been published. The majority of literature regarding equine drug metabolite profiles is derived from sports drug detection research and is generally targeted at detecting marker metabolites of drug abuse. However, the bulk of the literature on equine drug metabolism enzymology is derived from veterinary studies aimed at determining the molecular basis of metabolism. In one article, the phase 1 and 2 metabolisms of seven of the most important classes of drugs monitored in horseracing were reviewed, including: anabolic-androgenic steroids (AAS), beta₂-agonists, stimulants, sedatives/tranquilizers, local anesthetics, non-steroidal anti-inflammatory analgesics (NSAIDS)/cyclooxygenase-2 (COX-2) inhibitors, and opioid analgesics. A summary of the literature relating to the enzymology of drug metabolism in this species is also be presented. The future of equine drug metabolism in the area of doping research will be influenced by several factors, including: a possible move towards the increased use of blood and other alternative testing matrices; the development of assays based on intact drug conjugates; the increasing threat of 'designer' and herbal-based products; advances in the use of in vitro technologies; the increased use of liquid-chromatography/high-resolution mass spectrometry; and the possibility of screening using 'omics' approaches. Also, the recent cloning of a range of equine cytochrome P450 (CYP) enzymes opens up the potential for carrying out more detailed mechanistic pharmacological and toxicological veterinary studies [11310].

**Report from Iran**

One survey evaluated the use of prohibited substances cases in the first 2 years of
medication regulation in horseracing in Iran so that the impact of these regulations on the level of positive cases over the period could be assessed. A total of 656 horses that were winners or second in races were tested during the 2 year study. In the first year 354 horses (209 males and 145 females) and in the second year 302 horses (155 males and 147 females) were tested. In the 2 years, 306 were found to be positive. Urine samples were taken from candidate horses and sent to the Central Doping Laboratory. Blood samples were taken from those horses where a urine sample could not be taken within one hour. Detection and measurement of prohibited substances were carried out by ELISA, GC and HPLC using standard methods. Thirty-two percent of males were positive for prohibited substances, which was not significantly different from the percentage of females (26 %). In the second year, of the 302 horses tested for prohibited substances, 33.5% of males were positive, again similar to females (33 %). Almost 83 percent of horses tested positive for prohibited substances once in the first year, 15 percent tested positive twice and 2 percent tested positive 3 times. In the second year 78 percent tested positive once, 15 percent tested positive twice and 7 percent tested positive 3 times. Morphine was the most used prohibited substance and was detected 42 times during the survey, followed by caffeine and phenylbutazone. Morphine was also the most used drug in combination with other drugs in both years. It was concluded that in Iran morphine and caffeine were the most popular prohibited substances found in the measurements. As these substances were found in the environment and food stuffs, their presence in the samples may be due to unintentional feeding of contaminated materials (bread, hay and chocolate) [10295].

**Live stock**

In contrast to the use of hormonal doping agents in sports to enhance the performance of athletes, in the livestock industry hormonal growth promoters ("anabolics") are used to increase the production of muscle meat. This leads to international disputes about the safety of meat originating from animals treated with such anabolics. As a consequence of the total ban in the EU of all hormonal active growth promoters ("hormones") in livestock production, in contrast to their legal use [e.g. of five such hormones (17beta-estradiol, testosterone, progesterone, trenbolone and zeranol) as small solid ear implants and two hormones as feed additives for feedlot heifers (melengestrol acetate) and for swine (ractopamine) in the USA], the regulatory controls also differ sharply between the EU and the USA. In the EU the treatment of slaughter animals is the regulatory offence that has to be controlled in inspection programs. In the USA testing for compliance of a regulatory maximum residue level in the edible product (muscle, fat, liver or kidney) is the purpose of the inspection program (if any). The EU inspection programs focus on sample materials that are more suitable for testing for banned substances, especially if the animals are still on the farm, such as urine and feces or hair. In the case of slaughtered animals, the more favored sample materials are bile, blood, eyes and sometimes liver. Only in rare occasions is muscle meat sampled. This happens only in the case of import controls or in monitoring programs of meat sampled in butcher shops or supermarkets. As a result, data on hormone concentrations in muscle meat samples from the EU market are very rare and are obtained in most cases from small programs on an ad hoc basis. EU data for natural hormones in meat are even rarer because of the absence of "legal natural levels" for these hormones in compliance testing. With the exception of samples from the application sites – in the EU the site of injection of liquid hormone preparations or the site of application of "pour on" preparations – the hormone concentrations observed in meat samples of illegally treated animals are typically in the range of a few micrograms per kilogram (ppb) down to a few tenths of a microgram per kilogram. In the EU dozens of illegal hormones are used and the number of active compounds is still expanding. Besides estrogenic, androgenic and progestagenic
compounds also thyreostatic, corticosteroidal and beta-adrenergic compounds are used alone or in "smart" combinations. An overview is given of the compounds identified on the EU black market. An estimate is also given of the probability of consumption in the EU of "highly" contaminated meat from the application sites in cattle. Finally some data are presented on the concentration of estradiol in bovine meat from animals treated and not treated with hormone implants. These data are compared with the recent findings for estradiol concentrations in hen's eggs. From this comparison, the preliminary conclusion is that hen's eggs are the major source of 17alpha- and 17beta-estradiol in the consumer's daily "normal" diet [10299].

Proper identification of racehorses competing in an official race and maintenance of defensible chain of custody are important in doping control regulations. The purpose of one study was to develop a reliable multiplex PCR method for providing genetic evidence for matching donors to test samples by using short tandem repeat (STR) loci. Amplification of 21 STR loci from blood, urine or hair root was achieved in a single tube and STR length polymorphism was analyzed using fluorescent labeled capillary electrophoresis. This novel approach showed an allele confidence interval of 0.19-0.43 bp and size estimation error of 0-0.48 bp. In 90 thoroughbred (TB) and 171 standardbred (STB) horses, the method was highly discriminating and reproducible with probability of false identification of 1 in 10^{11} (TB) and 1 in 10^{13} (STB). All loci were highly polymorphic with an average probability of identity of 0.18 (TB) and 0.13 (STB), heterozygosity of 0.65 (TB) and 0.68 (STB), and polymorphism information content (PIC) of 0.62 (TB) and 0.69 (STB). The highest allele frequency also reflected the degree of polymorphism due to high correlation with PIC. To obtain evidence of sample tampering with human material, three human specific STR markers were included in the panel. This method is the first in the horseracing industry, specifically designed for racehorse identification and detection of equine sample contamination by human DNA [10300].

Recombinant human erythropoietin (rhEPO), darbepoetin alfa (DPO) and methoxy polyethylene glycol-epoetin beta (PEG-EPO) are synthetic analogues of the endogenous hormone erythropoietin (EPO). These erythropoiesis-stimulating agents have the ability to stimulate the production of red blood cells and are commercially available for the treatment of anaemia in humans. These drugs are understood to have performance-enhancing effects on human athletes due to their stimulation of red blood cell production, thereby improving delivery of oxygen to the muscle tissues. Although their effect on horses has not been proven, these substances were thought to be similarly performance enhancing and have indeed been applied covertly to horses. As such, these protein-based drugs are prohibited by authorities in both human and equine sports. The method officially adopted by the International Olympic Committee (IOC) and World Anti Doping Agency (WADA) for the confirmation of rhEPO and/or DPO (rhEPO/DPO) in human urine is based on electrophoresis in combination with Western blotting. A shortcoming of the WADA method is the lack of definitive mass spectral data for the confirmation of a positive finding. Recently, a liquid chromatography-tandem mass spectrometry (LC/MS/MS) method for the detection and confirmation of rhEPO/DPO in equine plasma was reported. However, we have not been successful in achieving the reported sensitivity. One paper presented a method for the detection and confirmation of rhEPO/DPO, as well as the newly released PEG-EPO, in equine plasma. The procedures involve immunoaffinity extraction using anti-rhEPO antibody-coated Dynabeads followed by trypsin digestion. The injected extract was further purified and concentrated using an on-line trap column in the nano-LC system. Detection and confirmation were achieved by monitoring a unique peptide segment of rhEPO/DPO/PEG-EPO using nano-liquid chromatography-tandem mass spectrometry equipped with a nanospray ionisation source operated in the selected reaction monitoring mode. rhEPO, DPO and PEG-EPO can be confirmed at 0.1, 0.2 and 1.0 ng/mL, respectively, in equine
Intersex conditions

Two standardbred racehorses that had been winning races while competing as mares underwent postrace drug testing and had serum testosterone concentrations above the acceptable limit for female racehorses. Initial physical examinations by the referring veterinarian revealed ambiguous external genitalia and suspected intra-abdominally located testes leading to a preliminary diagnosis of male pseudohermaphroditism. Horses were referred for further evaluation of sex. Physical examination of the external genitalia confirmed the findings of the referring veterinarian. Transrectal palpation and ultrasonography revealed gonads with an ultrasonographic appearance of testes. On cytogenetic analysis, both horses were determined to have a 64,XY karyotype and 8 intact Y chromosome markers and 5 SRY gene markers, which were indicative of a genetic male and confirmed an intersex condition. Additionally, both horses had some male-type behavior and endocrinologic findings consistent with those of sexually intact males. Taken together, these findings confirmed that both horses were male pseudohermaphrodites. Both horses returned to racing competition as males. This raises the possibility that intersex conditions may be more common in racing standardbreds than was previously suspected [11323].
FUTURUM

Sport celebrates differences in competitors that lead to the often razor-thin margins between victory and defeat. The source of this variation is the interaction between the environment in which the athletes develop and compete and their genetic make-up. However, a darker side of sports may also be genetically influenced: some anti-doping tests are affected by the athlete’s genotype. Genetic variation is an issue that anti-doping authorities must address as more is learned about the interaction between genotype and the responses to prohibited practices. To differentiate between naturally occurring deviations in indirect blood and urine markers from those potentially caused by doping, the "biological-passport" program uses intra-individual variability rather than population values to establish an athlete’s expected physiological range. The next step in "personalized" doping control may be the inclusion of genetic data, both for the purposes of documenting an athlete’s responses to doping agents and doping-control assays as well facilitating athlete and sample identification. Such applications could benefit “clean” athletes but will come at the expense of risks to privacy. One article reviewed the instances where genetics has intersected with doping control, and briefly discusses the potential role, and ethical implications, of genotyping in the struggle to eliminate illicit ergogenic practices [12508].

Besides "conventional" misuse of drugs such as erythropoietin and insulins, experts fear that therapeutics that are currently undergoing clinical trials might be part of current or future doping regimens, which aim for an increased functionality and performance or organs and tissues. The number of compounds and doping methods in sports is in a state of constant flux. In addition to “traditional” doping agents, such as anabolic androgenic steroids or erythropoietin, new therapeutics and emerging drugs have considerable potential for misuse in elite sport. Such compounds are commonly based on new chemical structures, and the mechanisms underlying their modes of action represent new therapeutic approaches arising from recent advances in medical research; therefore, sports drug testing procedures need to be continuously modified and complementary methods developed, preferably based on mass spectrometry, to enable comprehensive doping controls. It may be discussed not only emerging drugs that can be categorized as anabolic agents (selective androgen receptor modulators, SARMs), modulators of muscle fiber calcium channels, gene doping such as hypoxia-inducible factor (HIF) stabilizers and peroxisome-proliferator-activated receptor (PPAR)delta-agonists, and erythropoietin-mimetics (Hematide) but also compounds with potentially performance-enhancing properties that are not classified in the current list of the World Anti-Doping Agency. Compounds such as ryanodine-calstabin-complex modulators (benzothiazepines) may also be included [09404, 09405].

The number of compounds and doping methods in sports is in a state of constant flux. In addition to traditional doping agents, such as anabolic androgenic steroids or erythropoietin, new therapeutics and emerging drugs have considerable potential for misuse in elite sport. Such compounds are commonly based on new chemical structures, and the mechanisms underlying their modes of action represent new therapeutic approaches arising from recent advances in medical research; therefore, sports drug testing procedures need to be continuously modified and complementary methods developed, preferably based on mass spectrometry, to enable comprehensive doping controls. It was thus not only discussed emerging drugs that can be categorized as anabolic agents (selective androgen receptor modulators, SARMs), gene doping (hypoxia-inducible factor stabilizers, peroxisome-proliferator-activated receptor (PPAR)delta-agonists) and erythropoietin-mimetics (Hematide) but also compounds with potentially performance-enhancing properties that are not classified in the current list of the World Anti-Doping Agency. Compounds such as ryanodine-calstabin-complex modulators (benzothiazepines) may also be included [09404, 09405].
complex modulators (benzothiazepines) were also included, their mass spectrometric properties discussed, and current approaches in sports drug testing outlined [10302].

The practice of using prohibited substances to enhance sports performance (typically referred to as “doping”) is a very old practice that has been with us for centuries. To ensure harmonized, coordinated, and effective antidoping programs, the World Anti-Doping Agency (WADA) was created as an independent agency. Over the years, testing programs have continued to expand, and WADA-accredited laboratory testing has increased 40 percent over the last 3 years. The issue of sports doping has gained widespread attention, and the public seems to be continually reminded of athletes who dope and the potential growing problem of doping at all levels of competition. According to WADA statistics, only a small percentage of athletes who are tested test positive for prohibited substances. Establishing the prevalence of prohibited substances use by results of drug testing would be similar to establishing the prevalence of driving under the influence of alcohol by the number of arrests for drinking and driving. A common goal of all drug-testing programs is to level the playing field so that athletes do not feel they have to use performance-enhancing drugs or prohibited substances to be competitive. Drug-testing programs serve the dual purpose of identifying those who attempt to gain an advantage over their competitors by using prohibited substances or techniques, and deterring their use by athletes by increasing the risk with disciplinary actions. However, statistics are somewhat misleading, since they combine all sports and it is known that some sports have a higher incidence of performance-enhancing drug abuse than others. The statistics also combine in- and out-of-competition test results, and it is known that some drugs are not easily detected in competition. WADA figures reveal that 278 000 samples were tested in 2009, with the figures increasing marginally over the years. It is needed more-intelligent testing to target those athletes at risk for drug misuse. Obviously, more research into the use of surrogates as evidence for the use of prohibited substances, as well as the use of individual rather than population reference ranges, will aid detection. It is needed to fine-tune the perceived deterrence model to make it more effective. This is particularly challenging given the history of certain sports and the different sociological and cultural factors involved in international competition. Identification of the endogenous substances that control muscle development and function is important for the treatment of those who suffer from muscular dystrophies and other, similar conditions. As these substances are developed for therapeutic uses, they may also be used by athletes to enhance performance. Endogenous substances also present a challenge for drug-testing laboratories and programs. From a scientific viewpoint, the next two biggest challenges are hormone receptor modulators and protein pharmaceutical agents. There are dozens of structures for hormone receptor modulators that can affect a single biological pathway. The difficulty with protein therapeutics is their relatively short half-lives, making collection of a representative sample and assay sensitivity a challenge [11426].

**Continuing research**

Since 2001, USD 53 million has been spent on research that has successfully discovered detection methods for a new range of substances and doping methods. This investment will continue [12034].

**Criminals**

Doping substances are big business and the criminal underworld (often the same shady characters involved in illegal gambling and corruption) has created networks of supply, just
as they do for other illegal drugs. This is known from the partnerships World Anti-Doping Agency (WADA) has with Interpol and the World Customs Organization [12034].

Economy and techniques

Testing is – and always has been – the bedrock of the fight against doping in sport; science being used against science, with the hope that the science – that of the anti-doping community – one day becomes too sophisticated for athletes to risk doping. The anti-doping community accepts this is not the case right now. Analysis has its limitations and far too many athletes are able to weigh up the risk of getting caught against the financial rewards and glory that can be gained through doping, and come down on the wrong side. When that is allied to unscrupulous members of the athlete entourage, and with financial backing from the underworld, it can be seen the extent of the ill. Doping analysis faces an uphill struggle on many fronts. It is expensive, substances to be tested keep being added to the list and the costs involved in running a comprehensive testing program are getting greater, not smaller. Many anti-doping organizations are operating on small budgets (especially in relation to the size of the problem), having had them frozen or even cut in 2012, which obviously limits the ability to develop effective programs. Consideration must also be given to the sophistication of the doping substances and methods now available and used by the cheaters [12034].

Beyond the 2012 London Olympic Games researchers face two important additional challenges. First, it will be helpful to devise ways of reducing the cost of testing. More cost-efficient methods for detecting banned performance-enhancing drugs and other prohibited strategies would free up resources for more tests or other strategies to foster drug-free sport. Second, fresh ideas would be welcome for developing novel strategies for detecting efforts to tinker with human physiology, including techniques aimed at altering gene expression. There are many good reasons to want to keep performance-enhancing drugs out of sport: to protect the health of athletes, to promote fair competition so that honest athletes are not disadvantaged and to preserve what is good and valuable in sport. Everyone who participates in the effort to deter cheating, through education, research or the process of collection, testing and adjudication, is in a position to support honorable athletes in their pursuit of excellence. These are jobs worth doing well [12482].

Intelligens

While the anti-doping scientists are becoming more sophisticated, so too is the intelligence that exists within the testing community. This intelligence allows for more target testing, so that the money spent is better spent [12034].

What if current zero-tolerance anti-doping policy continues?

What can be foreseen over the next decades if the central tenet of the anti-doping movement – eradication of doping – continues to drive a global agenda of surveillance and suppression of doping and doping-like behaviour? It is expected that, in the short term, continued pressure from the WADA and the IOC, backed by the international UNESCO convention, will oblige an increasing number of nations to adopt specific anti-doping legislation, especially those who aspire to organize Olympic Games. There is an international tendency to combat doping and related activities like trafficking through criminal law (e.g. in Italy, France and
Slovenia), quite akin to what happened in the field of psychotropic drugs, thus criminalizing the use, possession, traffic and commerce of doping substances. This development will be accompanied by increasingly repressive measures worldwide. Increasing numbers of citizens will have to comply with compulsory drug testing for an increasingly long list of substances. Still, anti-doping arguments are frequently formulated which explicitly or implicitly ignore the actual practical consequences of actual anti-doping policies in and outside sports. Just as for the “war on drugs” the consequences of the “war on doping” should be fully taken into account when engaging into the debate on how to regulate the use of performance enhancing substances and methods in sports [12012].

The alternative proposed is regulation of drug use, based on human rights and public health principles, with a combination of pragmatic policies taking into account local socio-cultural and economic specificities, and continuously adapted to on-going developments. High on the list of policies proposed are treatment and harm reduction measures. Countries like the Netherlands, Switzerland and lately Portugal, where such policies have been put in place, have shown that these strategies are accompanied by a reduction in the cost to society and the individual, by decreasing drug-related mortality and morbidity, as well as crime and its associated costs, without an increase in the prevalence of illicit drug use. The difficulties of applying a model of regulation and harm reduction to sports are of course huge, but perhaps more in line with anthropological generalizations of a socio-culturally moulded, evolutionary defined ‘human nature’. The choice between fighting doping by all means vs. regulation and harm reduction is difficult, since neither will solve the problem; no ultimate solution exists, it will remain ‘messy’. In our view, regulation and harm reduction may come with less cost to society and the individual, as compared to a zero-tolerance approach, and therefore merits to be considered. There is really no ready-made blueprint to offer; if an easy way existed it would already have been in place [12012]

Social, policy, and public health perspectives on new psychoactive substances

New psychoactive substances pose a particular challenge to those formulating drugs policy and related public health responses. This paper outlines some of the main issues arising from their use, with a particular focus on user perspectives. Such substances are often (at least initially) produced and distributed for different reasons than controlled drugs. They emerge in users' repertoires undetected by most monitoring systems and general population drug surveys. While reasons for use by innovators and early adopters are often in the spirit of self-experimentation, such substances may rapidly diffuse to the recreational arena as a result of enthusiastic user propagation where they act as substitutes or complements to controlled drugs. The majority of substances are believed to be sourced, albeit not exclusively, from manufacturers based in China. They are retailed to consumers through the Internet and physical shops (such as “head” and “smart” shops), as well as traditional “street dealers” (although data on the significance of this latter route of supply are limited). The data required for risk assessment of the harms such substances may pose, as well as information required for accurate user-derived harm reduction advice, are often limited. Moreover, some involved in the commercial supply have deliberately misbranded products, including substituting the active substance, in apparent attempts to circumvent regulatory frameworks. This leaves users susceptible to both health and criminal justice harms. Despite various attempts to restrict the supply, they often continue to be available through the illicit market, although it is not yet possible to predict whether they will join other drugs such as MDMA and LSD as mainstays of the recreational pharmacopedia [11427].
Technology advancement

Recent events in the sporting world have made explicit the moral, political, and cultural characteristics of discussions surrounding the use of enhancement technology in sport. The landscape of sport technologies and policy has changed dramatically and it is reasonable to consider that further innovations are imminent. Elite sports constitute arenas for convergent technological applications where a range of applications demonstrates the embeddedness of sports within technological structures. The prospects for even more radical technologies to influence athletic performance grow continually as progress in nanotechnology, stem cells, and genetics gain strength. This growing role of technology within sport raises questions about its future direction, particularly how biology will relate to the “new biology of machines” [06168].

More complex challenges

In less than 10 years after the implementation of the World Anti-Doping Code and of the International Standard for Laboratories and its related Technical Documents, the analysis of human samples for the purpose of anti-doping testing has undergone a noticeable evolution. The research programs developed by the anti-doping organizations, and in particular the World Anti-Doping Agency (WADA), have created an unprecedented momentum in anti-doping science to strengthen the existing analytical methods, as well as to support the development and implementation of new and more sophisticated methodologies by the WADA-accredited laboratories. The integration of technical novelties into the analytical menus has been stimulated by the never-ending challenges posed by the adoption of more complex doping regimens by some athletes and their entourage. This increased sophistication of doping practices has also been reflected in the addition of new doping substances or methods on the WADA Prohibited Substances and Methods List. The integration of new anti-doping scientific paradigms with the development of the Athlete Biological Passport or the foreseen implementation of genomic- and proteomic-based tests constantly reshapes the environment of anti-doping analysis. It is also true that analysis faces challenges from the law, whereby data protection, the rights of athletes and opposition to aspects of the Code – for example, whereabouts – can hinder efforts to catch the dopers. One article provides a multiangle perspective on some of the key analytical challenges that anti-doping analytical science will face in 2012 and beyond [12509].

New classes of drugs

In 2009 and 2010, the first prohibited selective androgen receptor modulators (SARMs) and the gene doping substances AICAR and GW1516 were detected on the black market. All these substances are still in clinical trials and have not yet been approved as medications. From experience, we may expect that these substances will appear very soon on the dietary supplement market, with advertising that the SARM products will achieve anabolic effects whereas the gene doping substances will enhance endurance. If these substances are added to other supplement products without being declared on the label, new sources of risk for inadvertent doping will be created. The risk of inadvertent doping is predominantly connected to dietary supplements aggressively marketed for their physiological effects, for example, muscle gain and fat loss, but it cannot be confined exclusively to such products. Therefore, athletes should, in general, carefully consider the risks and benefits of dietary supplements. If use seems to be essential, athletes should purchase dietary supplements only from low-risk sources. However, these sources still cannot guarantee that dietary
supplements are free of risk, but they do offer a risk minimisation. Dietary supplements produced by pharmaceutical companies might represent an alternative as such products have not yet been found to be contaminated with doping substances [11425].

An important aspect of preventive doping research is the rapid implementation of tests for emerging drugs with potential for misuse into routine doping control assays. New therapeutics of different classes such as PPARdelta-agonists (e.g. GW501516), ryanodine-calstabin-complex stabilizers (e.g. S-107 and JTV-519), and selective androgen receptor modulators (SARMs, e.g. S-40503) are currently used for the treatment of particular medical conditions such as metabolic syndrome, cardiac arrhythmia, debilitating diseases and osteoporosis, respectively. Due to their being at an early stage of clinical trials and the limited availability of data on the metabolism and possible renal elimination of the active drugs, the development of protocols for doping control analyses of plasma specimens could be an option for the detection of the circulating agents. The mass spectrometric fragmentation of four emerging drug candidates (GW501516, S-107, JTV-519, and S-40503) was elucidated by positive electrospray ionization and collision-induced dissociation using a high resolution/high accuracy mass spectrometer. A screening and confirmation procedure was established based on liquid chromatography/tandem mass spectrometry requiring a volume of 100 microl of plasma. Proteins were precipitated using acetonitrile, the specimens were centrifuged and the supernatant analyzed using a triple-quadrupole mass spectrometer employing multiple reaction monitoring of diagnostic ion transitions. The method was validated with regard to specificity, limits of detection (0.4-8.3 ng/mL), recoveries (72-98 %), intraday and interday precisions (12-21 %), and ion suppression/enhancement effects [09304].

Further challenges have been, and still are, the flood of new medications that the pharmaceutical industry places on the market for good reasons, but that can be misused for doping purposes, the occurrence of food supplements that can be purchased over the counter but may contain doping substances, and truly illegal substances that are produced for doping purposes. The sophisticated doper can be given advice on microdosing at levels that are nearly impossible to detect, and guidance as to how a cocktail of designer drugs can create the desired performance-enhancing effect. The appearance of new potential doping substances has often been accompanied by comments suggesting that they would be impossible to detect at doping controls. Now it is heard the same comments with respect to the potential use of gene transfer for the purpose of doping ("gene doping"). Such comments are too often made by scientists who are not experts in the field, but their statements are taken by the general public as expert opinions. Instead, one should listen to the true experts such as those who came together at the WADA Gene Doping conference in Stockholm, Sweden, in 2005 and stated, “Scientific progress made through research supported by WADA and others suggest that new detection methods are likely to emerge, which will help to keep sport untainted by gene doping methods” [12005].

Synthol

Synthol consists usually of oil, benzyl alcohol and lidocain. It consists of 85 percent of oil (normally it is oil built by medium-length MTC chains because it gives the best effects), 7,5 percent of lidocain (painkiller), 7,5 percent of alcohol (to sterilize the mixture). Synthol is a substance used by body builders as a temporary implant which is injected deeply into the muscle. The enlargement effects are immediate. Synthol is used in small groups of muscles to enlarge their volume (for example triceps, biceps, deltoids, muscles of the calf). Some serious drawbacks can be visible while using synthol. The muscles deform and become unnaturally shaped. The side effects of synthol are manifold and they can also cause a damage of nerves, oil embolic of the pulmonary, occlusion of the pulmonary artery,
myocardial infarction, cerebral stroke and infectious complications [09306].

**Tolperisone**

The rate constants of spontaneous and hydroxide-catalyzed decomposition and the tautomer-specific protonation constants of tolperisone, a classical muscle relaxant were determined. A solution NMR method without any separation techniques was elaborated to quantitate the progress of decomposition. All the rate and equilibrium constants were determined at four different temperatures and the activation parameters were calculated. The molecular mechanism of decomposition is proposed [09307].

**5-hydroxytryptamine (5-HT) agonist**

A possible link between the neurotransmitter, 5-hydroxytryptamine (5-HT), plasma tryptophan, and branched chain amino acids concentration and exercise-induced fatigue is described by the central fatigue hypothesis. 5-HT receptors and neuroendocrine "challenge" tests, using prolactin release as an indirect measure of 5-HT activity were studied by recent investigations. In one study, the original hypothesis about the role of amino acids in increasing brain 5-HT with a neuroendocrine challenge test on elite athletes diagnosed with unexplained, underperformance syndrome (UUPS) was combined. There was an apparent increased sensitivity of 5-HT receptors in athletes with UUPS compared with fit, well-trained controls, as measured via increased prolactin release following a bolus dose of m-chlorophenylpiperazine, a 5-HT agonist. No changes were observed in plasma amino acid concentrations in either group. There is evidence that well-trained athletes have a reduced sensitivity of 5-HT receptors. The present study suggests that this adaptation may be lost in athletes with UUPS: this might explain some of their observed symptoms [10206].

**Prediction of futures anabolic androgenic steroids**
The aim of one work was to develop a flexible in vitro synthesis procedure, which can be applied in order to study and predict the metabolic patterns of new derivatives of anabolic androgenic steroids (AAS) with respect to most prominent target compounds for doping control purposes. Microsomal and S9 fraction of human liver preparations were used as a source of metabolising enzymes and the co-substrates of the synthesis mixture were selected to favour phase-I metabolic reactions and glucuronidation as phase-II conjugation reactions. Model compounds within the study were 4,9,11-trien-3-one steroids, structural derivatives of gestrinone and trenbolone, which both are included in the list of prohibited compounds in sports by the World Anti-Doping Agency (WADA). The correlation between in vitro metabolism of human microsomes and in vivo excretion studies in human was compared with gestrinone and subsequently, the applicability of the in vitro model for prediction of AAS metabolic pathways for new doping agents was evaluated. All the AAS examined within this study were successfully metabolised using the developed in vitro model, hydroxylation, reduction and glucuronide conjugation being the most prominent reaction pathways. Hydroxylated and glucuronide-conjugated metabolites of in vivo experiment with gestrinone were the same metabolites formed in the enzyme-driven process, thus showing good in vitro-in vivo correlation. Liquid chromatographic-mass spectrometric and tandem mass spectrometric methods were developed, relying on the positive polarity of electrospray ionisation, which also allowed the direct detection of intact glucuronide-conjugated AAS metabolites. Due to charge delocalisation and high proton affinity, the developed method was proven effective in the analysis of AAS metabolites bearing extensive conjugated double bond systems in their structures [08195].
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